Functional analyses on the nonspecific lipid transfer protein (nsLTP) of apple (*Malus domestica*)

Von der Naturwissenschaftlichen Fakultät, der Universität Hannover
Zur Erlangung des Grades eines
Dr. rer. nat.
genehmigte Dissertation

von
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Geboren am 8. August 1971
in El-Minia, Ägypten

2006

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Tag der Prüfung: 01. 02. 2006

Dedicated to

my late mother
who is still alive in my heart

Summary

Despite numerous studies on *in vivo* function of plant nonspecific lipid transfer proteins, their respective role is still quite unclear. This study was conducted to grasp the *in vivo* function of this puzzling nsLTP in apple (*Malus domestica* cv. Elstar).

In the case of apple scab disease in apple (*M*. *domestica* cv. Elstar) caused by the fungus *Venturia inaequalis*, it has been demonstrated recently (Gau et al., 2004) that the protein level of nsLTP has declined to undetectable level in the apoplast after infection with *V. inaequalis*. The same result has been observed after application of the non-pathogenic antagonist *Pseudomonas fluorescens* Bk3 on the leaves of apple trees (Kürcüoglu et al., 2004). This finding indicates that nsLTP is implicated in the infection process. However, the exact role of nsLTP in this scenario is still unclear.

Southern blot analysis showed that ns*ltp* of *M*. *domestica* susceptible cultivar Elstar as well the apple scab resistant cultivar Remo is a multigene family, at least ten copies have been detected in this study. Monitoring the transcript level via Northern blot and radioactive labeled probe of ns*ltp* revealed that the transcript level of the susceptible cultivar Elstar has drastically declined after infection within one day. For further investigations, the cDNA of ns*ltp* of both cultivars Elstar and Remo was amplified by RT-PCR, cloned and sequenced.

Sequence analysis revealed that apple ns*ltp* like other plant species ns*ltp* has eight conserved cysteine residues and it is free of tryptophan. Moreover, the first 24 amino acids in the N-terminal domain represent a putative leader sequence which is responsible for navigation of nsLTP to the secretory pathway. This was confirmed via particle bombardment and transient expression of the GFP tagged mature nsLTP. Deletion of the putative leader sequence resulted in the failure of mature nsLTP to enter the secretory pathway. In contrast it is expressed in the cytosol. Remarkably transient expression of nsLTP in fusion with GFP showed that nsLTP seems to be localized in different cell compartments, but mainly associated with the envelope membrane of the chloroplast. However GFP fluorescent signals could be detected in bodies like vesicles and probably in mitochondria, peroxisomes. Interestingly, under light conditions nsLTP has been localized exclusively in the chloroplast of the guard cell. Sequence evaluation of *M. domestica* nsLTP predicted a putative phosphorylation site that was confirmed through immunoblot using antibodies against

phosphorylated tyrosine residues. This finding indicates that nsLTP has a paramount key element that enables it to play a pivotal role in the plant cell.

The upstream region of ns*ltp* was amplified from the susceptible cultivar Elstar and the resistant cv. Remo by using Genome Walker Kit. Screening the nucleotide sequence of the upstream regions for the cis-acting regulatory elements showed that these regions are very rich in light responsive elements. Ten motifs have been recorded in both susceptible and resistant apple cultivars. Promoter activity studies on these upstream regions using particle bombardment and DsRed revealed that both of them could drive the expression machinery for the DsRed marker gene under light conditions. However in the case of Remo the expression was higher than in Elstar. These results confirmed the close relation between the presence of the light responsive elements predicted by computer program and chloroplast localization on one hand and the light dependence of the promoter activity on the other hand.

Based on our observations it can be suggested that nsLTP might act as a transporter for lipid *in vivo*. Since lipids can be involved in several fundamental functions within the plant cell, nsLTP can also be involved in the same functions. In general nsLTP could be involved in photosynthesis, signal transduction, vesicle trafficking, secretion, cytoskeletal rearrangement, growth and development, seed germination, organ differentiation, pollination, responses to biotic and abiotic stresses and programmed cell death.

Key words: apoplast, non-specific lipid transfer protein, *Malus domestica*, *Venturia inaequalis*, green fluorescent protein, red fluorescent protein.

Zusammenfassung

Die vielfältigen *in vivo* Funktionen des nicht-spezifischen Lipidtransproteins (nsLTP) in Pflanzen sind trotz intensiver Untersuchungen nach wie vor unklar.
Für das Apfelschorf suszeptible Kultivar *Malus domestica* cv. Elstar konnte gezeigt werden, dass während der Infektion mit dem pilzlichen Pathogen *Venturia inaequalis* das nsLTP im Apoplasten der Pflanzen auf einen nicht nachweisbaren Spiegel sinkt (Gau et al. 2004). Die gleichsinnige Beobachtung über die Abnahme des nsLTP nach der Applikation des nicht-pathogenen Antagonisten *Pseudomonas fluorescens* Bk3 wurde von Kürcüoglu et al. 2004 gezeigt. Diese Ergebnisse lassen vermuten, dass das nsLTP in den Infektionsprozess involviert ist, jedoch ist die exakte Rolle des nsLTP immer noch unklar.
Die Southern Blot Analyse ergab, dass das ns*ltp* in den Apfelschorf-suszeptiblen und resistenten Kultivaren Elstar und Remo mit mindestens zehn Kopien vertreten ist und somit zu einer Multigen-Familie gehört. Untersuchungen des Transkriptlevels mittels Northern Blot Analyse mit einer radioaktiv markierten Sonde des ns*ltp*s zeigten, dass einen Tag nach der Infektion der Transkriptlevel des *nsltps* drastisch gesunken ist. Um dieses Ergebnis zu bestätigen wurde die cDNA des nl*tps* aus beiden *M. domestica* cv. Elstar und Remo mittels PCR amplifiziert, geklont und sequenziert.
Die Sequenzanalysen zeigten, wie aus anderen Pflanzenarten bekannt, ein tryptophanfreies nsLTP mit acht konservierten Cysteinresten. Weiterhin zeigten die ersten 24 Aminosäuren der N-terminalen Domäne eine putative Leadersequenz, welche verantwortlich für die Weiterleitung des nsLTP in den sekretorischen Transportweg ist. Die zelluläre Lokalisation des nsLTP wurde durch transiente Expression des mit GFP markiertem nsLTP untersucht. Die Eliminierung der putativen Leadersequenz führte dazu, dass das nsLTP nicht zum sekretorischen Transportweg weitergeleitet wurde, stattdessen aber im Cytosol lokalisiert blieb. Die Experimente über die transiente Expression des GFP markierten nsLTP zeigten weiterhin, dass das mit GFP markierte nsLTP im Gegensatz zu der vorhergesagten apoplastidären Lokalisation mit verschiedenen Zellkompartimenten assoziiert ist. Hauptsächlich war es mit der Envelop-Membran des Chloroplasten assoziiert aber auch in Vesikeln, Mitochondrien und Peroxisomen. Interessanterweise wurde das nsLTP unter Lichtbedingungen

ausschließlich in die Chloroplasten der Schließzellen transportiert. Die durch Sequenzevaluierung vorhergesagte Tyrosinphosphorylisierungstelle wurde in einem Immuno-Blot mit Antikörpern gegen phosphorylisierte Tyrosinreste bestätigt und deutet auf eine mögliche regulatorische Rolle des nsLTP in der Pflanzenzelle hin.

Die up-stream Region von dem suzeptiblen Kultivar Elstar und dem resistenten Kultivar Remo wurde mit dem Genome Walker Kit amplifiziert. Das Screening der Nucleotidsequenzen der up-stream Region nach cis-aktiven regulatorischen Elementen zeigte, dass diese Region eine hohe Anzahl an lichtregulierten Elementen aufweist. Jeweils zehn Motive wurden in beiden Kultivaren Elstar und Remo gefunden. Die nachfolgenden Untersuchungen über die Promotoraktivität dieser up-stream Region mittels transienter Expression mit dem DsRed Reportergen ergaben, dass beide Apfelsorten in der Lage waren, das DsRed - Markergen unter der Kontrolle der up-stream Region der Kultivare Elstar und Remo unter Lichtbedingungen zu exprimieren. Die Expression in Remo war jedoch stärker als in Elstar. Diese Ergebnisse bestätigen die enge Beziehung zwischen der großen Anzahl lichtabhängiger Elemente die mitttels Datenanalyse gefunden wurden und der Assoziation des nsLTP mit dem Chloroplasten. Auf diesen Beobachtungen basierend könnte man annehmen, dass nsLTP *in vivo* als Transporter für Lipide dient. Da Lipide in vielen verschiedenen und grundlegenden Prozessen der Pflanzenzelle eine Rolle spielen, kann man annehmen, dass das nsLTP in die gleichen Prozesse involviert ist. Weiterhin könnte das nsLTP in Prozessen wie der Photosynthese, der Signaltransduktion, den Vesikelprozessen, der Sekretion, der Reorganisation des Cytoskeletts, des Wachstums und der Entwicklung, der Samenkeimung, der Organdifferenzierung, der Bestäubung, bei biotischem und abiotischem Stress sowie im programmierten Zelltod eine Rolle spielen.

Stichworte: apoplast, non-specific Lipid Transfer protein, *Malus domestica*, *Venturia inaequalis*, green fluorescent protein, red fluorescent protein.

List of abbreviations

APS	Ammonium persulphate
ATP	Adenosine triphosphate
BAP	6-Benzylaminopurin
bp	base pair
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate (X-phosphate) 4-toluidine salt
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CCD	Charge coupled device
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CTAB	Hexadecyltrimethylammonium bromide
cv.	Cultivar
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DMF	N,N-Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotriphosphate
DPI	Diphenyleneiodonium chloride
DsRed	*Discosoma* sp. red fluorescent protein
DTT	Dithiothreitol
ESI-Q-TOF	Electron spray ionisation quadrupole time of flight
GA_3	Gibberellic acid
GFP	Green fluorescent protein
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazine ethansulfonate
IBA	Indole-3-acetic acid
IWF	Intercellular washing fluid
IPTG	Isopropyl-ß-D-thiogalacto pyranosid
Kbp	Kilobase pair
kDa	KiloDalton
LB medium	Lauri Bertani medium
M	Molar
µM	Micromolar
mA	Miliamper
MBP	Maltose binding protein

MCS	Multiple cloning site
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	Messenger RNA
NAA	1- Napthalene acetic acid
NBT	Nitro blue -tetrazolium
NC	Nitrocellulose
ng	Nanogram
ns-LTP	Nonspecific lipid transfer protein
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCI	Phenol chloroform isoamylalcohol
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PCD	Programmed cell death
PEG	Polyethylenglycol
PMSF	Phenylmethylsulfonylfluorid
PRs	Pathogenesis-related proteins
psi	Pound per square inch
PVDF	Polyvinylidenefluoride
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Round per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
RubisCO	Ribulose-1,5-bisphosphat-Carboxylase-Oxygenase
SDS	Sodium dodecyl sulfate
Sec	Second
TAE	Tris-acetate-EDTA buffer
Taq	DNA polymerase from *Thermus aquaticus*
TEMED	*N,N,N',N'*- tetramethylethylenediamine
UV	Ultra violet

V	Volt
W	Watt
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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1 Introduction

1.1 Apple and apple scab disease

Apple (*Malus domestica*) is one of the most widely grown fruits in the world, and susceptible to several fungal and bacterial diseases, among which scab is the most severe and economically important. The major economic loss by apple scab is attributed to the reduction in fruit quality of scabbed fruits. But the disease can affect the tree in several ways. For example, severe infection of leaves will cause defoliation and subsequently reduction in tree vigor, which may restrict or prevent the fruit bud formation for the next year (MacHardy 1996). Under some circumstances, the losses from apple scab can be 70 percent or more of the total fruit value (Agrios 1997).

Although cultivars exhibit different degrees of susceptibility, none is immune (Komjanc et al., 1999). Apple scab exists worldwide, however it is more severe in areas with cool, moist spring and summers (Agrios 1997). In regions with semiarid conditions, scab lesions may be so few as to be undetected in most years. The first report of apple scab was from Sweden in 1819, and it was nearly 15 years before a second report, from Germany was published (MacHardy 1996).

The causal agent of apple scab is the highly sophisticated fungus *Venturia inaequalis*. A taxonomic classification of fungi includes *V. inaequalis* in the subdivision Ascomycotina, class Loculoascomycetes, order Pleosporales, and family Venturiaceae. Loculoascomycete fungi have bitunicate asci and the ascocarp is an ascostroma. In the Pleosporales, the ascocarp is a peritheciod pseudothecium that contains cylinderical asci and persistent pseudoparaphyses. Including the genus *Venturia* into the family Venturiaceae is based on pseudothecium and ascospore characteristics.

This fungus attacks exclusively members of the genus *Malu*s, cultivated varieties of apple as well as crab apple (MacHardy 1996).
The life cycle of *V. inaequalis* (Figure 1.1) begins with overwintering of the pathogen as an immature pseudothecia in the dead leaves on the ground of apple orchards. In late winter and spring, pseudothecia become mature, and the discharge of the ascospores as a primary inoculum, may continue for 3 to 5 weeks after petal fall.

During this period, buds start to open, initiating vulnerable sites for the primary infection. Ascospores germinate and cause infection when kept wet at temperature ranging from 6 to 26 °C the fungus grows between the cuticle and the outer cell wall of the epidermal cells. Lesions appear within 8 to 15 days, contributed to the production of enormous numbers of conidia by the subcuticular mycelium, causing rupture of the cuticle layer.

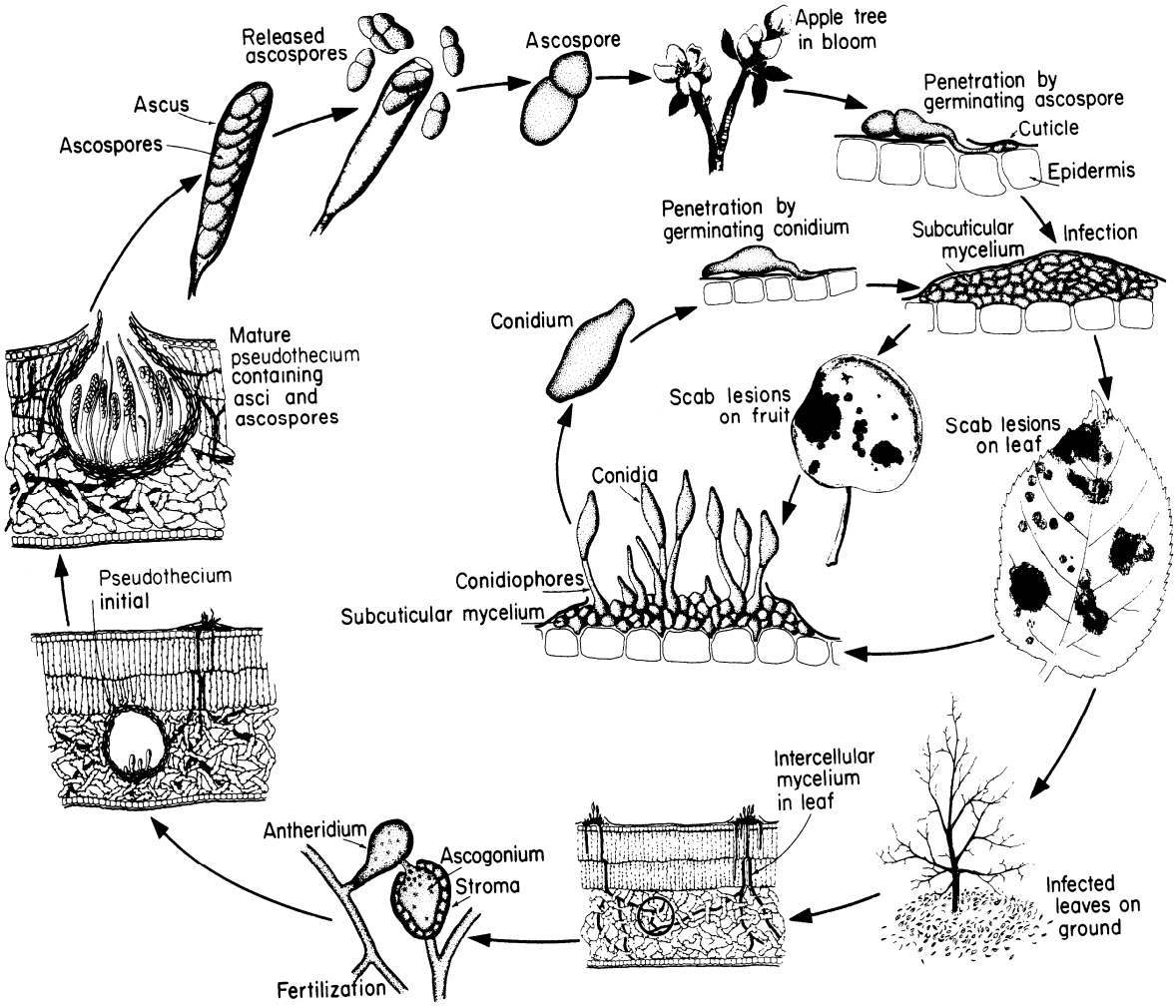

Figure (1.1): Life cycle of *Venturia inaequalis*

Source: (Agrios 1997)

These conidia continue to cause secondary infections during wet weather throughout the growing season. After infected leaves fall to the ground in autumn, the mycelium invades the interior of the leaf tissues forming pseudothecium, which maintain the fungus through the winter (Agrios 1997).

1.2 Apoplast and fungal growth

A crucial point in this life cycle, unlike obligate fungal parasites, *V. inaequalis* does not develop haustoria to obtain nutrients, nor does it grow intercellularly in host tissue forming subcuticular stroma (Figure 1.2). However, the cell wall breakdown by cell wall degrading enzymes provides a portion of these nutrients. As well as the extracellular melanoprotein that is produced by the fungus itself causes modification of the plant solute transport mediated possibly by membrane damage (MacHardy 1996). These growth sites represent a part of the apoplast.

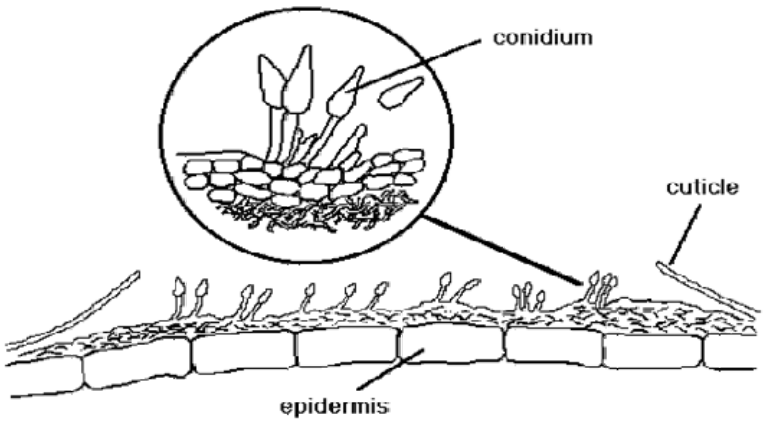

Figure (1.2): Growth pattern of *V. inaequalis* within the host tissues

The term "apoplast" was coined by the German botanist Ernst Münch in 1930 to describe the dead compartment in the plant body. Now, this dead compartment includes the interfibrillar and intermicellar space of the cell walls, the xylem as well as the gas and water filled intercellular space in its entirety. The border of the apoplast is formed by the outer surfaces of plants. Solutes or microorganisms adhering to these surfaces are not, however, apoplastic (Sattelmacher 2001). It has been suggested to consider the apoplast as the internal environment of the plant body. Like animal cells those of plants are surrounded by a liquid medium in the apoplast that is defined as apoplastic fluid. This internal environment has several important functions in our bodies; likewise, the apoplast is important and has numerous functions for plant development and performance (Sakurai 1998).

Apoplast forms a continuous, yet structured space between plant cells and constitutes a considerable part of the plant's body, namely between 5 and 10% of the plant's mass (Winter et al., 1993). The apoplast fluid is made up by the transpiration stream and its chemical composition is most likely achieved via water-, ion-, and

protein transporters (Hoson 1998; Sakuria 1998; Sattelmacher 2001) that may have a tissue-specific distribution. More recently, evidence has been obtained that the apoplast has an important function during plant defence and contains quite a number of proteins. More than 200 proteins (Robertson et al. 1997) are exported from the interior of the cells to their outside mostly via the endoplasmic reticulum and the plasma membrane, but also via different, and not completely unravelled routes via the plasma membrane as suggested for yeast (Gozalbo et al., 1992).

The apoplast responds to various environmental signals in diverse ways. Before the final response is induced, plant must perceive such stimuli, transform the signals, and transduce them to the site of reaction. It has been suggested that the apoplast is involved in the response as well as in the perception and transduction of environmental signals with the plasma membrane. The mechanism by which the response of the apoplast to environmental signals is brought about is summarized by Hoson in1998 (Figure 1.3).

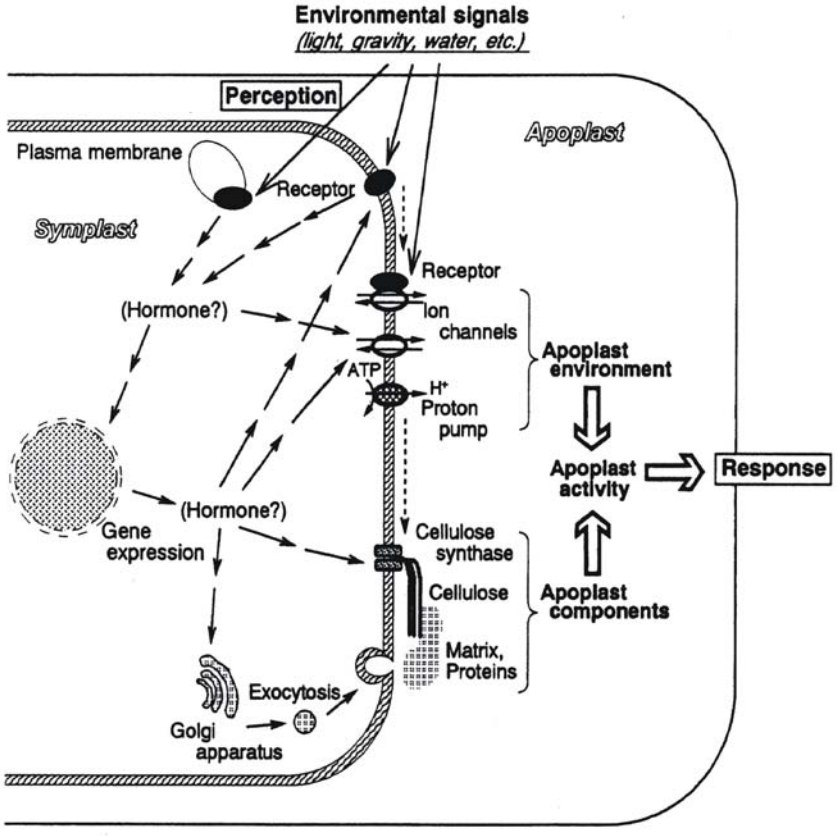

Figure (1.3): Schematic representation of the apoplast response to environmental signals.

Source: (Hoson 1998)

Various environmental signals as well as microorganisms are recognized by a specific receptors located in the plasma membrane or within the symplast. This

recognition process triggers a cascade of signals through the cytosol destinated to the nucleus. Within the nucleus, these signals activate specific genes to be expressed; the products of these genes can be transported to the apoplast via exocytosis. The transportation process leads to change the apoplast components that modify the apoplast environment and activity as a final response to the signals.

1.3 Interaction between *V. inaequalis* and apple within the apoplast

To understand more about the interaction between host and pathogen, it was necessary to analyze the protein contents in the apoplast as a first line of defense in the plant. The protein content and composition of this fluid were analysed by SDS PAGE (Figure 1.4). This analysis lead to the finding that a group of newly synthesized and/or highly expressed proteins have been exported to the apoplast after infection (Gau et al., 2004). The isoelectric focusing gel electrophoresis revealed that the majority of the apoplastic proteins have an acidic isoelectric point. The sequences of these newly synthesized and highly expressed proteins were determined by electron spray ionisation quadrupole time of flight mass spectroscopy (ESI-Q-TOF). Homology research in databases confirmed the presence of ß-1,3-glucanase, chitinase, thaumatin-like protein and a cysteine protease. This group of proteins belongs to pathogenesis-related proteins family (PRs). These results were corroborated by Western blot detection against some of these proteins. In contrast to these observations, non-specific lipid transfer protein with 9 kD molecular mass has declined drastically to non-detectable level within the first week after infection. The comparison with the intercellular washing fluid (IWF) of resistant apple cultivar (*M. domestica* cv. Remo) that bears resistance against apple scab, powdery mildew, and fire blight with uninfected susceptible cultivar (*M. domestica* cv. Elstar), showed a large difference between both of them. Moreover and more interestingly the protein pattern of the resistant cultivar showed high homology to those of the IWF from the infected susceptible cultivar Elstar. This observation indicates the constitutive expression of at least some of the pathogenesis-related genes in the resistant cultivars.

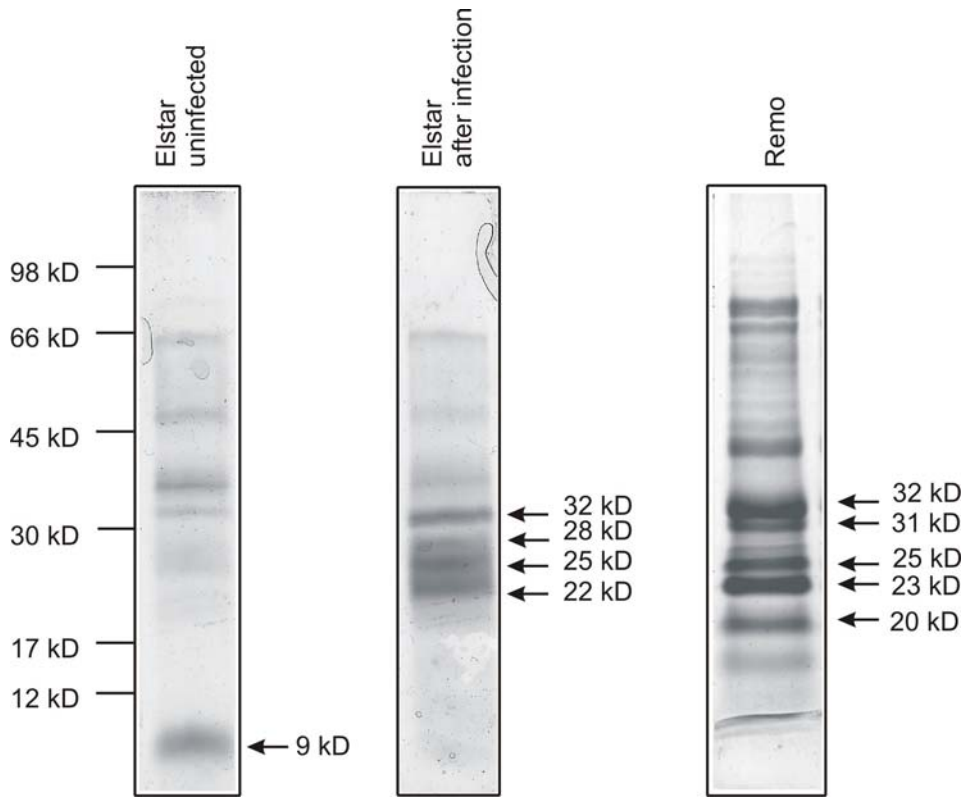

Figure (1.4): Preparative SDS-PAGE separation of apoplastic fluid from *M. domestica* cv. Elstar healthy leaves, *V. inaequalis* infected Elstar leaves and healthy Remo. Arrows indicate bands that were analyzed by ESI-QTOF mass spectrometery for further analyses. Each lane was loaded with 50 µg protein (Gau et al., 2004).

The main interest was to focus on the role of nsLTP in the infection process and to elucidate the possible *in vivo* function or functions of this puzzling protein in apple. As well as to answer the question; why the level of nsLTP has been declined to undetectable level in the apoplast of the susceptible apple cultivar leaves after infection with the fungus *V. inaequalis*.

1.4 Plant lipid transfer proteins (LTPs)

Plant lipid transfer protein has been discovered by Kader in 1975. Since this time several groups are investigating this group of proteins. Different approaches have been adopted to elucidate the possible function or functions for this enigmatic group. Until now the clear image of LTP is missing from the impressive number of articles regarding plant lipid transfer protein. Generally LTPs have the ability to transfer lipids between membrane vesicles *in vitro* (Yamada 1992; Bourgis and Kader 1997). Particularly nsLTPs exhibit a broad range of binding affinities to several classes of

phosphlipids and/or glycolipids and lack any specificity toward fatty acids or cutin monomers (Helmkamp 1986; Wirtz and Gadella 1990; Douliez et al., 2001). The nsLTP group has been identified in different plant species including monocots, dicots and gymnosperms (Kader 1996).

In the case of scab disease in apple (*M*. *domestica* cv. Elstar) caused by *V. inaequalis*, it has been suggested recently (Gau et al., 2004) that the nsLTP might be implicated in the infection process. However, the exact role of nsLTP in this scenario is elusive. Different functions beside lipids transfer have been proposed for nsLTP, including involvement in epicuticular wax biosynthesis (Sterk et al., 1991) and antimicrobial function (Molina et al., 1993; Segura et al., 1993; Cammue et al., 1995; Nielsen et al., 1996; Kristensen et al., 2000). On the other hand the *in vivo* function of nsLTPs is considered to be controversial (Canevacini et al., 1996). In addition, it has been shown that nsLTPs can be induced in response to different environmental stress factors, such as cold and drought stress (Ouvrard et al., 1996), heat shock and salt stress (Torres-Schumann et al., 1992). The spatial, developmental, drought and ABA-induced expression of three ns*ltp* family members in tomato were documented (Treviño and O`Connell 1998). In strawberry nsLTP was identified in response to ABA, wounding and cold stress (Yubero-Serrano et al., 2003).

Interestingly, the expression profile of nsLTPs was documented for different organs in different plant species as tissue-specific and developmentally regulated (Sterk et al., 1991; Fleming et al., 1992; Thoma et al., 1994). During the development of tracheary elements (TE) in zinnia, secretion of a TED4 protein that encoded for *ltp* into the medium, inhibit proteasome activity to protect the neighbouring cells. The depletion of TED4 protein from the culture medium results in an increase in mortality of other living cells (Endo et al., 2001).

Recently, *ltp* in *Euphorbia lagascae* seedling were found at high concentration in the inner region close to the cotyledon, and a smaller amount in the outer region of the endosperm (that must undergo programmed cell death). It has been proposed that *ltp* are involved in the recycling of endosperm lipids, or protecting the growing cotyledons from proteases released during programmed cell death PCD (Eklund and Edqvist 2003).

1.5 Structure of plant LTP

The number of amino acids residues varies from 91 to 95 of the mature plant LTP (Kader 1996). They lack of tryptophan and have eight conserved cysteine residues that are engaged in four disulfide bridges formation. Investigations on wheat LTP using different approaches revealed that this protein mainly constitutes from helical segments which connected by disulfide bridges (Simorre et al., 1991; Désormeaux et al., 1992). Based on nuclear magnetic resonance (1H NMR) data a model of wheat LTP has been built (Gincel et al., 1994), this model has been checked by crystallographic studies on wheat and rice (Pebay-Peyroula et al., 1992; Hwang et al., 1993). Structural studies of LTP revealed that the four helices form hydrophobic cavity, which runs through the protein (Lee et al., 1998). This cavity can accommodate fatty acids, acyl-CoA or phospholipids as shown in Figure 1.5 (Pons et al., 2003). The binding activity of plant LTP toward fatty acids lack specificity (Edqvist and Farbos 2002). This unspecificity was contributed to the involvement of non-specific van der Waals interactions that causes flexibility of the ligand-binding activity (Han et al., 2001).

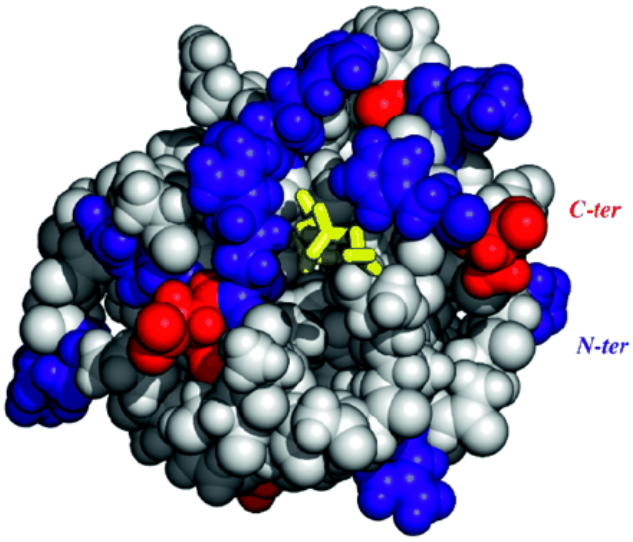

Figure (1.5): Model representation of the ns-LTP2 structure from wheat: Positively charged residues are coloured blue and negatively charged residues are coloured red; the phospholipid is represented in licorice mode. It can be seen how the terminal glycerol group sticks out of the molecular surface and how the basic residues surround the phosphate group in yellow (Pons et al., 2003).

1.6 nsLTP as allergene

In human healthy diet, fruits are considered to be a key element. However, in some individuals it can cause sever allergic reactions. Generally food allergy resulted from the improper reaction of the immune system toward specific protein, and once sensitized, the individual prone to allergic syndromes by homologous proteins of other food via cross reactivity (Gao et al., 2005). Interestingly, nsLTPs have been characterized as true plant food allergens not correlated with birch pollinosis (Fernandez-Rivas et al., 1997; Pastorello et al., 1999a; Sánchez-Monge et al., 1999; Asero et al., 2000). nsLTP belongs to food allergy class in which the immune reaction takes place in the gastrointestinal tract (Gao et al., 2005). This classification reflects the stability of nsLTP to proteolytic attack and food processing to reach the gastrointestinal immune system. Moreover, it has been reported that nsLTPs are insensitive to oxidative attack that usually destroys the allergenicity of birch pollen-related fruit allergens (van Ree 2002). Regarding heat, it has been also suggested that LTP is heat stable (Asero et al., 2000; Lindorff-Larsen et al., 2001). In many species, nsLTP has been identified as allergen, including peach (Pastorello et al., 1999b; Ballmer-Weber 2002), apple (Pastorello et al., 1999a; Diaz-Perales et al., 2002a), apricot (Pastorello et al., 2000a, 2000b), plum (Pastorello et al., 2001), cherry (Scheurer et al., 2001), hazelnut (Pastorello et al., 2002), walnut (Pastorello et al., 2004), chestnut (Diaz-Perales et al., 2000), grape (Pastorello et al., 2003), maize (Pastorello et al., 2000a), asparagus (Diaz-Perales et al.,2002b), and lettuce (Asero et al., 2000; Miguel-Moncín et al., 2003).

1.7 Distribution and localization of nsLTPs

The nsLTP group has been identified in different plant species including monocots, dicots and gymnosperms (Kader 1996). Interestingly the abundance of LTP was high in young broccoli leaves (Pyee et al., 1994). Similar observation has been reported in tobacco (Fleming et al., 1992), in barley (Molina and Garcia-Olmedo 1993), sugar beet (Nielsen et al., 1996), and apple (Koutb 2003).

In animal, nsLTP has been purified form bovine, rat, and human with a molecular weight of 14 kD and isoelectric point from 8.6 to 9.6 (Bloj and Zilversmit 1977; Crain and Zilversmit 1980; Noland et al., 1980; Poorthuis et al., 1981; Traszkos and Gaylor 1983; Westerman and Wirtz 1985; van Amerongen et al., 1987).

Plant nsLTP, in contrast with mammalians they exhibit a large variation in their primary structure, with a similarity between 30 to 50 % (Tchang et al., 1988).
Regarding localization, in general plant LTPs have been purified from the extracellular medium e.g. barley LTP was found to be secreted into aleurone cell culture (Mundy and Rogers 1986), several isoforms of grapevine LTP have been purified from the extracellular medium of somatic embryo culture (Coutos-Thevenot et al., 1993). In addition immunocytochemical studies revaled the localization of LTP1 was localized in the cell wall in *Arapidopsis* (Thoma et al., 1993), barley (Molina et al., 1993), partial localization to the cell wall in castor bean (Tsuboi et al., 1992), in the cell wall of epidermal cells of maize coleoptiles (Sossountzov et al., 1991).

1.8 Mode of action of LTP

It is clear that LTPs facilitate the movement of phospholipids between membranes. The question now is how this group of proteins performs this activity? One mechanism has been suggested for the phosphatidylcholine-specific LTP from mammalian cells, which suggests a phospholipids-LTP complex that *per se* interacts with the membrane and replaces its bound phospholipids by another molecule from the membrane (Wirtz, 1991). Comparable events have been proposed for plant LTP (Kader et.al, 1982; 1984). Despite the apparent similar mode of action between animal and plant nsLTP, there is no sequence homology in the amino acids (Wirtz 1991). Another model for action has been suggested that LTPs contain a hydrophobic cavity that can accept one acyl chain but not a phospholipids molecule (Shin et al., 1995). This binding activity facilitates the extraction of the phospholipid when LTP interacts with membrane surface (Figure 1.6).

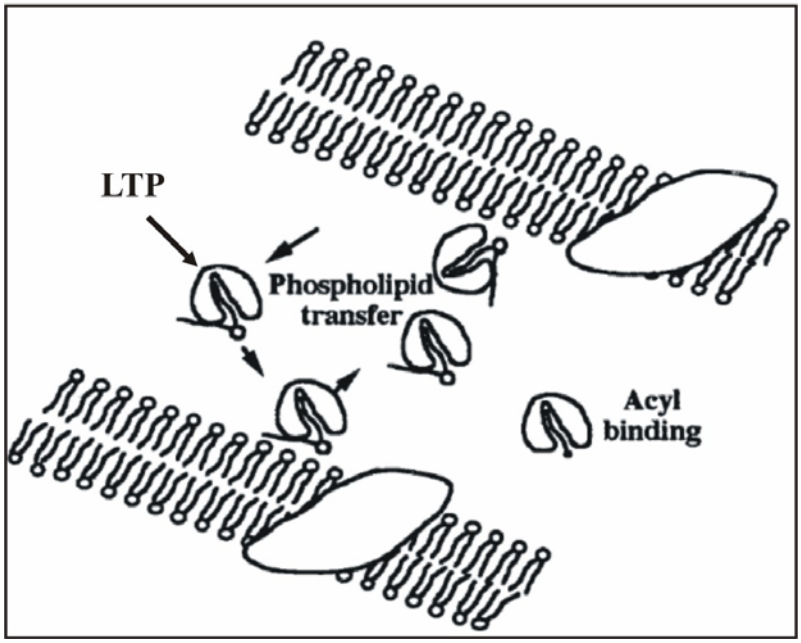

Figure (1.6): Diagrammatic representation for the postulated mode of action of plant lipid transfer protein (Kader 1996).

1.9 nsLTP substrates

In the vegetative plant cell, lipids constitute around 5 to 10 % of the dry weight, and almost all of this weight is localized in the membranes (Ohlrogge and Browse 1995). The most abundant lipids in most cells those are derived from the fatty acids and glycerolipid biosynthetic pathway (Ohlrogge and Browse 1995). Moreover fatty acids are the precursor for cutin and epicuticular wax synthesis that protect the plant against biotic and abiotic stresses (Schnurr et al., 2002). Unlike animals and fungi plant fatty acids synthesis mainly takes place within the plastid. Therefore plants must have mechanisms to export fatty acids from plastid to other sites in the cell. In general lipids represent the structural basis for cell membranes and fuel for metabolism. Particularly lipids in plant play a fundamental role in different cellular processes including photosynthesis, signal transduction, vesicle trafficking, secretion, cytoskeltal rearrangement (Welti and Wang 2004), growth and development, seed germination, organ differentiation, pollination and responses to biotic and abiotic stresses (Wallis and Browse 2002; Wang 2002; Farmer et al., 2003; Lindsey et al., 2003; Meijer and Munnik 2003; Sperling and Heinz 2003). The lipid transfer activity of plant lipid transfer protein is clear *in vitro* but the same activity *in vivo* still speculative.

1.10 Putative functions proposed for plant LTP

Based on the *in vitro* activity of LTP for transferring lipids between donor and acceptor it has been hypothesized in several reviews that LTPs could be involved in different functions, where lipid movement is thought to be important (Kader et al., 1982; Arondel and Kader 1990; Yamada 1992).

It has been suggested that LTPs are implicated in different biological functions including cutin formation, embyrogenesis (Sterk et al., 1991), defense reactions (Garcia-Olmedo et al., 1995), adaptation to different environmental stresses (Plant et al., 1991; Hughes et al., 1992; White et al., 1994) and PCD (Eklund and Edqvist 2003). Kader 1996 in his review suggested possible *in vivo* functions for plant LTPs presented below in Figure (1.7), however this diagram was crowded by question marks.

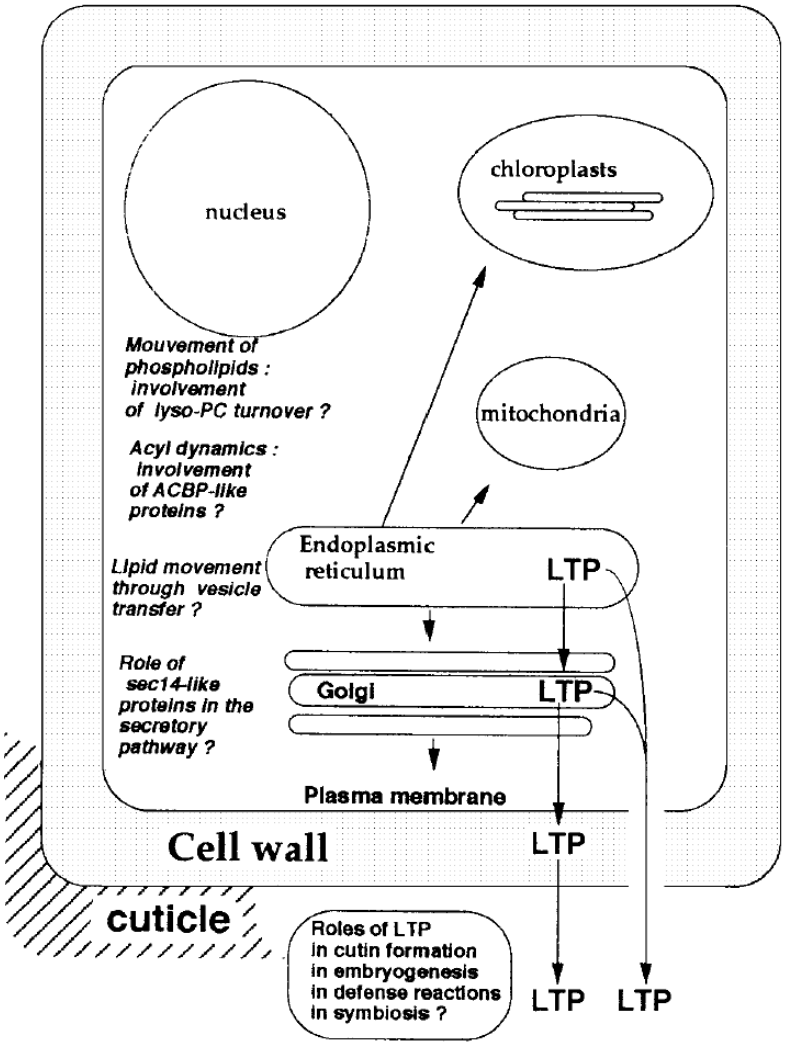

Figure (1.7): Diagrammatic representation shows the possible *in vivo* functions of plant lipid transfer proteins (Kader 1996).

Aim of the research

Despite the intensive investigations on plant nonspecific lipid transfer proteins (nsLTPs), their *in vivo* function is still elusive. It has been reported that the protein level of nsLTP of apple scab susceptible cultivar *Malus domestica* cv. Elstar has been found to decline to undetectable level in the apoplast after infection with *Venturia inaequalis*, the causal agent of apple scab (Gau et al., 2004). Concomitantly the nsLTP has not been detected in the apoplast of the apple scab resistant cultivar *M*. *domestica* cv. Remo.

The main task for the current study is to grasp the *in vivo* function of nsLTP in *M. domestica* and its implication in the infection process. To achieve this goal the copy number of this gene will be deterimend via southern blot analysis as well as the transcript level of ns*ltp* will be investigated before and after infection in the susceptible apple cultivar Elstar.

Furthermore, the cDNA of ns*ltp* will be amplified, cloned and sequenced. Sequence evaluation will also be done for the retrieved sequence. In most of the cases the function of a protein is closely correlated with the localization, therefore the subcellular localization study of this protein will be done with the transient expression of the nsLTP in fusion with GFP by using particle bombardment transformation.

In an attempt to understand the regulation pattern of ns*ltp*, the upstream regions from susceptible and resistant apple cultivars will be isolated and analyzed. Promoter activity will be confirmed via insertion of these upstream regions as artificial promoters for DsRed and transient expression experiment under different conditions. The upstream region as well as the transcriped region of ns*ltp* will undergoes methylation analyses to determine if the epigenetic information has influence on the regulation pattern of ns*ltp*.

Biochemical characterization of the nsLTP will be done to study the phosphorylation status of the amino acid residues (serine, therionine and tyrosine) as one of the most important characters of the posttranslational modifications in protein synthesis. This is an attempt to investigate the impact of the posttranslational modifications on the *in vivo* function/s of nsLTP.

2 Materials and Methods

2.1 Artificial infection of apple trees

2.1.1 Plant propagation

Apple trees (*Malus domestica*) were cultivated as described by Gau et al., (2002). Three different cultivars were used for cultivation including *M. domestica* cv. Elstar, Gloster, Holsteiner Cox, and Remo. The original *in vitro* cultures were kindly provided by Dr. I. Szankowski. These sterile and genetically identical plants were subcultured regularly on media see table 2.1. After six weeks plants were transferred to rooting media containing 1 x Murashige & Skoog medium (Murashige and Skoog 1962) including vitamins, 3 % sucrose, 1.5 µM indolebutyric acid and 0.7 % plant agar. After rooting, the plants were planted into soil and adapted to greenhouse conditions. Apple trees were grown in the green house at approximately 24 °C and light-dark cycle about 12 h. During this period the plants were illuminated with fluorescent tubes (Radium white) at approximately 80 µmoles m^{-2} s^{-1} under a light/dark regime of 12 h.

	Perales Medium	Cl-Medium	Puite& Shaart Medium
MS-medium+Vitamins	1 X MS	1 X MS	1 X MS modified
Sucrose	3 %	3 %	3 %
Myoinositol	0.01 %	0.01 %	0.01 %
BAP	3.1 µM	4.4 µM	3.1 µM
NAA	0.5 µM	-	-
GA_3	2.8 µM	-	-
IBA	-	0.5 µM	-
Plant agar	0.8 %	0.8 %	0.8 %
pH	5.8	5.8	5.8
Apple cultivar	Gloster	Holsteiner Cox	Elstar

Table (2.1): Media used for *in vitro* cultivation of different apple *M*. *domestica* cultivars.

2.1.2 Propagation of *Venturia inaequalis*

A strain of *Venturia inaequalis*, isolated from leaves of *M. domestica* cv. Elstar in Biologische Bundesanstalt (Dossenheim, Germany) and designated as a strain no. 15, (kindly provided by Dr. K. Schulze) was cultivated as previously reported by Parker et. al.,(1995). Cellophane membranes with approximately 8 cm in diameter were soaked in distilled water overnight and autoclaved. Subsequently, PDA medium was prepared and pH value was adjusted to 5.6. After autoclaving, approximately 20 ml of the hot medium was poured in each Petri dish. After solidification, the agar surface was covered with a sterile cellophane membrane. Finally each plate was inoculated with 0.5 ml of conidial suspension and incubated at 18 °C in the dark for one week.

PDA medium: 39.0 g potato dextrose agar (Duchefa, Netherlands) per liter distilled water.

2.1.3 Conidia harvest

Cellophane membranes were removed from the PDA plats surface under sterile conditions, and transferred to 250 ml bottle. Afterwards 50 ml sterile distilled water was added to the membranes and shaken for 5 min at 270 rpm. The suspension was filtered through a nylon membrane filter (69 µm pore size) and adjusted to a concentration of 10^5 conidia per ml as determined by a hematocytometer.

2.1.4 Inoculation of apple trees

Apple trees (*M. domestica* cv. Elstar) were grown in the green house under the previously described conditions. The trees were sprayed with the conidial suspension of *V. inaequalis* (1x 10^5 conidia per ml) on both upper and lower leaves surfaces. For mock infection, other apple trees were sprayed by distilled water. Subsequently, the inoculated plants were kept for three days at 100 % relative humidity, under transparent plastic tents at 19 °C (to facilitate the penetration of conidia for cuticle layer). After three days the plastic tents were removed.

2.1.5 Isolation of the intercellular washing fluid (IWF)

For the isolation of IWF the infiltration/centrifugation technique (Hogue and Asselin 1987) with slight modifications was used. The leaves were cut from the trees by a razor blade and its weight was determined. The harvested leaves were submerged in PBS buffer or water in a plastic box, and covered with a sieve. Infiltration with PBS buffer was done for 4 min. Leaves were dried with tissue papers and inserted into homemade holder as illustrated in Figure 2.1. Centrifugation was done for 5 min at 100 xg in an HS-4 rotor (Sorvall) to remove the excess of liquid. Subsequently, the IWF samples were collected by centrifugation step at 4 °C for 20 min at 700 xg. The obtained apoplastic washing fluid was stored at –20 °C.

PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4.

2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

2.2.1 Determination of protein concentration by Bradford method

Calibration curve was prepared by using a standard protein sample BSA (Bovine Serum Albumin, Sigma). Afterwards 20 µl from IWF samples were added to 80 µl PBS buffer, and 100 µl PBS buffer in a separate tube as a blank. One ml of Bradford reagent was added to each sample and as well as the blank, mixed very well and after 5 min the absorbance was measured by spectrophotometer at 595 nm according to Bradford (1976). The corresponding concentrations were calculated by the aid of calibration curve.

Bradford reagent: 100 mg Coomassie Brilliant Blue G-250 (CBB) was dissolved in 50 ml 96 % Ethanol and 100 ml 85 % H_3PO_4, Fill up to 1 liter with distilled water. After dissolving of CBB the solution were filtrated through a paper filter (Schleicher& Schull, Dassel, Germany).

Figure (2.1): Homemade holder for apoplastic fluid isolation from leaves; **A:** Holder without plant leaves; **B:** holder with plant leaves before fixing; **C:** Fixed plant leaves within the holder; **D:** Holder with plant leaves, ready for centrifugation.

2.2.2 Gel preparation

As described by Schägger and von Jagow (1987), nine percent separating gel was prepared by mixing gel contents and poured it into the gel cassette (size 8 x 6 x 0.075 cm). For polymerization the separating gels were covered with n-butanol. After polymerization the isobutanol was removed, and the gel cassette was fixed into electrophoresis cell. The contents of 4 % stacking gel were mixed and poured above separating gel. A comb with ten teeth was immersed in the stacking gel. After polymerization of the stacking gel, cathode buffer was added and the comb was removed. The wells were washed by cathode buffer and samples were loaded to the wells. The gel was let run at 25 mA by using electrophoresis power supply (EPS 600, Pharmacia, Sweden).

Separating gel contents 9 % (20 ml)		**Stacking gel contents 3 % (6.5 ml)**	
6.67 ml	3X Gel buffer	1.6 ml	3X Gel buffer
2.67 m	100 % glycerol	0.86 ml	Acrylamide solution 2
4.5 ml	Acrylamide solution 1	4.0 ml	Distilled H_2O
6.05 ml	Distilled H_2O	4 µl	TEMED
10 µl	TEMED	40 µl	10 % APS*
100 µl	10 % APS*		

*Freshly prepared.

Sample buffer 2X: 124 mM Tris pH 6.8, 20 % glycerol, 10 % 2-mercaptoethanol, 10 % SDS, and 0.02 % Coomassie Brilliant Blue G.
Gel buffer 3X: 2.9 M Tris, 0.3 % SDS, adjust pH to 8.45 with HCl
Acrylamide solution 1: 40 % acrylamide and 1.6 % bisacrylamide.
Acrylamide solution 2: 30 % acrylamide and 0.8 % bisacrylamide**.**

Anode buffer 10X: 2 M Tris, pH 8.9, fill up with distilled water.
Cathode buffer 10X: 1 M Tris, 1 M Tricin, 1 % SDS, pH 8.25, and fill up with distilled water.

2.2.3 Gel staining

After separation, the gel was stained with CBB solution overnight with shaking. The gel was washed several times with de-staining solution until protein bands appeared.

Coomassie Brilliant Blue solution: 0.01 % CBB R-250, 10 % Acetic acid, and 40 % Methanol in distilled H_2O.
Destaining solution: 10 % Acetic acid, and 40 % Methanol in distilled H_2O.

2.3 Protein samples preparation for mass spectrometry (Jensen et al., 1998)

2.3.1 Washing and destaining

The target bands were excised from SDS gel by using a scalpel. Excised gel pieces were put in reaction tubes and washed two times for 15 min with shacking with double volume of deionized water/acetonitrile 1:1 (v/v). Washing solution was discarded and gel pieces were incubated for 5 min in one volume acetonitrile at RT. Acetonitrile solution was replaced by one volume of 100 mM ammoniumbicarbonate and tubes were mixed and incubated for 5 min at RT. After addition of one volume acetonitrile to ammoniumbicarbonate, tubes were mixed and again incubated for 15 min at RT with shaking. The whole liquid was removed and gel pieces were dried under speed vacuum.

2.3.2 In gel digestion

The dried gel pieces were gradually quenched by adding 10 µl trypsin (10 ng/µl in 50 mM ammoniumbicarbonate) and incubated in ice for 10 min. Quenching step was repeated several times until saturation of gel pieces with trypsin solution. The excess of trypsin solution was discarded and gel pieces were soaked in excess of 25 mM ammoniumbicarbonate solution. Samples were incubated overnight at 37 °C.

2.3.3 Extraction of peptide fragments from gel

After overnight digestion, samples were shortly centrifuged and the supernatant was removed. Afterwards gel pieces were sonicated by using ultrasonic for 2 min and the resultant liquid was collected in a new tube. Gel pieces were incubated for 20 min in one volume 25 mM ammoniumbicarbonate with shaking. After addition of one volume acetonitrile and incubation for 15 min under shaking, mixture was sonicated for 2 min. The supernatant was collected in the same reaction tube. Gel pieces were incubated two times for 15 min in 5 % (v/v) formic acid/ acetonitrile (1:1) and liquid was collected in the same tube. The whole collected supernatant was centrifuged und vacuum for 10-15 min at 60 °C. Finally samples with volume 10-15 µl were ready for sequence and stored at -20 °C.

2.3.4 Sequencing

The extracted solutions were combined and concentrated with ZipTips C18 (Millipore). *De novo* sequencing was done on a quadrupole/time-of-flight hybrid mass spectrometer (Q-TOF2 Micromass, Waters, Manchester, United Kingdom) in positive-ion mode. Amino acid sequences were identified by homology search by using the program PeptideSearch (EMBL, Heidelberg, Germany).

2.4 Detection of nsLTP phosphorylation by immunological assay

2.4.1 Protein electro-blotting

After separation, gel was disassembled and subsequently assembled in the transfer cassette in the following order; cathode side, plastic support with holes, scotch-brite pad, 1 layer of Whatman 3 mm filter paper, protein gel, PVDF* membrane (Schleicher & Schull, Dassel, Germany), one layer of 3 mm filter paper, scotch-brite pad, plastic support with holes and anode side. The cassette was submerged in the electro-transfer buffer within the electro-blotting cell. Blotting was done for 1 h at 400 mA at 10 °C. The procedure was done according to Towbin et al. (1979).

* Treated with methanol for 3 sec, 5 min in water and 5 min in transfer buffer.

Transfer buffer: 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0,01 % SDS, 20 % Methanol, pH 9.9.

2.4.2 Membrane development

After the transfer of protein onto the NC membrane, membranes were incubated for 1 h with agitation in the blocking solution. The membranes were incubated overnight at 4 °C with agitation with 10 ml of the first antibody (rabbit polyclonal anti-phosphoserine, rabbit polyclonal anti-phosphothreonine and mouse monoclonal anti-phosphotyrosine (ZYMED®Laboratories Inc.) 1: 10000 diluted in 5 % nonfat powder milk). The membranes were washed two times for 10 min with PBST buffer. Membranes were incubated with 10 ml of the second antibody (IgG anti rabbit and anti-mouse coupled with alkaline phosphatase, Sigma, Munich, Germany) with the dilution 1: 20000 in blocking solution for 1 h. Membranes were washed shortly for two times for, followed by four times for 6 min. Equilibration of the membranes was done

by incubation with 10 ml TMN buffer for 2 min. Finally membranes were developed with 10 ml of TMN buffer containing 0.4 mM NBT and 0.3 mM BCIP as a final concentrations. After the desired intensity was produced, the development was stopped by washing the membranes excessively with distilled water.

PBST buffer: PBS buffer with 1% tween 20
TMN buffer: 100 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, pH 9.5.
Blocking solution: 5 % nonfat dry milk and 0.05 % Tween-20 in PBS buffer.
NBT stock solution: 61 mM Nitro Blue Tetrazolium in 70 % DMF.
BCIP stock solution: 115 mM 5-Bromo-4-chloro-3-indolylphosphate in 70 % DMF.

2.5 Isolation of ns*ltp* cDNA

2.5.1 Isolation of the total RNA

Approximately 5 g of stored leaves samples at -80 °C after IWF collection were ground well in liquid nitrogen in precooled mortar and pestle. The obtained powder was mixed with 15 ml of prewarmed (50 °C) lysis buffer and 15 ml of PCI solution and shaken for 20 min. The mixture was centrifuged for 20 min at 13000 xg. The supernatant was removed and mixed with 15 ml of PCI solution and again centrifuged for 20 min at 13000 xg. Supernatant was mixed with 0.75 % volume of 8 M LiCl and stored overnight at 4 °C. Solution was centrifuged for 20 min at 13000 xg and 4 °C. After removal of the supernatant, pellet was mixed with 5 ml distilled water, 500 µL 3 M Na-acetat pH 5.2 and 5 ml of cold (-20 °C) 96% ethanol, and stored for 1 h at –20 °C. After centrifugation for 20 min at 4 °C and 13000 xg, the pellet was washed with 10 ml 70 % ethanol (-20 °C) and centrifuged again for 20 min. Supernatant was carefully removed and pellet was dried by speed vacuum. Eventually the pellet was dissolved in 1 ml autoclaved distilled water and kept at –80 °C until use.

Lysis buffer: 600 mM NaCl, 20 mM EDTA, 4% SDS, 100mM Tris-Cl pH 8.
PCI: 25 volume Phenol: 24 volume Chloroform: 1 volume Isoamylalcohol, by 100 mM Na-Acetat pH 4.5 and stored in dark at 4 °C.

2.5.2 Determination of quantity and purity of DNA and RNA

DNA and RNA isolations were diluted 1:100 and the absorptions were measured at 230 nm 260 nm and 280 nm. DNA and RNA concentrations were determined by the following formulas;

$$\frac{E_{260} \times 50 \times \text{dilution factor}}{1000} = \mu g \text{ DNA} / \mu l$$

$$\frac{E_{260} \times 40 \times \text{dilution factor}}{1000} = \mu g \text{ RNA} / \mu l$$

The quotients E_{260}/E_{280} and E_{260}/E_{230} give information about contamination with proteins and polysaccharides respectively. A quotient between 1.8 and 2 shows a sufficient purity.

2.5.3 Determination of RNA pattern in agarose gel

1.5 % agarose gel was used for this purpose. An appropriate amount of agarose was melted in autoclaved water 1 min in a microwave (600 W). After cooling down to ~ 60 °C, 1% MOPS, pH 7.0 and 3.4 % Formaldehyd were added. Afterwards, solution was poured into the gel cassette. From each RNA sample, 2 µl were taken and mixed with 18 µl Northern-Mix. The mixture was centrifuged and incubated at 65 °C for 15 min. Samples were chilled in ice, centrifuged and loaded to the gel. Samples were electrophortically separated after addition of 1x MOPS as a running buffer. RNA was visualized in UV light.

MOPS 10X: 0.2 M MOPS, 0.05 M sodium acetate and 0.01 M EDTA, pH 7.0
Northern Mix: 5 ml Solution 1 + 1 ml Solution 2 + 40 µl Ethidiumbromid, 5 mg/ml.
Solution 1: 1x MOPS pH 7, 6.5 % Formaldehyd, 50 % Formamid.
Solution 2: 0.25 % BPB, 0.25 % Xylene Cyanol, 50 % Glycerol, 1 mM EDTA, pH 8.0

2.5.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed by using peqGold M-MuLV Reverse Transcriptase RNase H^- (Peqlab-Biotechnologie GmbH, Germany). Appropriate amounts of RNA samples

were mixed (0.005-0.25 µg/µl total RNA, final concentration) with 1 µl of Oligo $(dT)_{23}$ primer (0.5µg/µl) and 1 µl deoxynucleotide mixture (500 µM each dNTP as a final concentration). Afterwards an appropriate volume of water was added to make the final volume 10 µl. The subsequent steps were carried out in the thermocycler (PTC 200, Biozym, Oldendorf, Germany). The reaction mix was incubated initially at 70 °C for 10 min and then chilled at 0 °C. A second reaction mix consisted of 2 µl of 10x buffer for M-MuLV-RT, 1 µl RNase inhibitor (20u/µl), 1 µl M-MuLV reverse transcriptase and 6 µl of water was added to the first reaction. The first strand of cDNA was built by incubation the mixture at 50 °C for 50 min. For PCR, the first reaction mix was prepared using 5 µl of cDNA template from RT- reaction, 10 pmol of each primer (nsLTP forward 5'-ATG GCT AGC TCT GCA GTG AC-3' 4 µl 2.5 mM dNTP mix and sterile distilled water to make up to 25 µl per reaction. Afterwards, a second reaction mix containing one unit of red *Taq* polymerase (Sigma, Munich, Germany), 5 µl of 10 x complete buffer and 19.5 µl of sterile distilled water per reaction was added to the first reaction mix at 80 °C (hot start). The whole reaction was denatured initially for 3 min at 94 °C. The thermocycler was adjusted as follows for 36 cycles: denaturation at 94 °C for 30 sec, annealing at 46-56 °C for 1 min, extension at 72 °C for 2.5 min and a final extension for 3 min at 72 °C for proof-reading.

Forward primer for ns*ltp*: 5′-ATG GCT AGC TCT GCA GTG AC-3′
Reverse primer for ns*ltp*: 5′-TAC TTC ACG GTG GCG CAG TT-3′

2.5.5 Agarose gel electrophoresis

1.5 % agarose gel was used for purification of cDNA. An appropriate amount of agarose was melted in 1x TAE electrophoresis buffer for 1 min in a microwave (600 W). After cooling down to ~ 50 °C, solution was poured into the gel cassette. For each 10 µl aliquots of sample 5 µl sample buffer was added and separated at 100 V for 30-45 min in an electrophoresis apparatus (BioRad, Munich, Germany) using 1 x TAE as buffer system. DNA ladder 100 bp (Gene Ruler, Fermentas, Munich, Germany) was used as size standard. Gel was incubated for 30 min in ethidium bromide solution (0.5 µg /ml). Nucleic acids were visualised in UV light.

TAE buffer (50x): 40 mM Tris, 10 mM sodium acetate, 1 mM EDTA, with acetic acid adjust the pH to 7.8.

2.5.6 Purification of PCR products

E.Z.N.A. Cycle-Pure Kit (PeQLab-Biotechnologie GmbH) was used to purify the PCR products. PCR products were purified before cloning to remove any contaminants that may affect the cloning step. To the total volume of PCR products 4-5 volumes of CP buffer were added. Subsequently, 750 µl of the mixture was loaded to the column fitted to a collecting tube and centrifuged at RT for 1 min at 16000 xg. DNA-wash buffer (with 1.5 volumes of ethanol) 750 µl was added to the column and was centrifuged for 1 min at 16000 xg. This step was repeated. In all the centrifugation steps the solution collected in the collecting tubes was discarded. The column was centrifuged again to dryness. The dried column was placed on a sterile reaction tube and 50 µl of sterilized distilled water was added directly on to the membrane of the column, incubated at RT for 2-3 min and centrifuged at 16000 xg to elute DNA.

2.5.7 Cloning of ns*ltp* cDNA in pNEB193

The cDNA of ns*ltp* was cloned in plasmid vector (pNEB193, New England Biolabs). Linearization of the plasmid and ligation were done in one step, by mixing a purified plasmid and PCR products with 1:3 ratio. Then restriction enzyme *Sma*I, T4 ligase, 1x reaction buffer, 0.5 mM ATP and 1 mM DTT were added to the reaction tube. The volume was adjusted to 20 µl by sterile distilled water. Reaction tubes were incubated overnight at 4 °C.

2.5.8 Electrocompetent *E. coli* cells

One ml of overnight culture on LBG medium was inoculated in 250 ml LBG medium, and incubated at 37 °C with shaking. The optical density was measured at 600 nm (0.5 – 0.6). Culture was cooled down in ice for 15 min. After centrifugation for 10 min at 4000 xg, the pellet was resuspended in 5 ml distilled water, and subsequently filled up to 250 ml with distilled water. Centrifugation was done again for 10 min at 4000 xg, and the pellet was resuspended in 250 ml distilled water (this step was repeated once again). Pellet was resuspended in 25 ml 15 % glycerol and centrifugation was

done for 30 min at 4000 xg. Finally the pellet was mixed with 1.5 volume of 15 % glycerol, and 100 µl aliquots were stored at – 80 °C.

LBG (per liter): 10 g NaCl, 10 g tryptone, 5 g yeast extract, 1 g glucose, pH 7.5.

2.5.9 Transformation of *E. coli*

100 µl of electrocompetent cells were mixed with 3 µl ligate in a precooled electroporation cuvette. Electroporation was done in BTX cell at 1.25 kV. After electroporation, 900 µl LB medium without antibiotic were added to cell suspension and incubated for 90 min. Eventually, bacterial cells were cultivated on LBA plates containing appropriate antibiotic for selection.

LBA (per Liter): 10 g NaCl, 10 g tryptone, 5 g yeast extract, 20 g agar, pH 7.5.

2.5.10 Screening the transformed colonies

Several white and as well as blue colonies were picked and subcultured overnight in liquid LB medium in the presence of 100 µg/ml ampicillin. Afterwards, plasmid was isolated from these cultures by using HB-lysis.

2.5.11 HB-lysis and plasmid preparation

For isolation of the plasmid, 1.5 ml of overnight culture was centrifuged for 2 min at 16000 xg. Pellet was resuspended in 300 µl Buffer 1, and another 300 µl Buffer 2 were added. The mixture was incubated for 5 min at room temperature. After mixing with an additional 300 µl Buffer 3, mixture was incubated for 10 min in ice. Afterwards mixture was centrifuged for 10 min at 16000 xg and RT. The supernatant was transferred and recentrifuged for 10 min at 16000 xg. From this supernatant, 800 µl were mixed with 700 µl 2-Propanol and centrifuged for 30 min at 16000 xg. Pellet was incubated with 500 µl cold 70 % ethanol for 2 min subsequently centrifugation was done for 10 min at 16000 xg and RT. After drying the pellet with speed vacuum, pellet was resuspended in 50 µl distilled water. Plasmid DNA was dissolved at 50 °C for 10 min, and stored at – 20 °C.

Buffer 1: 50 mM Tris-Cl pH 8.0, 10 mM EDTA.

Buffer 2: 200 mM NaOH, 1 % SDS.

Buffer 3: 2.55 M K-Acetat pH 4.8 (adjust with acetic acid).

2.5.12 Sequencing

Plasmids which harbouring the right fragments were digested by using restriction enzymes from the MCS of the vector. After selection of the right plasmid, the plasmid preparation was sent to a company for sequencing (Sequence Lab, Göttingen, Germany).

2.6 Northern blot analysis

The total RNA from apple plants was extracted as described in (2.5.1). RNA from mock infected Elstar plants was taken as a control. Leaves from artificially infected plant were harvested at different intervals; 1, 3, 5 days, 1 and 2 weeks. Harvested materials were immediately frozen in liquid nitrogen and stored at -80 °C for RNA extraction. Ten microgram from the total RNA were separated on 1.5 % RNA agarose gel according the description in (2.5.3). The separated RNA was blotted to a nylon membrane as described for southern blot in (2.7.5). Membranes were fixed at 80 °C for 2 h and used after blocking for hybridization or kept in dark at room temperature for later use.

2.6.1 Radioactive labeling

Around 50 ng of PCR products of ns*ltp* extracted by phenol/chloroform were mixed with 10 µl random primer. Mixture was boiled in water bath for 5 min and then centrifuged for few seconds. 10 µl of 5X buffer and 5 µl of radioactive $dCTP^{32}$ were added to the mixture. After addition of 1 µl of Klenow enzyme, reaction tube was incubated for 30 min at 37 °C. The reaction was stopped by adding 2 µl of stop-Mix and followed by 100 µl TNE buffer.

TNE buffer: 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA.

2.6.2 Separation of the labeled probe

Column for separation was prepared by using 1 ml Sephadex G50 in TE buffer and a filter in the bottom with 35 µm for pore diameter. The column was centrifuged at 1500 xg for 5 min in reaction tube and the flow through was discarded. One washing step was done by 100 µl TNE buffer and centrifugation at 1500 xg for 5 min. Labeling reaction was loaded to the column and centrifugation at 1500 xg was done for 5 min in a new reaction tube. The collected sample was immediately used for hybridization.

Prehybridization solution (100 ml): 5 ml 100X Denhard solution, 25 ml 20X SSPE, 2 ml 10 % SDS, 2 ml Salmon sperm DNA (10 mg/ml).
Hybridization solution: 10 ml prehybridization solution, radioactive probe.
100X Denhard: 2 % PVP 10, 2 % BSA (filter sterilized), 2 % Ficoll 400.
20 X SSC: 0.3 M Na-Citrat pH 7.0, 3 M NaCl.
20 X SSPE: 3.6 M NaCl, 0,2 M NaH_2PO_4, 20 mM EDTA, pH 7.4.

2.6.3 RNA-cDNA hybridization

Membranes were incubated with the prehybridization solution for 2h at 60 °C. Hybridization was done with the radioactive labeled probe in 20 ml of hybridization solution at 55 °C overnight. The hybridized membranes were washed two times 50 ml of 2X SSC, 0.2 % SDS at RT for 15 min. Again membranes were washed two times with 50 ml of 1XSSC, 0.2 % SDS.

2.6.4 Autoradiography

Radioactivity signals were detected by overlaying a Kodak® X-Omat LS film on the membrane overnight at -80 °C. T Films were incubated in the developing solution for 1 min and washed by water. Finally membranes were fixed in the fixation solution for 3 min.

2.7 Southern blot analysis

2.7.1 Genomic DNA extraction

Around 2 g of leaf materials from *M*. *domestic*a were harvested and ground in a pre cooled mortar in the presence of liquid nitrogen. The fine powder was transferred

equally into six 2ml tubes. Preheated 800 µl CTAB buffer were added to each tube mixed and incubated for 30 min at 65°C. Eight hundred µl of CI Mix were gently mixed to avoid genomic DNA sharing. Samples were centrifuged for 10 min at 10000 xg. Thereafter the aqueous phase was transferred into a new tube, this step was repeated several times to obtain a clear sample. For DNA precipitation 2/3 volume of a precooled at -20°C isopropanol was added and gently mixed. Afterwards samples were incubated overnight at 4°C. Centrifugation for 10 min at 13000 xg was done. Supernatant was removed and pellet was washed by 200 µl WB. The washing buffer was carefully removed and pellet was resuspended in 200 µl TE buffer supplemented with RNase A (final concentration 10 µg/ml). After incubation for 30 min at 37°C, 100 µl of 7.5 M ammonium Acetate and 750 µl Ethanol were added and gently mixed. At room temperature samples were centrifuged for 10 min at 13000 xg and subsequently the supernatant was completely removed and pellet was resuspended in a suitable volume of sterile distilled water.

CTAB-buffer: 3 % CTAB, 1.4 M NaCl, 0.2 % ß-Mercaptoethanol*, 20 mM EDTA, 100 mM Tris-HCl pH 8,0, 1,0 % PVP-40.
* Add CTAB and ß-Mercaptoethanol after autoclaving.
CI Mix: 24 ml Chloroform, 1 ml Isoamylalcohol.
Wash buffer (WB): 76 % Ethanol, 10 mM Ammoniumacetate.
TE-buffer: 10 mM Tris-HCl. pH 8,0, 1 mM EDTA.
RNase A: 10 mg/ml Stock solution in distilled water.

2.7.2 Genomic DNA digestion

Around 50 µg extracted genomic DNA were digested with restriction enzymes in 500 µl assay for 2 h at 37 °C. Afterwards digestion assay was vortexed at low speed, centrifuge for few seconds and incubated overnight at 37 °C.

2.7.3 Precipitation of digested DNA

Digested DNA was precipitated by adding 50 µl Na-actate pH 5.2 and 800 µl cooled 96 % Ethanol. The mixture was centrifuged for 10 min at 16000 xg. Pellet was washed with 70 % Ehanol at 16000 xg for 5 min. To dry the pellet, tubes were

incubated for 20 min at 37 °C. Dried pellet was dissolved in appropriate volume of TE buffer at 4 °C overnight.

2.7.4 Separation of DNA on agarose gel

Dissolved DNA was mixed with the loading buffer and loaded in 0.8 % agarose. Gel was let to run overnight at 20 mA.

2.7.5 DNA transfer to nylon membrane

The stained DNA separated fragments were denatured by washing two times for 20 min by the denaturation solution with gentle agitation. After washing the gel by distilled water, it was neutralized by washing two times for 20 min with the neutralization solution and again washed by distilled water. The blotting cassette was assembled in the following order; 3 mm filter paper, DNA gel, nylon membrane, three layers of 3 mm filter paper, around 10 cm of tissue papers. The cassette was put on one layer filter paper that was submerged in both ends in 20X SSC solution. Above the tissue papers, around 500 g weight was put to facilitate the capillarity transfer overnight at room temperature. Membranes were fixed at 80 °C for 2 h in oven. Membranes were used immediately for hybridization or kept in dark at room temperature for later use.

Denaturation buffer: 1.5 M NaCl, 0.5 M NaOH.
Neutralization buffer: 1 M Tris-HCl pH 8.0, 2 M NaCl.
20 X SSC: 0.3 M Na-Citrat pH 7.0, 3 M NaCl.

2.7.6 DNA labeling

The ns*ltp* was amplified by PCR and subsequently the products were extracted by phenol/chloroform. Around 1 µg of purified DNA were denatured by boiling in water bath at 95 °C for 10 min and immediately cooled in ice. To the freshly denatured DNA 2 µl of 10X DIG high prime, 4 µl of 5X decamer nucleotides as a random primer and 1 µl of Klenow enzyme were added. The total volume was adjusted to 15 µl by distilled water. Mixture was incubated for 20 h at 37 °C.

2.7.7 PCR DIG labeled probe synthesis

The full length of ns*ltp* including the upstream region was amplified by using PCR DIG probe synthesis Kit (Roche, Penzberg, Germany). By using forward and reverse primers of ns*ltp* (2.5.4) the PCR reaction mixture was prepared according to Kit instructions.

2.7.8 Hybridization

Membranes were placed in the hybridization tubes DNA-side in and incubated with 20 ml of the prehybridization solution for 2 h in rotor oven at 40 °C. Solution was discarded and membranes were incubated overnight with 20 ml of the hybridization solution DIG labeled probe 3:1 PCR DIG probe and Klenow DIG probe in the prehybridization solution at 42 °C. Labeling process was tested by agarose gel, the labeled material should have a larger size than the nonlabeled one.

Prehybridization solution (High-SDS-Formamid-solution) 100 ml: 41 ml 100 % deionized formamid, 16.6 ml 30 X SSC, 5 ml 1 M Sodium phosphate pH 7.0, 20 ml 10 % Blocking solution, 1 ml 10 % N-Laurylsarcosine, 16,5 ml 40 % SDS.
Hybridization solution: DIG labeled probe in prehybridization solution
30 X SSC: 4.5 M NaCl, 0.45 M Na-Citrat.

2.7.9 Visualization of the probe-target hybrids

After hybridization, membranes were washed with 100 ml of 2X SSC/0.1 % SDS two times for 5 min at room temperature. Solution was discarded and membranes were again washed two times with 100 ml of 0.5X SSC/0.1 % SDS for 15 min at 68 °C. Membranes were washed once for 5 min with 100 ml of washing buffer. To quench the membrane background, membranes were incubated with blocking solution for 30 min at room temperature. After discarding of the blocking solution, membranes were incubated with 50 ml Anti-Digoxigenin-AP 75 mU/ ml in blocking solution for 30 min. Membranes were equilibrated with 20 ml of the detection buffer for 5 min. Detection of the membranes was done by distribution of around 20-60 drops (1-2 ml) of CDP-Star diluted 1:100 in detection buffer on a membrane. Membranes were put between

two plastic sheets. After removing the excess of detection solution, plastic sheets were sealed avoiding any air bubbles.

Maleic acid buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5 (adjust with solid NaOH).
Washing buffer: 0.1 M Maleic acid, 0.15 M NaCl; pH 7.5, 0,3 % (v/v) Tween 20.
Blocking solution: 10 X Blocking reagent diluted 1:10 in Maleic acid buffer.
Detection buffer 1 X: 100 mM Tris, 100 mM NaCl, pH 9.5.

2.7.10 Autoradiography

Chemiluminescent signals were detected by overlaying Kodak® X-Omat LS film on the membrane for appropriate time at RT. Films were incubated in the developing solution for 1 min and washed by water. Finally membranes were fixed in the fixation solution for 3 min.

2.8 Determination of ns*ltp* intron/exon structure by PCR

To achieve this goal, a standard PCR reaction was done by using the genomic DNA from different apple cultivars as a template. In one reaction tube 1 µl from diluted DNA, forward and reverse primer and mixture of dNTPs (10 mM) were added and subsequently the whole volume was completed to 26 µl by distilled water. In another reaction tube, 5 µl of 10X PCR reaction buffer and 2 µl *Taq* polymerase were added and volume was filled to 25 µl with distilled water. Contents of both tubes were mixed immediately before launching PCR. The whole reaction was denatured initially for 3 min at 94 °C. The thermocycler was adjusted as follows for 36 cycles: denaturation at 94 °C for 30 sec, annealing at 46-56 °C for 1 min, extension at 72 °C for 1.5 min and a final extension for 3 min sec at 72 °C for proof- reading. Subsequently 5 µl of the PCR reaction were separated on 1.5 % agarose gel, stained in Ethidium bromide and finally analyzed under UV light.

2.9 Upstream regions amplification

The upstream regions in two different apple cultivars (Elstar and Remo) were amplified by using Universal Genome Walker^{TM} Kit, (BD Bioscieces Clontech, USA). The principal of this kit is presented in Figure 2.2. DNA from apple tissue was extracted as described in (2.7.1).

2.9.1 Genomic DNA digestion

Around 2.5 µg genomic DNA were mixed with 8 µl restriction endonulase (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I) in presence of the suitable buffer. The total assay volume was adjusted by deionized water to 100 µl. After incubation of the tubes at 37 °C for 2 h, the mixture was vortexed at low speed for 5-6 sec. Again tubes were incubated at 37 °C overnight (16-18 h). To check the complete digestion of genomic DNA 5 µl were run on a 0.5 % agarose gel.

2.9.2 Genomic DNA purification

To each digestion assay 95 µl phenol 80 % equilibrated to pH 8.0 were added and vortexed at low speed for 5-6 sec. Tubes were spun briefly to separate the aqueous and organic phases. The aqueous phase was transferred into a new tube and the organic phase was discarded. After addition of 95 µl of chloroform, tubes were shortly vortexed and briefly spun. Again the upper layer was transferred into new tube. For DNA precipitation two volumes (190 µl) of ice cold 95 % ethanol and 1/10 volume (9.5 µl) of 3 M NaOAc pH 5.2 were added and vortex was done at low speed for 5-6 sec. Centrifugation was done at 16000 xg for 10 min. Subsequently, supernatant was removed and pellet was washed by centrifugation in 100 µl of ice cold 70 % ethanol for 5 min at 16000 xg. The supernatant was decanted and pellet was air dried and dissolved in 20 µl TE buffer pH 7.5. Vortex at low speed was done for 5-6 sec and 1 µl from the mixture was run on 0.5 % agarose gel to determine the approximate quantity of purified DNA.

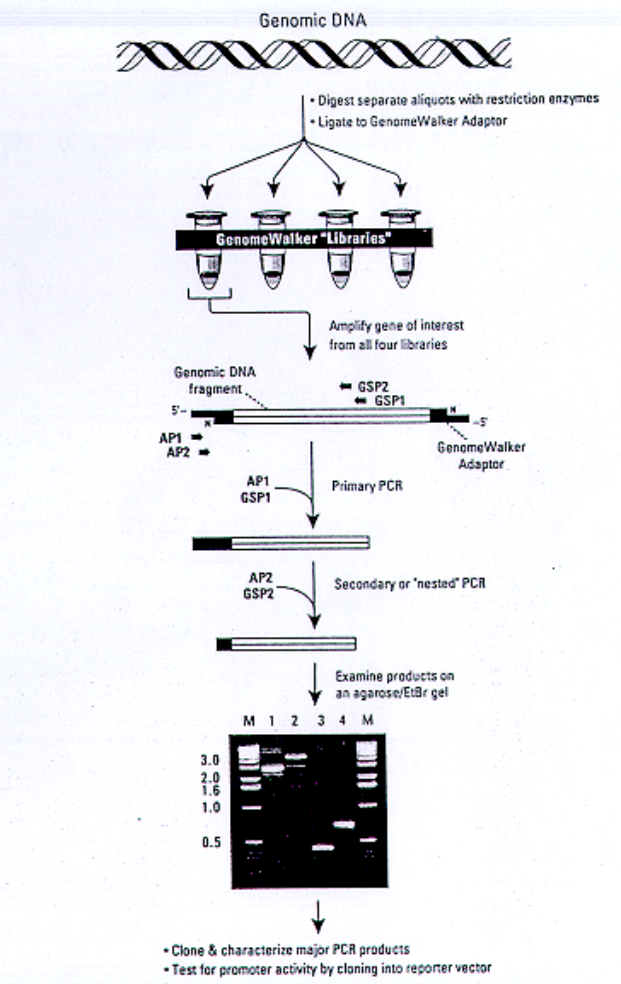

Figure (2.2): Schematic diagram shows principal steps in Genome Walker Kit.

2.9.3 Ligation of genomic DNA to Genome Walker adaptor

To construct each library, 4 µl of digested purified DNA were mixed with 1.9 µl GenomWalker adaptor (25 µM), 1.6 µl 10X ligation buffer and 0.5 µl T4 DNA ligase (6 units/µl). Mixture was incubated at 16 °C overnight. The reaction was stopped by incubation at 70 °C for 5 min.

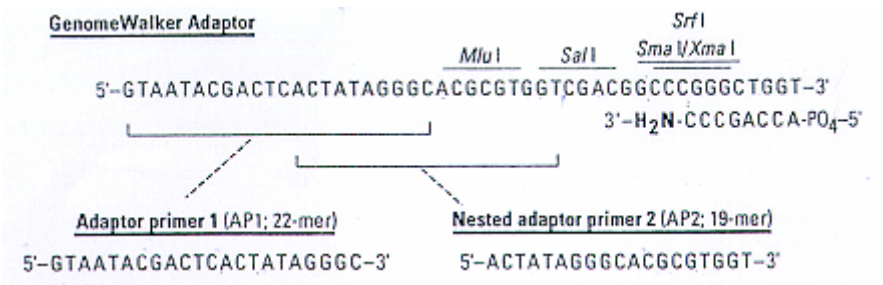

Figure (2.3): Adaptors and adaptors primers sequence used in Genome Walker Kit.

2.9.4 PCR-based DNA walking in GenomWalker libraries

To amplify the upstream region and as well as the down stream region, different primers according to the recommendations provided by the company, two gene specific primers for ns*ltp* (GSP1 and GSP2) were designed by using Primer package. In order to amplify more from the upstream region, the same procedure has been repeated by using the corresponding gene specific primers for upstream II region.

Upstream I GSP1:	5'-CAT GAG CAA CGC TCA CCG CCA TGC ACA A-3'
Upstram I GSP2:	5'-AGC AAG GTT GGT CAC TGC AGA GCT AGC C-3'
Upstream II GSP1:	5'-GAA TAG TCG AAC TAA GGG TAT TGT GGT C-3'
Upstram II GSP2:	5'-CAA CTT TTG TGG CCA CGT TTA CGT GTT T-3'
Downstream GSP1:	5'-CCA GAC TGC TTG CAA CTG CCT GAA GAA T-3'
Downstream GSP2:	5'-CTC CAC CAA CTG CGC CAC CGT GAA GTA G-3'

2.9.4.1 Primary PCR

The primary PCR master mix was prepared as follow:

37.8	µl	deionized H_2O
5	µl	10X *Tth* PCR Reaction Buffer
1	µl	dNTP (10 mM for each)
2.2	µl	Mg $(OAc)_2$ (25 mM)
1	µl	adaptor primer 1 (10 µM)
1	µl	gene specific primer 1 (10 pmole)
1	µl	of each DNA library
1	µl	Advantage Genomic Polymerase Mix (50X)
50	µl	

Tubes were mixed by vortexing without introducing bubbles. The thermocycler (PTC 200, Biozym, Oldendorf, Germany) was programmed as follow:

- 7 cycles

  94 °C 25 sec

  72 °C 3 min

- 32 cycles

  94 °C 25 sec

  67 °C 3 min

- 67 °C for an additional 7 min after the final cycle.

To analyze the primary PCR products, 10 µl were run on 1.5 % agarose gel.

2.9.4.2 Secondary PCR

The secondary PCR master mix was prepared as follow:

39	µl	deionized H_2O
5	µl	10X PCR Reaction Buffer
1	µl	dNTP (10 mM for each)
1	µl	adaptor primer 2 (10 µM)
1	µl	gene specific primer 2 (10 pmole)
1	µl	Each DNA library
2	µl	*Taq* polymerase
50	µl	

Tubes were mixed by vortexing without introducing bubbles. The thermocycler was programmed as in the primary PCR. Ten µl of the PCR products were analyzed on 1.5 % agarose gel.

2.9.4.3 PCR products cloning and sequencing

The obtained fragments by secondary PCR were immediately ligated in pGEM T-vector. Three µl of the PCR products were mixed with 1 µl pGEMT-vector and 1 µl of T4-DNA ligase in presence of ligation buffer. The ligation assay was incubated overnight at 4 °C. From this ligation 3 µl were used to transform *E. coli* strain XL1-Blue and subsequently the blue-white screening was done as described in (2.5.10). To confirm the positive transformed colonies, plasmid was isolated and digested by *Nco*I and *Not*I and subsequently the right plasmid was sent for sequencing.

2.10 Promoter activity test

2.10.1 PCR amplification of the upstream region

The forward and reverse primers* were designed containing *Bam*HI and *Eco*RI restriction sites. Genomic DNAs of Elstar and Remo were used as a template for PCR. The essential elements for promoter were amplified as fragments with the size 351 bp and 357 bp for Elstar and Remo respectively. The amplified fragments were purified from agarose gel as described in (2.5.6). The purified upstream regions were cloned in pGEMT vector as intermediate step. After blue white screening, the right clones were digested with *Bam*HI and *Eco*RI.

*Elstar forward primer:	5′ TCC CTT AGA ATT CAA AAT AG 3′
*Elstar reverse primer:	5′ AAA AGC TTA AAA AGG ATC CG 3′
*Remo forward primer:	5′ GGG CTG GTC CTC GAA TTC AC 3′
*Remo reverse primer:	5′ GTG TAT GAG TAA TGG ATC CG 3′

2.10.2 Insertion of the upstream region in front of DsRed marker gene

The vector pe35AscloptRed containing the DsRed gene (kindly provided by Prof. Dr. E. Maiß) was digested by *Bam*HI and *Eco*RI to remove the double 35S promoter. The digested upstream regions from pGEMT vector were ligated with the linerazied pe35AscloptRed vector by using T4-DNA ligase. Transformation of *E. coli* strain was done by using 3 µl from the ligation assay and electroporation at 1.25 kV. The right clones were selected and used for particle bombardment and transient expression in apple leaves.

2.10.3 Negative control plasmid

For negative control preparation, the double 35S promoter was removed from the pe35AscloptRed vector by digestion with *Bam*HI and *Eco*RI. The linear plasmid was purified from agarose gel. To religate the linear plasmid, the two sticky ends were filled by treatment with Klenow enzyme in the presence of dNTPs for 30 min. Klenow enzyme was inactivated by incubation of the reaction assay for 15 min at 85 °C. The

treated linear plasmid was incubated overnight at 4 °C with T4-DNA ligase in presence of 5X ligation buffer containing PEG, ATP and DTT. After transformation, the right plasmid was selected and used as a negative control.

2.11 Subcellular localization of nsLTP

2.11.1 Overexpresion of ns*ltp* in *E. coli*

2.11.1.1 Amplification of ns*ltp*

To amplify the ns*ltp*, the vector pNEB 193 harboring the ns*ltp* was used as a template for PCR by forward and reverse primers containing an *Eco*RI site for the direct integration into the pMal c2X expression vector. Subsequently PCR products were purified as described in (2.5.6).

2.11.1.2 Cloning of ns*ltp*

The vector pMAL-c2X was lineraized by using *Xma*I to insert ns*ltp* downstream from the maIE gene, which encodes for maltose binding protein (MBP). PCR product with blunt ends was ligated with the linear pMAL-c2X by using T4-DNA ligase in presence of DTT, ATP and PEG. 3 µl from the ligation assay were used to transform Epicurian Coli® BL21-CodonPlus™ (DE3)-RIL (Stratagene, Netherlands). Blue-white screening and digestion were done to select the right clone.

2.11.1.3 Insert orientation

Since the PCR amplified ns*ltp* has blunt ends, it was necessary to confirm the orientation of the gene in the expression vector. To prepare sample for sequence, around 3 µg of plasmid DNA in reaction tube were dried in a speed vacuum. Tubes was closed, labeled and sent for sequence. Retrieved sequence was checked by computer program to determine the orientation.

2.11.1.4 Expression of ns*ltp* in *E. coli* BL21-CodonPlus™ (DE3)-RIL

The ns*ltp* was expressed in fusion to maltose binding protein as inclusion bodies in the cytocol. According to the protocol provided by the company, 10 µl from *E. coli* stock culture stored at -80 °C were inoculated in 10 ml LB medium (containing 100

µg/ml ampicillin and 34 µg/ml chloramphenicol) and incubated overnight at 37 °C. In the second day 2.5 ml from the overnight culture were centrifuged and the supernatant was discarded. Pellet was resuspended in 1 ml NZY medium containing the two antibiotics. This 1 ml was inoculated in 250 ml NZY medium containing the same antibiotics and incubated at 37 °C. The OD was measured at several intervals at 600 nm until it reached to 0.5. Subsequently the expression system was induced by addition of 1 mM IPTG and the culture was incubated at 37 °C overnight.

NZY Broth (per liter): 5 g NaCl, 2 g $MgSO_4.7H_2O$, 5 g yeast extract, 10 g NZ amine (casein hydrolysate), pH 7.5.

2.11.1.5 Bacteria harvest

Bacterial culture was centrifuged for 15 min at 6000 xg. Pellet was resuspended in 20 ml 20 mM Hepes pH 7.0. To minimize the damage of protein contents those released from bacterial cell by chemical treatment, bacterial suspension was passed through the French Press two times at 20000 psi. To remove the cell debris the solution was centrifuged for 15 min at 900 xg. The supernatant was again centrifuged for 30 min at 10000 xg and dialyzed overnight at 4 °C against dialysis buffer containing 20 mM HEPES, 1mM ß-mercaptoethanol and PMSF.

2.11.1.6 Chromatography and elution

Column with 1 cm diameter was filled (around 6 cm) with amylase resin and equilibrated with the dialysis buffer. Subsequently, the supernatant was loaded to the equilibrated column and let to flow through. Protein concentration was measured by photometer. The flow through was collected and stored at -20 °C. Afterwards column was washed three times by dialysis buffer. The expressed protein was eluted by application of 10 mM maltose and 2 ml fractions were collected and stored at -20 °C.

2.11.1.7 Antisera production against the recombinant protein

A polyclonal antiserum against the recombinant protein (nsLTP in fusion with the MBP) was raised in goat as described in Singh et al., 2005. An appropriate amount of the recombinant protein (500 µg) was dialyzed against 10 mM sodium phosphate

buffer (pH 7.0) in the presence of 0.05 % SDS. After dilution with an equal volume of Freund's complete adjuvance, the emulsion was divided into two equal parts and injected subcutaneously into a goat and after three weeks into the hingleg muscles. The bleeding was has been done after the second immunization by three weeks.

2.11.1.8 Determination the produced antibody specificity

To determine the specificity of the produced antibody that was raised in goat against the recombinant protein nsLTP in fusion with the maltose binding protein (MBP), IWF samples from young and old healthy apple leaves were collected and used for western blot. The procedure was done as described in 2.4 with some modifications including the use of antigoat peroxidase (with dilution 1:20000) as a second antibody, development of the membrane. For development membranes were incubated with 5 ml mixture 1:1 of solutions A and B for 1 minute. The membrane was dried and the light emission was detected by using a CCD-camera.

Solution A: 1 ml 250 mM Luminol in DMSO, 0.44 ml 91 mM p-cumaric acid in DMSO, 10 ml 1 M Tris pH 8.5 fill up to 100 ml with distilled water.

Solution B: 10 ml 1 M Tris pH 8.5, 61 µl 30% H_2O_2 fill up to 100 ml with distilled water.

2.11.2 Transient expression of ns*ltp* in fusion with GFP

2.11.2.1 N-terminal fusion of the ns*ltp* with GFP

The vector pBSK that harboring the EGFP gene was kindly provided by PD Dr. Jutta Papenbrock. Two clones were provided, one as an N-terminal fusion and the other one as a C-terminal fusion. The cassette for N-terminal fusion was as follow –*Hind*III-2x35S promoter- "MCS"- GFP- polyA- EcoRI- in pBSK. On the other hand the cassette for C-terminal fusion was as follow; -*Hind*III-2x35S promoter-GFP-"MCS"-polyA-EcoRI- in pBSK. In both cases the MCS was having *Bgl*II and *Nco*I as a restriction sites (Figure 2.4).

2.11.2.1.1 PCR amplification of ns*ltp*

To amplify the full length of ns*ltp*, two specific primers* with *Nco*I and *Bgl*II restriction sites were designed by using Primer package. To enhance the activity of the cleavage close to the end, we added additional nucleotides around the restriction sites to have the activity of 90 % for *Bgl*II and 75 % for *Nco*I. PCR was performed and subsequently PCR products were used for further steps.

* ns*ltp* full length forward primer:	5' GGT ACC CAT GGC TAG CTC TG 3'
* ns*ltp* full length reverse primer:	5' CCA GAT CTC ACG GTG 3'
* ns*ltp* mature forward primer:	5' GTT GCC ATG GCC ATA AC 3'
* ns*ltp* mature reverse primer:	5' CCA GAT CTC ACG GTG 3'
* ns*ltp* truncated forward primer:	5' GGT ACC CAT GGC TAG CTC TG 3'
* ns*ltp* truncated reverse primer:	5' GGT AGA TCT GCT CTA CTT GTA GG 3'

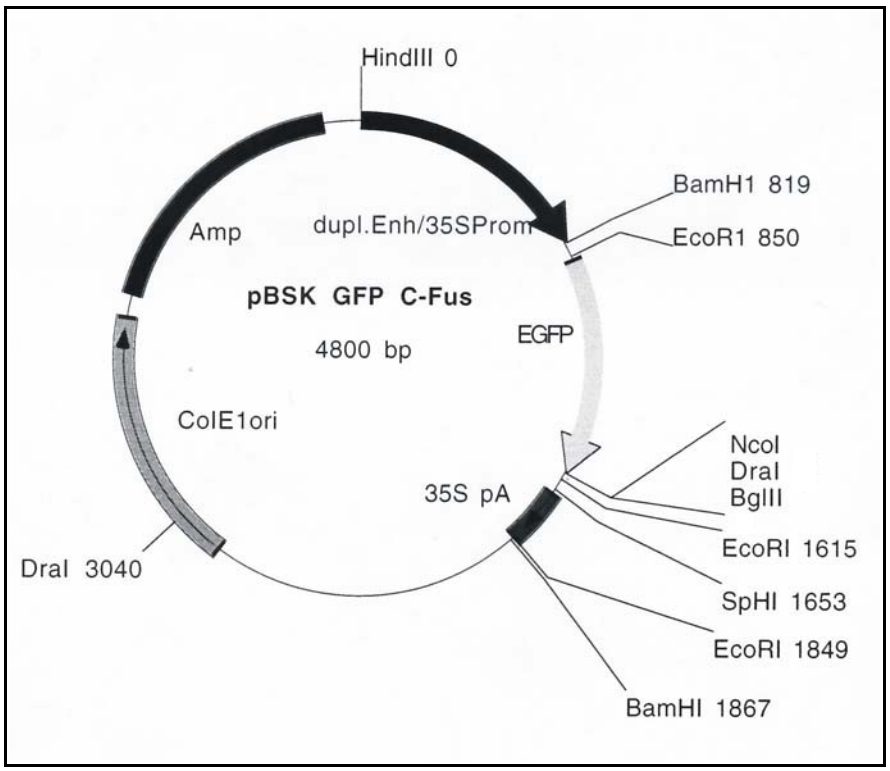

Figure (2.4): Map of Bluescript vector with EGFP under the control of double 35S promoter. This vector has been used for C-terminal fusion with EGFP gene.

2.11.2.1.2 Cloning of ns*ltp* in pBSKGFPN-terminal

To facilitate this cloning step an intermediate step was introduced, 3 µl of pure PCR fragment were ligated in pGEMT-vector in presence of ligation buffer and T4 DNA ligase. Ligation assay was incubated overnight at 4 °C.
An appropriate volume of the ligation assay (3 µl) was used for transformation of XL1-Blue as descried in (2.5.10). After blue-white screening, the right colony was cultivated overnight in LB medium with ampicillin 100 µg/ml at 37 °C. Plasmid preparation was done according to (2.5.11).

2.11.2.1.3 Vector backbone and ns*ltp* purification

The vector pBSKGFPN-terminus clone and as well as the pGEMT-vector that harbors the ns*ltp* were digested with *Nco*I and *Bgl*II in the presence of 2X of Tango^{+} buffer (Fermentas, Munich, Germany). To purify the linear vector and ns*ltp* from the digestion assay, the corresponding DNA fragments were extracted by Gel Extraction kit (The Geneclean II® Kit Bio 101 Inc.). DNA fragments were excised from ethidium bromide-stained agarose gel with a sharp scalpel under long-wave UV light. One volume of gel was melted in three volumes of 5 M NaI solution at 50 °C for 5 min. The solution was incubated with 10 µl of glassmilk suspension for 5 min with gentle shaking. Suspension was centrifuged for 5 sec and supernatant was removed. Pellet was washed three times by 700 µl of ice cold NEW WASH. Eventually to elute DNA, pellet was resuspended in 20 µl sterile distilled water and incubated at 55 °C for 2 min. Centrifugation for 30 sec was done and supernatant was carefully collected.

2.11.2.1.4 N-terminal ligation of ns*ltp* with GFP and transformation of *E. coli*

The right clone of pGEMT-vector containing ns*ltp* was digested with *Bgl*II and *Nco*I. Fragment corresponds to ns*ltp* was purified from agarose gel. A ligation assay was prepared, with tacking into consideration the ratio between the backbone and the insert (1:3). Transformation and screening were done.

2.11.2.2 C-terminal fusion of the ns*ltp* with GFP

In this case, PCR products of ns*ltp* were precipitated and purified as described in (2.5.6). Purified products were directly subjected to enzymatic digestion overnight with *Nco*I and *Bgl*II in the presence of 2X of 10X Tango^{+} (Fermentas, Munich, Germany) at 37 °C. The digestion assay was separated on 1.5 % agarose and band was excised from the gel and DNA was extracted using sigma kits. Column was firstly equilibrated by 100 µl sterile distilled water and centrifugation for 5-6 sec. Water was collected and removed from the tube. The excised agarose was loaded to the column and immediately centrifugation was done for 10 min at maximum speed. Diluted collected DNA was concentrated by speed vacuum.

2.11.2.3 Preparation of samples for sequencing

After selection of the right clone, a plasmid preparation was done for this clone. The purified plasmid was subjected to enzymatic digestion by using *Nco*I and *Bgl*II. After confirmation of ns*ltp* presence in the construct via enzymatic digestion, around 3 µg of the undigested purified plasmid were dried in a speed vacuum in 1.5 ml tube. On the other hand, around 120 pmole of GFP primer* were also dried by the manner. The tubes were labeled and closed by parafilm and finally sent for sequencing.

***GFP primer for sequence**

GFP reverse primer for GFPN-terminal fusion:

5'-GCT TGC CGT AGG TGG CAT CGC CCT-3'

GFP forward primer for GFPC-terminal fusion:

5'-CTG GAG TTC GTG ACC GCC GCC GG-3'

2.11.2.4 Particle bombardment preparations

Plant materials

Leaves from different cultivars of apple *Malus domestica*. cv. Elstar, Holsteiner Cox and Gloster were harvested from greenhouse and *in vitro* plants for testing the efficiency of transient expression.

2.11.2.4.1 Gold particle solution (Micro-carrier) preparation

Forty mg from gold particle (Bio-Rad) with a diameter of 0.4 to 1.2 µm were suspended in 1 ml 96 % Ethanol and centrifuged for short time. Supernatant was removed and gold particles were washed two times in 1 ml 96 % Ethanol and once in 1ml of distilled water. Finally gold particles were resuspended in 1 ml sterile distilled water and divided into 50 µl aliquots and stored at -20°C.

2.11.2.4.2 Coating gold particles with DNA

One tube of gold particles was thawed and Ultra-sonicated for 10 min. Appropriate amounts of plasmid DNA (2-5 µg) were added, and immediately vortexed very well was done to ensure a good contact in between DNA and particles. Drop by drop an aliquot of $CaCl_2$-Solution was added. Promptly one sperimidine aliquot was added and the solution was mixed by vortexing for 1 min. After short centrifugation for 5-6 sec, the supernatant was removed and subsequently particles were washed two times with 250 µl 96 % Ethanol. Eventually particles were resuspended in 85 µl 96 % Ethanol and stored at 4°C.

$CaCl_2$-Solution

0.3 M $CaCl_2$-Solution was prepared in distilled water and autoclaved. 50 µl aliquots were stored at -20°C.

Spermidine

A spermidine solution with a concentration 0.1 M was prepared in sterile distilled water as a stock solution. Then aliquots of 0.01 M in 20 µl were stored at -80°C.

2.11.2.4.3 Plasmid preparations

Plasmid was purified from *E. coli* XL1-Blue strain via QIAGEN or HB-Lysis preparations, in case of HB-lysis plasmid preparation was sometimes treated with RNAase and sometimes not.

2.11.2.4.4 Media for transient expression of ns*ltp* in fusion with GFP

0.5 % plant agar was dissolved in distilled water and autoclaved. Plant agar was transferred into Petri dishes and let to solidify. Plates were stored at 4°C.
Sometimes wetted filter papers were used instead of plant agar.

2.11.2.4.5 Macro-carrier preparation

Macro-carriers and macro-carrier stainless steel holders were sterilized by soaking in 96 % Ethanol for short time. Subsequently they were transferred to filter papers and covered with Petri dish lids for drying. After drying the macro-carriers were fitted into their stainless steel holders. On the other hand the gold particles again were sonicated for 3 min. From the upper part of the sonicated particles 5 µl were taken, and put in the middle of the macro-carrier. The macro-carriers were let for 5-10 min for drying before bombardment.

2.11.2.4.6 Particle-gun shooting

PDS-1000/He Biolistic®, Particle Delivery System from Bio-Rad was cleaned by 70% Ethanol before use. A rupture disk with 1350 psi was loaded in the equipment. The macro-carrier launch assembly was assembled by first, laying in place a stopping screen followed by an inverted macro-carrier holder, which is held by screwing on the launch assembly lid. The launch assembly was slit into place immediately below the helium nozzle. Opened Petri dish containing the target, centered, lower side up and fixed with plastic stencil leave, was slit onto the third shelf at 6 cm. The chamber was closed and the vacuum was pulled until it reached 27-28 inches of Hg, then the vacuum was held. The gun was fired until the rupture disk was ruptured, afterwards the chamber was vented. Plates containing the bombarded leaves were transferred and incubated for 48 h in the growth chamber. For the next shooting, the ruptured disk, macro-carrier and stopping screen were replaced.

2.11.2.4.7 Microscopy

After 48 h incubation, a part of the leaf was transferred from Petri dish, placed on a glass slide and subsequently covered with 50 % glycerol and then covered with glass cover. Slides were examined by using excitation filter 565/30 nm, beam splitter 585 nm and emission filter 620/60 nm (see table 2.2).

Table (2.2): Filter system for the reporter proteins; filter 09 (487909 – 0000) ZEISS, was used for GFP and 41021 for DsRed.

Filter system	GFP (filter 09) nm	DsRed (filter 41021) nm
Excitation Filter	BP 450-490	HQ 565/30
Beam Splitter	FT 510	Q 585 lp
Emission Filter	LP 520	GQ 620/60

2.12 Methylation anlysis of apple ns*ltp*

The methylation pattern of ns*ltp* was detected via methylation sensitive restriction enzymes and southern blot**.** Around 70 µg genomic DNA of different Elstar and Remo samples were used for southern blot using *Msp*I (methylation insensitive) and its isoschizemer *Hpa*II (methylation sensitive) and *Ava*II. The whole procedure was done as described in (2.7). Only the purification step for digested genomic DNA was done by using phenol/chloroform as described in (2.9.2).

2.13. Bioinformatics tools

Different bioinformatics tools were used in this study for DNA analysis, including DNASTAR, Clonemanger and Primer packages. The retrieved cDNA sequence was submitted to the NCBI database http://www.ncbi.nlm.nih.gov/ for homology search and vector contamination detection. The net sequences were used for promoter predictions by using different packages available. The retrieved sequence of cDNA was analyzed by using DNASTAR program. An open reading frame (ORF) was found via CLONEMANAGER program. This ORF was translated, and the deduced amino

acids were used for homology research in the database. Alignment of deduced amino acids with different amino acids sequence in different plant species was done by DNASTAR program. Protein similarity among several plant species has been done by using multiple alignment tools ClustalW available in EMBL http://www.ebi.ac.uk/clustalw/#. The amino acids sequence was used to predict the protein localization site according to the rules described by von Heijne (1983) by PSORT program available in http://psort.ims.u-tokyo.ac.jp/form.html. In order to predict the possible phosphorylation sites, deduced amino acids sequence was submitted to the NetPhos 2.0 prediction server (Blom et al., 1999; http://www.cbs.dtu.dk/services/NetPhos/). For the upstream regions analysis we used promoter prediction program that was available in http://www.fruitfly.org, and http://genes.mit.edu. To determine the cis-acting regulatory elements those distributed in the upstream regions we analyzed these sequences by using the PlantCARE database available at http://intra.psb.ugent.be:8080/PlantCARE/.

3 Results

3.1 nsLTP in the apoplastic fluid of the resistant apple cultivar Remo

It has been shown previously that nsLTP declined to undetectable level in the apple scab susceptible cvltivar Elstar, therefore It was necessary to confirm whether the resistant apple cultivar Remo contains the nsLTP in its apoplastic fluid or not. The IWF from young leaves of healthy plants of *M*. *domestica* cv. Elstar and Remo was collected by infiltration/centrifugation technique (Hogue and Asselin 1987). The IWF was collected from the leaves by using water and as well as PBS as extraction buffer. SDS PAGE analysis of these four samples (Figure 3.1) revealed that the level of nsLTP in the IWF of the resistant cultivar is much less present, in comparison with the susceptible cultivar in the case of using PBS for IWF collection. The result showed that the level of nsLTP was very high in the susceptible cultivar Elstar when water was used to collect the IWF in comparison with water. On the other hand the putative nsLTP band in the resistant apple *M*. *domestica* cv. Remo was not detected when the PBS was used to collect the IWF. To confirm the SDS PAGE analysis, the putative nsLTP band in Remo sample was excised from gel and prepared for sequence by mass spectrometry.

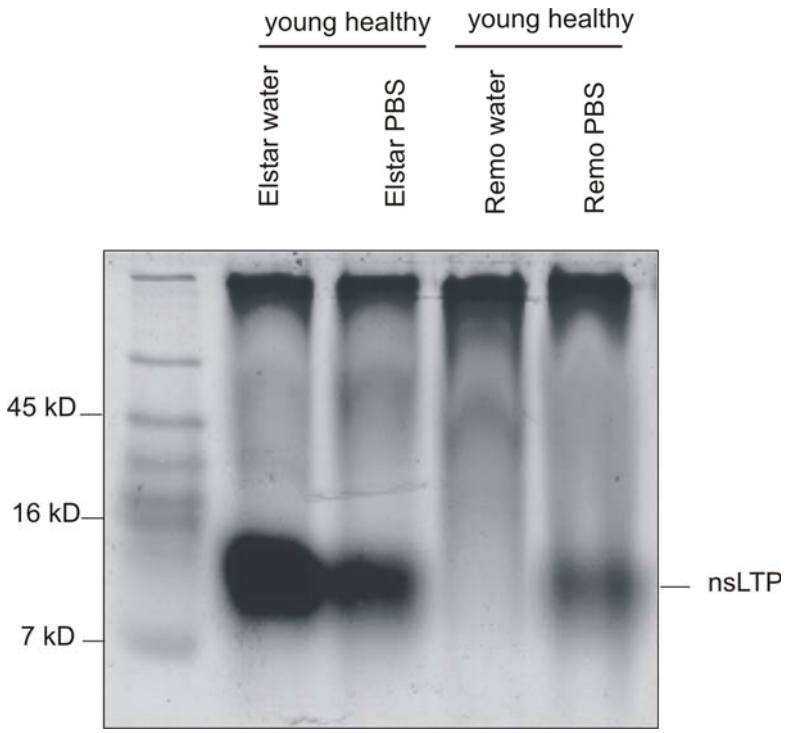

Figure (3.1): separation of IWF samples on SDS PAGE; IWF from healthy *M. domestica* cv. Elstar and Remo young leaves extracted by water and PBS buffer. Each lane was loaded with 50 µg protein based on Bradford determination.

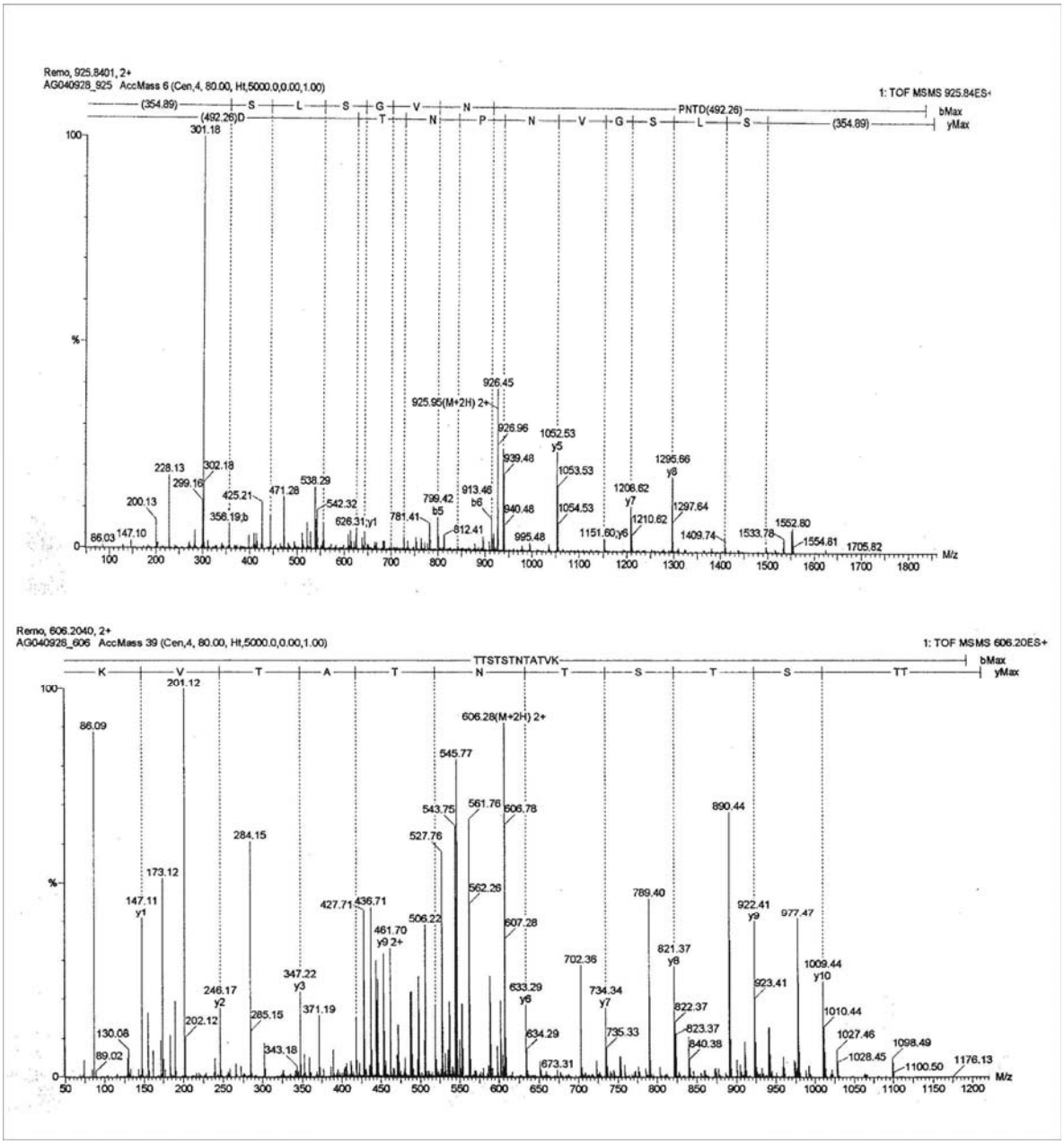

Figure (3.2): Corresponding amino acids sequences which retrieved from mass spectrometry analysis.

The ESI Q-TOF mass spectroscopy analysis revealed that the *de novo* sequence of the tryptic fragments from a 9 kD band from the apoplastic fluid of the resistant apple cv. Remo has significant homolgy to the nsLTP from *M*. *domestica* (Table 3.1).

Table (3.1): ESI Q-TOF mass spectroscopy of tryptic peptide fragments and identification of the derived peptide sequences from cv. Remo by homology search. § NCBI accession number; # Swiss Prot accession number.

Charge	Mass [D]	Derived amino acid sequence	Sequence similarity	Organism	Accession number
2+	925.84	SLSGVNPNTD	Mald3	*Malus domestica*	AJ277164.1§
2+	606.2	TTSTSTNTATVK	nonspecific lipid transfer protein	*Malus domestica*	Q9M5X7#

3.2 Isolation of cDNA of apple ns*ltp* by RT-PCR

3.2.1 Cloning and sequencing

By using total RNA preparation from young uninfected leaves and the corresponding primers in RT-PCR, we could amplify a fragment of cDNA from the susceptible apple *M. domestica* cv. Elstar and the resistant one Remo with a size approximately 350 bp (Figure 3.3). For amplification of this fragment in *E. coli*, fragment was purified and cloned in pNEB 193 vector. After cloning the cDNA of ns*ltp*, the right plasmid was purified and sent for sequencing. The retrieved sequences were analyzed by using different programs.

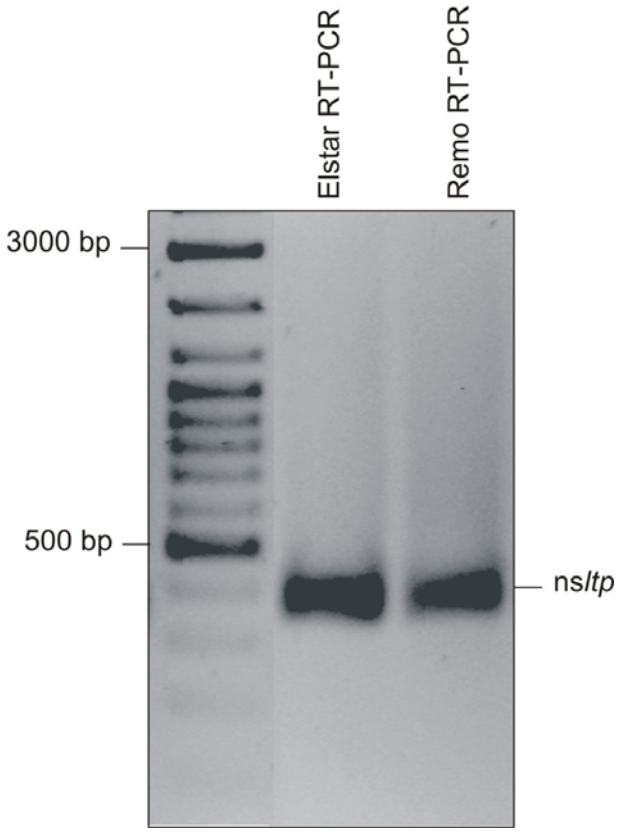

Figure (3.3): ns-*ltp* cDNA on 1.5 % agarose gel after RT-PCR from total RNA of *M. domestica* cv. Elstar and Remo.

```
  1   atggctagct ctgcagtgac caagcttgct ttggtggtgg ccttgtgcat
        M  A  S   S  A  V   T  K  L  A   L  V  V   A  L  C

 51   ggcggtgagc gttgctcatg ccataacatg tggccaagtg accagcagcc
      M  A  V  S   V  A  H   A  I  T   C  G  Q  V   T  S  S

101   ttgcgccatg cattggctac gtgaggagtg gcggagctgt ccctccagct
       L  A  P   C  I  G  Y   V  R  S   G  G  A   V  P  P  A

151   tgctgcaatg gaatcagaac cattaacggc ttggccagga ccaccgctga
        C  C  N   G  I  R   T  I  N  G   L  A  R   T  T  A

201   ccgccagact gcttgcaact gcctgaagaa tcttgccggc agcatcagtg
      D  R  Q  T   A  C  N   C  L  K   N  L  A  G   S  I  S

251   gtgttaaccc taacaatgca gcagggcttc ctggaaagtg tggagtcaac
       G  V  N   P  N  N  A   A  G  L   P  G  K   C  G  V  N

301   gtcccctaca agatcagcac ctccaccaac tgcgccaccg tgaagtaa
        V  P  Y   K  I  S   T  S  T  N   C  A  T   V  K  -
```

Figure (3.4): Nucleotide sequence of *M*. *domestica* cv. Elstar cDNA and deduced amino acid sequence of ns*ltp*. The putative signal peptidesare underlined.

```
  1   atggctagct ctgcagtgac caagcttgct ttggtggtgg ccttgtgcat
        M  A  S   S  A  V   T  K  L  A   L  V  V   A  L  C

 51   ggcggtgagc gttgctcatg ccataacatg tggccaagtg accagcagcc
      M  A  V  S   V  A  H   A  I  T   C  G  Q  V   T  S  S

101   ttgcgccatg cattggctac gtgaggaatg gcggagctgt ccctccagct
       L  A  P   C  I  G  Y   V  R  N   G  G  A   V  P  P  A

151   tgctgcaatg gaatcagaac cattaacagc ttggccagga ccaccgctga
        C  C  N   G  I  R   T  I  N  S   L  A  R   T  T  A

201   ccgccagact gcttgcaact gcctgaagaa tcttgccggc agcatcagtg
      D  R  Q  T   A  C  N   C  L  K   N  L  A  G   S  I  S

251   gtgttaaccc taacaatgca gcagggcttc ctggaaagtg tggagtcaac
       G  V  N   P  N  N  A   A  G  L   P  G  K   C  G  V  N

301   gtcccctaca agatcagcac ctccaccaac tgcgccaccg tgaagtaa
        V  P  Y   K  I  S   T  S  T  N   C  A  T   V  K  -
```

Figure (3.5): Nucleotide sequence of *M*. *domestica* cv. Remo cDNA and deduced amino acid sequence of ns*ltp*. The putative signal peptides are underlined.

3.2.2 Sequencing evaluation of ns*ltp*

An open reading frame was found that contains 349 bps and encodes a protein with a calculated molecular mass of 11.4 kD and an isoelectric point 8.79. Homology search in database revealed that the amino acid sequence showed homology to nonspecific lipid transfer protein of *M. domestica* cv. Golden Delicious, NCBI accession number AF221502 with a similarity 100 %. Amino acid sequence of ns-LTP exhibits conservation of eight cysteine residues and absence of tryptophan residue. PSORT analysis showed that the possible cleavage site of the ns-LTP of *M. domestica* cv. Elstar and Remo could be located after amino acid number 24 (von Heijne 1983). Moreover, PSORT analysis revealed that the precursor of ns-LTP does not contain H/KDEL (endoplasmic reticulum retention signal) site. This analysis showed that the probability of the localization site is 82 % outside the plasma membrane. After cleavage of the signal peptide of ns-LTP, the mature protein has a predicted molecular mass 9 kD and a calculated isoelectric point 8.9. The isoelectric point of ns-LTP is not affected by removing the 24 amino acids of the signal peptides.

3.2.3 Alignment of the deduced amino acid sequences from Elstar and Remo

The deduced amino acid sequences from the susceptible apple *M*. *domestica* cv. Elstar and from the resistant cultivar Remo as ell as from cultivar Golden delicious were aligned by using DNASTAR program. Alignment result shows that amino acid sequence of Elstar nsLTP is identical to that of Golden delicious. However there are two substitutions have been found within the 115 amino acid residues in the resistant cultivar Remo. The first substitution is the serine residue in Elstar has been substituted by asparagine in Remo at position 43. The second substitution is the amino acid residue glycine in Elstar has been changed to serine in Remo at 60 (Figure 3.6).

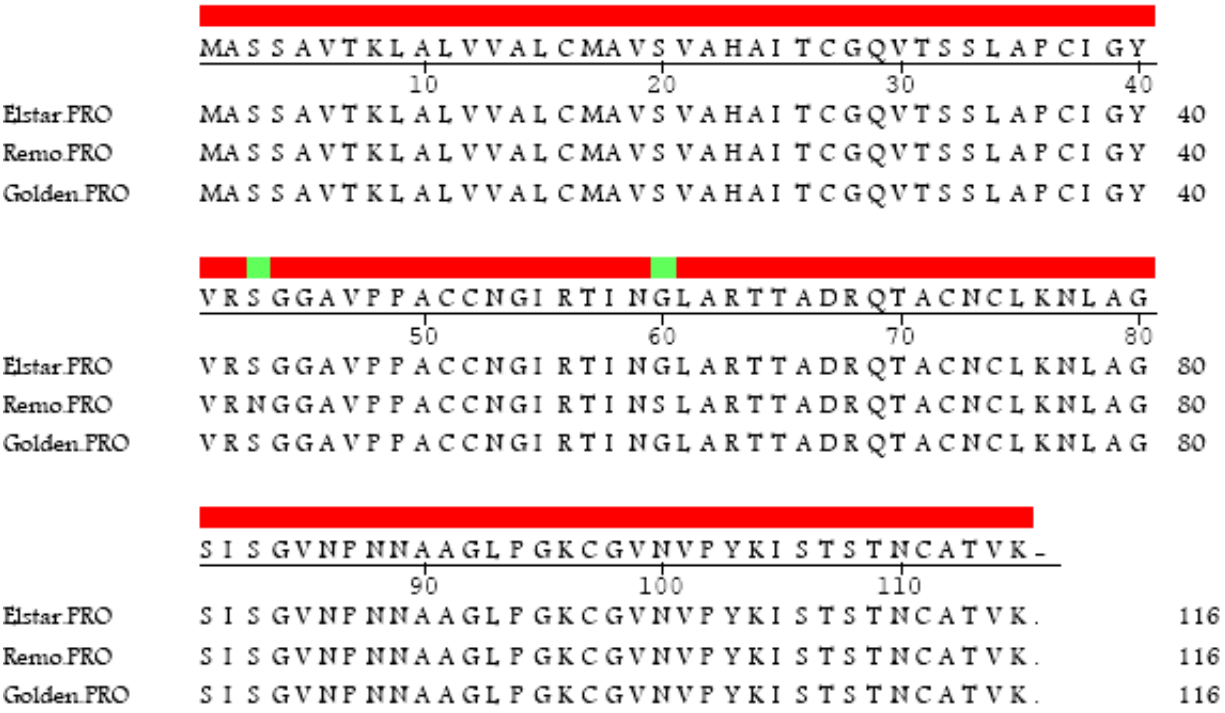

Figure (3.6): Alignment of the deduced amino acid sequences of nsLTP from three different apple *M*. *domestica* cv. Elstar, Remo and Golden delicious. Green colour indicated the substitution.

3.3 Apple ns*ltp* is an intronless gene in different apple cultivars

By the amplification of the cDNA of ns*ltp*, one can not decide whether this gene contains an intron or not. To determine the structural pattern of ns*ltp*, the genomic DNA of different apple cultivars was extracted and used as a template for PCR. Amplification of ns*ltp* was done by using the same primers that have been used in RT-PCR. The amplified fragment in all of the tested cultivars has the same size of the cDNA that was amplified by RT-PCR with approximately 350 bp (Figure 3.7). This result indicates that apple ns*ltp* in *M. domestica* cv. Elstar, Gloster, Holsteiner Cox and Remo has one exon with one open reading frame encoding for 115 amino acids therefore it is an intronless gene.

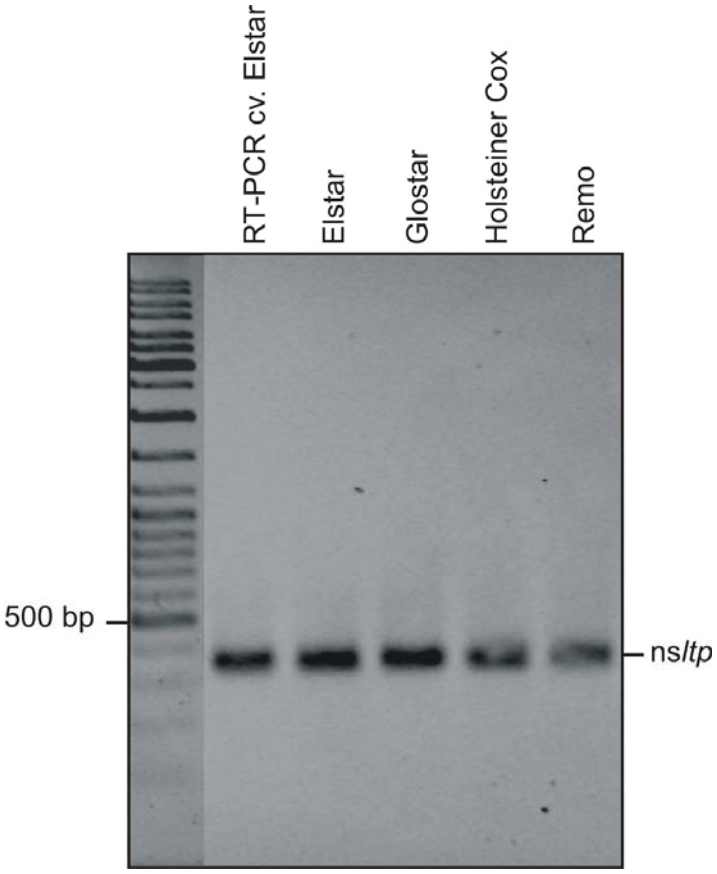

Figure (3.7): Amplification of ns*ltp* from cDNA in *M. domestica* cv. Elstar and from genomic DNA

3.4 Homology search of the nucleotide sequence of ns*ltp*

The nucleotide sequence of the retrieved cDNA was submitted with different sequences of the cDNA of plant lipid transfer protein to determine the degree of homology among different plant species. Multiple alignments of the DNA sequences were done by using ClustalW program available in the EMBL. Several plant species have been chosen for this analysis in addition to the apple *M. domestica cv*. Elstar and Remo. Results revealed that the plant ns*ltp* within these species has a size ranging from 348 bp to 366 bp. (Figure 3.8).

```
Brassica        ---------ATGGCTGGTCTAATGAAGTTGGCATGCTTGATC---TTCGCCTGCATGAT- 47
Corylus         ---------ATGGGTAGCCTTA---AGTTGGTATGCGCGGTC---CTCTTGTGCATGAT- 44
Phaseolus       ---------ATGGCCACCCTCA---ACTCTGCGTGCGTCGTTGCCGTGCTGTGCTTGGT- 47
Vigna           ---------ATGGCCAGCATCA---AGTGTGCGTGCGTCGTTGCCCTCCTGTGCTTGGT- 47
Retama          ---------ATGGCAAGCATCA---AGGTTGCATGTGTGGTT---CTGATATGCATGGTT 45
Citrus          ---------ATGGCTGCCCTCA---AGCTTGTTTCAGCTTTG---GTTCTGTGCATGTT- 44
Davidia         ATGGGTAGGTCAGGAGTGGTGATTAAGGTGGGTATTGTGGCGGTGCTGATGTGCATGGT- 59
Vitis           ATGGGTAGCTCCGGAGCTGTGA---AGCTAGCTTGTGTGATGGTG---ATATGCATGGT- 53
cv. Elstar      ATGGCTAGCTCTGCAGTGACCA---ACCTTGCTTTGGTGGTGGCCT---TGTGCATGGC- 53
cv. Golden      ATGGCTAGCTCTGCAGTGACCA---AGCTTGCTTTGGTGGTGGCCT---TGTGCATGGC- 53
cv. Remo        ATGGCTAGCTCTGCAGTGACCA---AGCTTGCTTTGGTGGTGGCCT---TGTGCATGGC- 53
Pyrus           ATGGCTAGCTCTGCAGTGATCA---AGCTTGCTTTGGTGGTGGCCT---TGTGCATGGC- 53
Prunus          ATGGCTTACTCTGCCATGACTA---AGCTTGCTTTGGTGGTGGCCT---TGTGCATGGT- 53
Fragaria        ATGGCTATCTCTACTGCTATGA---AGCTTTCTTTGGTCGCTCTCT---TGTGCATCGT- 53
Lilium          ---------------------------ATGGCTCGCTCCTCCGCCGTCTGCTTCCTCCTC 33
Hordeum         ATGGCGGCTCCGAGGGGTGCGG---CACTGGTGCTGGCGATGGTGCTCGCGGCCATGGTG 57
Triticum        -----------ATGGCTCGCAC---TACAGCTACTAA-GCTCGTGCTGGTCGCCCTGGTG 45
Oryza           ATGGCCCGTGCACAG-CTGGTG---T-----TGGTCGCCCTCGTGGCAGCGGCTCTGCTC 51
Zea             ATGGCTCGCACGCAATCTGCCG---TA---GCGGTCGCCGTGGTGGCCGCGGTGCTGCTG 54
                                                                         *

Brassica        --CGTGGCCGGTCCAATCACATCGAACGCGGCTCTGAGTTGTGGCACCGTTAGCGGCTAC 105
Corylus         --GGTGGCCGCACCCGTTGCCCGG---GCGTCCCTGACATGCCCACAGATAAAAGGCAAC 99
Phaseolus       --GGTGCTGACGGCACCCACTGCACATGCCGCCATCTCCTGCGGCCAGGTCACAAGTTCC 105
Vigna           --GGTTGCCACCGCACCCACGGCACATGC---CATCACCTGCGGCCAGGTCGCAAGTTCC 102
Retama          ATGGTGGGTGCTGCACCCATTGCACAGGC---CATAACATGTGGACAGGTGGTCAGCAAC 102
Citrus          --GGTGACTGGTCCCCTGAGTGCTCAGGC---CATAACATGTGGGCAGGTGAGTGGCTCA 99
Davidia         --GGTGAGTGCACCCCA---TGCCGAAGCGGCGATAACATGCGGCACGGTGACAGTCAGC 114
Vitis           --GGTGGCGGCACCGGCGGTTGTGGAAGCAACCGTAACATGTGGTCAGGTGGCATCTGCC 111
cv. Elstar      --GGTGAGCGT---------TGCTCATGC---CATAACATGTGGCCAAGTGACCAGCAGC 99
cv. Golden      --GGTGAGCGT---------TGCTCATGC---CATAACATGTGGCCAAGTGACCAGCAGC 99
cv. Remo        --GGTGAGCGT---------TGCTCATGC---CATAACATGTGGCCAAGTGACCAGCAGC 99
Pyrus           --TGTCAGCGT---------TGCTCATGC---CATAACATGTAGCCAGGTGAGCGCCAAC 99
Prunus          --TGTGAGCGTGCCCAT---TGCTCAAGC---CATAACATGTGGCCAAGTGTCCAGCAGC 105
Fragaria        --GGTTGCTTTGCCCAT---AGCCCAAGC---CATCACCTGCGGCCAAGTAGCGAGTAGC 105
Lilium          CTCCTCGCCTTCCTCATTGGCACAGCCTCGGCAATCACCTGTGGTCAGGTTGACTCTGAC 93
Hordeum         GTGGCGCCGCC---TGTGACGGTGCGCGCGGCCATATCGTGCTCGGCGGTGTACAGCACG 114
Triticum        GCGGCACTCCTCCTTGTAGCCTCCGACGCGGCAATCTCCTGCGGCCAGGTGAGCTCCGCC 105
Oryza           CTGGCGGGCCCACACACCACCATGG---CCGCCATCAGCTGCGGCCAGGTCAACTCCGCC 108
```

```
Zea             CTGGCAGCGGCGGCGACGACCTCGGAGGCCGCCATCACCTGCGGGCAGGTGAGCTCCGCC 114
                                        *     *    **        *
Brassica        GTGGCACCGTGCATTGGCTACCTGGCCCAGAATGCGCCGGCCCTTCCCAGAGCGTGCTGC 165
Corylus         CTCACGCCATGCGTGCTCTACCTGA---AGAACGGCGGCGTTCTTCCTCCCTCTTGCTGC 156
Phaseolus       CTGGCTTCATGCATCCCTTTCCTCA---CGAAAGGTGGGGCGGTGCCAGCGTCATGCTGC 162
Vigna           CTCACTTCATGCATCCCATTCATCA---CCAAGGGTGGGATCGTGCCGCCGTCATGCTGC 159
Retama          CTAACCCCATGCATCACATACCTGC---AGAGAGGTGGAGCTGTTCCCGGACAATGCTGC 159
Citrus          TTGGCACCATGCATCGGTTTCTTGA---GATCGGGCGGCCCTATACCTATGCCATGCTGC 156
Davidia         CTGGCACCGTGCTTGAACTACCTGA---AGAAGGGTGGTCCGGTGCCGCCAGCCTGCTGC 171
Vitis           TTGAGCCCGTGCATTAGCTACTTGC---AGAAAGGTGGTGCAGTGCCAGCTGGGTGCTGC 168
cv. Elstar      CTTGCGCCATGCATTGGCTACGTGA---GGAGTGGCGGAGCTGTCCCTCCAGCTTGCTGC 156
cv. Golden      CTTGCGCCATGCATTGGCTACGTGA---GGAGTGGCGGAGCTGTCCCTCCAGCTTGCTGC 156
cv. Remo        CTTGCGCCATGCATTGGCTACGTGA---GGAATGGCGGAGCTGTCCCTCCAGCTTGCTGC 156
Pyrus           CTTGCACCATGCATTAACTACGTGA---GGAGTGGCGGAGCTGTCCCTCCAGCTTGCTGC 156
Prunus          CTTGCACCATGCATACCCTACGTGA---GAGGCGGTGGAGCTGTGCCTCCAGCTTGCTGC 162
Fragaria        ATTTCACCTTGCGTAAACTACGTGA---AGAGTGGCGGCGCTGTCCCTGCCGCTTGCTGC 162
Lilium          CTCACCTCCTGCCTTGGCTATGCTA---GAAAGGGCGGGGTCATCCCACCGGGCTGCTGC 150
Hordeum         CTGATGCCGTGCCTG--CAGTACGTGCAGCAG-GGCGGGACGCCGGCCCGGGGCTGCTGC 171
Triticum        CTTGGCCCGTGCCTTTCCTATGCACGCGGCAACGGCGCCAGCCCGTCTGCGGCCTGCTGC 165
Oryza           GTGTCGCCCTGCCTCAGCTACGCCCGCGG------GCTCCGCCCGTCGGCGGCCTGCTGC 162
Zea             ATCGCGCCCTGCCTCTCCTACGCCCGCGGCACGGGGTCCGGCCCCTCCGCGTCCTGCTGT 174
                 *     * *** *                                 *       *****
Brassica        AGCGGCGTTACTAGTCTA-AACAACCTGGCCCGTACAACCCCAGACCGTCAGCAAGCTTG 224
Corylus         AAGGGCGTCAGGGCTGTA-AACGACGCCTCCAGGACCACGTCCGACCGCCAGTCCGCTTG 215
Phaseolus       TCCGGAGTGAGGTCCCTC-AACGCCGCCGCAAAGACCACCCCAGACCGCCAGGTC---TG 218
Vigna           GCCGGAGTGAAGTCCCTC-AACGCCGCCGCAAAGACCACCCCAGACCGCCAGGCCGTGTG 218
Retama          AACGGAGTCAAGACACTC-GTGTCGTCTGCTCAGACCACTGCTGATAAACAGACTGCGTG 218
Citrus          AACGGCGTCAGGTCTCTC-AACGCTGCCGCCAGAACCACACCTGACCGCCAAACTGCATG 215
Davidia         AACGGAATTAAGTCCCTC-AACGCGGCGGCCAAGACCACAGCTGACCGCCAGGCTGCTTG 230
Vitis           AGCGGGATTAAGAGCCTC-AACAGCGCGGCCAAGACCACAGGTGATCGCCAGGCCGCTTG 227
cv. Elstar      AATGGAATCAGAACCATT-AACGGCTTGGCCAGGACCACCGCTGACCGCCAGACTGCTTG 215
cv. Golden      AATGGAATCAGAACCATT-AACGGCTTGGCCAGGACCACCGCTGACCGCCAGACTGCTTG 215
cv. Remo        AATGGAATCAGAACCATT-AACAGCTTGGCCAGGACCACCGCTGACCGCCAGACTGCTTG 215
Pyrus           AATGGAATCAAAACCATT-AACGGCTTGGCCAAGACCACCCCTGACCGCCAGGCTGCTTG 215
Prunus          AACGGCATTAGGAACGTC-AACAACTTGGCAAGGACCACCCCTGACCGCCAGGCCGCTTG 221
Fragaria        AACGGAATTAGATCCCTT-AACAGCGCGGCTAAGACCACTGCTGACCGCCAGACCACCTG 221
Lilium          GCGGGTGTGAGGACCCTT-AACAACTTAGCCAAGACCACTCCTGATCGCCAGACTGCATG 209
Hordeum         GCCGGCATCCAGAACCTG-CTGGCCGAGGCCAACAACAGCCCCGACCGCCGCACCATCTG 230
Triticum        AGCGGCGTCAGGAGATTGGCCGGCCAAGTGCAG-ACTGCCGCTGACAAGAAAGCGGCGTG 224
Oryza           AGCGGCGTCAGGAGCCTC-AACTCCGCCGCCAGCACCACCGCCGACCGCCGCACCGCCTG 221
Zea             AGCGGCGTCAGGAACCTC-AAGAGCGCCGCCAGCACCGCCGCCGACAGGCGCGCCGCCTG 233
                   **  *         *                   *         **             **
Brassica        CCGTTGCCTTGTAGGAGCCGCTAACGCCTT---GCCTACTATCAACGCTGCCCGTGCAGC 281
Corylus         CAACTGCTTGAAAGATACAGCCAAAGGCAT---CGCTGGCCTCAACCCTAATCTTGCTGC 272
Phaseolus       CAACTGCCTGAAATCCGCCGCCGGTGCCAT---CCCTGGATTCAATGCTAACAACGCAGG 275
Vigna           CAATTGCCTCAAAAGCGAGGCCGGCAGGAT---CGGTGGATTCAATGCTAACAACGCAGC 275
Retama          CAATTGCCTCAAATCCACTGCTGCTACCAT---CCCTAATATAAATTTTGGGAATGCAGG 275
Citrus          CAACTGCTTGAAGCAAGCCGCCGGATCAAT---CCCTAACCTCAACCTTAATAATGCTGC 272
Davidia         TAATTGCCTGAAAACAGCTTCTACCAGCAT---CGCTGGCATCAACCTGAGCTATGCTTC 287
Vitis           TAAGTGCTTGAAAACCTTTTCTAGTTCCGT---CTCTGGCATCAATTACGGTCTTGCAAG 284
cv. Elstar      CAACTGCCTGAAGAATCTTGCCGGCAGCAT---CAGTGGTGTTAACCCTAACAATGCAGC 272
cv. Golden      CAACTGCCTGAAGAATCTTGCCGGCAGCAT---CAGTGGTGTTAACCCTAACAATGCAGC 272
```

```
cv. Remo        CAACTGCCTGAAGAATCTTGCCGGCAGCAT---CAGTGGTGTTAACCCTAACAATGCAGC 272
Pyrus           CAACTGCCTGAAGAACCTTGCTGGCAGCGT---CAGTGGTGTTAACCCTGGCAATGCCGA 272
Prunus          CAACTGCCTGAAACAGCTTTCCGCCAGCGT---CCCCGGAGTCAACCCTAACAATGCCGC 278
Fragaria        CAATTGCCTCAAACAGGCCTCCGGTGCCAT---CAAAGGACTCAACCCTAACCTTGCAGC 278
Lilium          CAACTGCCTCAAGTCTCTGGTGAACCCCAG---CCTTGGCCTCAATGCTGCTATCGTCGC 266
Hordeum         CGGCTGCCTCAAGAACGTCGCCAACGCCGCCCCCGGCGGGAGCGAGATCACCCGCGCCGC 290
Triticum        CCTGTGCATCAAGAGTGCTGCC------------GGTGGGGTCAAAGAAGGCACGGCCGC 272
Oryza           CAACTGCCTCAAGAACGTTGCCGGCAGCAT---CAGCGGCCTCAACGCCGGCAATGCCGC 278
Zea             CAACTGCCTCAAGAACGCCGCCAGGGGCGT---CAGCGGCCTCAACGCCGGCAACGCCGC 290
                    *** *                                   *         *

Brassica        TGGACTTCCTAAGGCATGTGGAGTCAACATTCCTTACAAGATCAGCAAAACCACCAACTG 341
Corylus         TGGCCTCCCCGGCAAGTGTGGTGTCAACATTCCTTACAAGATCAGCCCCTCCACCAACTG 332
Phaseolus       CATACTCCCGGGCAAGTGTGGCGTCAGCATCCACTACAACATCAGTACCTCCACCAACTG 335
Vigna           CATACTCCCGGGCAAGTGTGGCGTCAGCATCCCCTACAAGATCAGCACCTCCACCAACTG 335
Retama          GTCACTCCCTGGCAAATGCGGGGTCAACCTCCCTTACAAGATCAGCCCCTCCACCAACTG 335
Citrus          TGGTCTTCCAGGAGCTTGTGGAGTCAGCATTCCTTACAAGATCAGCACCTCCACTGACTG 332
Davidia         CAGCCTCCCTGGCAAATGTGGTGTCAACGTTCCCTACAAGATCAGCCCTAGCACTGATTG 347
Vitis           TGGGCTTCCGGGCAAGTGTGGTGTCAGCGTTCCTTACAAGATCAGCCCCTCCACTGACTG 344
cv. Elstar      AGGGCTTCCTGGAAAGTGTGGAGTCAACGTCCCCTACAAGATCAGCACCTCCACCAACTG 332
cv. Golden      AGGGCTTCCTGGAAAGTGTGGAGTCAACGTCCCCTACAAGATCAGCACCTCCACCAACTG 332
cv. Remo        AGGGCTTCCTGGAAAGTGTGGAGTCAACGTCCCCTACAAGATCAGCACCTCCACCAACTG 332
Pyrus           ATCGCTTCCTGGAAAGTGTGGAGTCAACGTCCCCTACAAGATCAGCACCTCCACCAACTG 332
Prunus          AGCGCTTCCCGGCAAGTGTGGAGTTAGTATTCCTTACAAGATTAGCGCCTCCACCAACTG 338
Fragaria        TGGGCTTCCAGGCAAGTGTGGAGTCAACGTTCCGTACAAGATCAGCACCTCCACCAACTG 338
Lilium          CGGCATCCCCGGCAAGTGCGGCGTCAACATCCCCTACCCGATCAGAATGCAGACTGATTG 326
Hordeum         CGCGCTCCCGTCCAAGTGCAACGTCAACCTCCCCTACAAGATCAGCCCCAGCGTTGACTG 350
Triticum        AGAGATCCCCTCCAAGTGCCGCGTCAGCGTCCCCTACAAGATCAGCTCAACTGTGAACTG 332
Oryza           CAGCATCCCCTCCAAGTGCGGCGTCAGCATCCCCTACACCATCAGCCCCTCCATCGACTG 338
Zea             CAGCATCCCCTCCAAGTGCGGCGTCAGCATCCCCTACACCATCAGCACCTCCACCGACTG 350
                     * **       **    ** *   * *  ***   ** **           * **

Brassica        CAACAGTGTGAAATGA----- 357
Corylus         CAACAACGTGAAGTGA----- 348
Phaseolus       CGCTACTATCAAGTTTTGA-- 354
Vigna           CGCTAGTATCAAGTTTTGA-- 354
Retama          CGCCAGCATCAAGTTCTGA-- 354
Citrus          CTCTAAGGTCAGGTGA----- 348
Davidia         CTCCAAGGTGCAGTGA----- 363
Vitis           CTCCAAGGTGACTTGA----- 360
cv. Elstar      CGCCACCGTGAAGTAGG---- 349
cv. Golden      CGCCACCGTGAAGTAA----- 348
cv. Remo        CGCCACCGTGAAGTAA----- 348
Pyrus           CGCCACCGTGAAGTAA----- 348
Prunus          CGCCACCGTGAAGTGA----- 354
Fragaria        CGCTGCCGTGAAGTGA----- 354
Lilium          CAACAAGGTGAGGTAA----- 342
Hordeum         CAACTCGATCCACTGA----- 366
Triticum        CAATAAGATCTAG-------- 345
Oryza           CTCCAGGG--AACTAATCTGA 357
Zea             CTCCAGGGTGAACTAA----- 366
```

Figure (3.8): Multiple alignment of nucleotide sequence of ns*ltp* from different plant species**.** Asterisk means conservative residue.

The phylogenetic tree presented in Figure 3.9 can be classified into three major group based in their degree of homology in the cDNA of the ns*ltp* nucleotide sequences. The first major group includes *Brassica napus*, *Corylus avellana*, *Citrus sinensis*, *Retama raetam*, *Phaseolus vulgaris*, and *Vigna radiate*. Interestingly three cultivars of apple (*M. domestica* cv. Elstar, Remo and Golden Delicious) are located in a separated group with *Davidia involucrate*, *Vitis vinifera*, *Fragaria x ananassa Prunus persica*, *Pyrus communis*, within the tree. The third major group contains the monocotyledonous plants including; *Lilium longiflorum*, *Hordeum vulgare*, *Triticum aestivum*, *Oryza sativa*, and *Zea mays*.

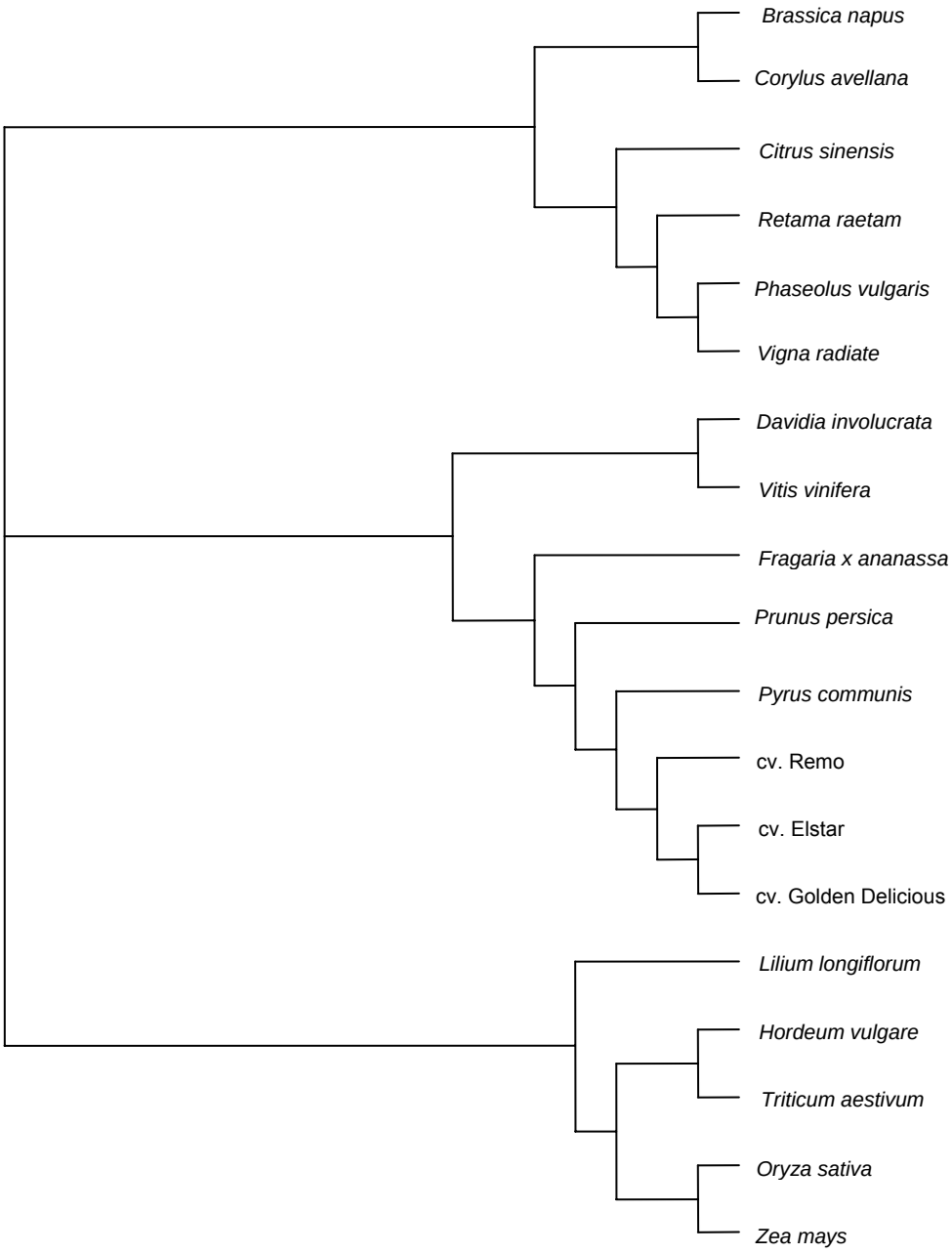

Figure (3.9): Phylogenetic tree of the nucleotide sequences of the ns*ltp* within different plant species, using ClustalW method (phylip)**.** *Brassica napus* (AJ245873); *Citrus sinensis* (AF369931); *Corylus avellana* (AF329829); *Davidia involucrata* (AY059472); *Fragaria x ananassa* (DQ066731); *Hordeum vulgare* (AF109195); *Lilium longiflorum* (AF171094); *Malus domestica* cv. Golden Delicious (AF221502); *Oryza sativa* (AY327042); *Phaseolus vulgaris* (U72765); *Prunus persica* (AY792996); *Pyrus communis* (AF221503); *Retama raetam* (AF439280); *Triticum aestivum* (AY789644); *Vigna radiate* (AY300806); *Vitis vinifera* (AF467946); *Zea mays* (U66105). All accession numbers belong to NCBI database.

3.5 Multiple sequence alignment of the amino acid sequence of nsLTP

To show the degree of relationship among apple nsLTP and different other plant species, amino acids sequences from database were used to determine the distance between species based on the homology of nsLTP. The phylogenetic tree was built by using multiple alignment program ClustalW (Figure 3.10). From this analysis it can be concluded that the vast majority of these 33 plant species (monocot, dicot and gymnosperm) contain 8 conserved cysteine residues in the nsLTP. Only in three species (Zinnia, Capsicum and Picea) the distribution and the number of cysteine residues are different. In addition, the length of nsLTP was ranging from 82 to 183 amino acids.

```
Arabidopsis       ------------------MAFA--LRFFTCLVLTVCIVAS-----VDAAISCGTVAGSL 34
Brassica          ------------------MASA--LSFFTCLVLTVCIVAS-----VDAAISCGTVTSNL 34
Cicer             ------------------MAS---MKVVCVALIMCIVIAP----MAESAITCGRVDTAL 34
Medicago          ------------------------------------------------SCGTVTGAL 9
Euphorbia         MKLKTHIHQSHITRNIIYNMAG---IKVAVLV-VALMVVASG---MYANAITCGQVSSSL 53
Vigna             ------------------MAS---LKVACMVAVVFMVVVSA---HMAHAITCGQVASSL 35
cv. Elstar        ------------------MAS---SAVTNLALVVALCMAVS----VAHAITCGQVTSSL 34
cv. Remo          ------------------MAS---SAVTKLALVVALCMAVS----VAHAITCGQVTSSL 34
Fragaria          ------------------MAS---STAMKLSLVALLCIVVAL--PIAQAITCGQVASNI 36
Hordeum           ------------------MARSAATQLVLVAMVAAMLLVAAD-----AAISCGQVSSAL 36
Triticum          ------------------MARTAATKLVLVALVAAMILAASD-----AAISCGQVSSAL 36
Sorghum           ------------------MAR----LAVAIAVVAAVVVVLAA-TTSEAAISCGQVSSAI 36
Zea               ------------------MART--QSAVAVAVVAAVLLLAAAATTSEAAITCGQVSSAI 39
Oryza             ------------------MAR---AQLVLVALVAAALLLAGP-HTTMAAISCGQVNSAV 37
Citrus            ------------------------------------------------ITCGQVTGSL 10
Daucus            ------------------MGVL--RSSFVAMMVMYMVLATTP--NAEAVLTCGQVTGAL 37
Beta              ------------------MASSAFVKFTCALVMCMMVAAP----LAEA-ITCGLVASKL 36
Spinacia          ------------------MASSAVIKLACAVLLCIVVAAP----YAEAGITCGMVSSKL 37
Lycopersicon      ------------------MEMVS--KIACFVLLCMVVVAP----HAEA-LTCGQVTAGL 34
Solanum           ------------------MEMFG--KIACFVLLCMVVVAP----HAEA-LSCGQVTSGL 34
Capsicum          ------------------MEMVG--KIACVVLLCMVVVAP----HAEA-LTCGQVQSRM 34
Nicotiana         ------------------MEIAG--KIACFVVLCMVVAAP----CAEA-ITCGQVTSNL 34
Atriplex          ------------------MASSVVFKLACAVFMCMLVAAP----HAEA-LTCGQVTSSM 36
Gossypium         ------------------MASSMSLKLACVAVLCMVVGAP----LAHGAVTCGQVTSSL 37
Vitis             ------------------MGSSGAVKLACVMVICMVVAAPA---VVEATVTCGQVASAL 38
Helianthus        ------------------MAK-MAMMVLCAGVTCMVVGAP-----YTEALSCGQVSSSL 35
Avicennia         ----------------MEGMNKSMCIIVVVAVLAAWVV--P----HGEAAISCGTVASKL 38
Prunus            ----------------MASSGQLLKLVCLVAVMCCMAVGGP----KAMAAVSCGQVVNNL 40
Pisum             ------------------MSK---VVLISVMTACMLISSSYG----KATLTCEQVTIWL 34
Pinus             ----------------MAEKKSRSVSGAVICVCIWACMGVFLHQPASAALDCNTIIQQI 43
Ricinus           ------------------------------------------------AVPCSTVDMKA 11
Zinnia            ----------------MAATTTTILLTISFALTNLYFVESAHQTGAPAPAADCSTVILNM 44
Picea             ----------------MDSRRLKRSGIVCMVLMSMLMLVVCE------DSDNTACLSSL 37

Arabidopsis       APCATYLSKGGL---VPPSCCAGVKTLNSMAKTTPDRQQACRCIQSTAKS--ISGLNPSX 89
Brassica          APCAVYLMKGGP---VPAPCCAGVSKLNSMAKTTPDRQQACKCLKTAAKN--VN---PSL 86
Cicer             APCLGYLQG-GPGP-SAQ-CCGGVRNLNSAAVTTPDRQAACNCLKSAAGS--ISRLNANN 89
Medicago          SPCIAYLRG-GAGP-SPA-CCAGVKRLNAAATTTPDRQAACNCLKSAAGA--ISGLNAST 64
Euphorbia         APCVNFLKS-GGAP-SPQ-CCNGLGGMVNQAKSTADKQAACNCLKTAAKN--MPGLNPAN 108
Vigna             APCISYLQK-GGVP-SAS-CCSGVKALNSAASTTADRKTACNCLKNLAGP--KSGINEGN 90
cv. Elstar        APCIGYVRS-GGAV-PPA-CCNGIRTINGLARTTADRQTACNCLKNLAGS--ISGVNPNN 89
cv. Remo          APCIGYVRN-GGAV-PPA-CCNGIRTINSLARTTADRQTACNCLKNLAGS--ISGVNPNN 89
Fragaria          SPCLTYVKS-GGAV-PAA-CCSGIRNLNGMAKTTADRQAACNCLKQAAGG--IKGLNPNL 91
Hordeum           RPCISYASGNGGIL-PPA-CCSGVKRLAGAAQSTADKQAACKCIKSAAG-----GLNAGK 89
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Triticum          TPCVAYAKGSGTSP-SGA-CCSGVRKLAGLARSTADKQATCRCLKSVAG-----GLNPNK 89
Sorghum           ALCLSYARGQGFAP-SAG-CCSGVRSLNSAARTTADRRAACNCLKNAARG--ISGLNAGN 92
Zea               APCLSYARGTGSGP-SAS-CCSGVRNLKSAASTAADRRAACNCLKNAARG--VSGLNAGN 95
Oryza             SPCLSYPRG-GSGP-SAA-CCSGVRNLNSAASTTADRRTACNCLKNVAGS--ISGLNAGN 92
Citrus            APCIVYLRSGG--P-IPVPCCNGVRSLNAAARTTPDRQTACNCLKQAAGS--IPNLNPNN 65
Daucus            APCLGYLRSQVNVP-VPLTCCNVVRGLNNAARTTLDKRTACGCLKQTANA--VTGLNLNA 94
Beta              APCIGYLQGAPG---PSAACCGGIKSLNSAAASPADRKTACTCLKSAAT--SIKGINYGK 91
Spinacia          APCIGYLKGGP----LGGGCCGGIKALNAAAATTPDRKTACNCLKSAAN--AIKGINYGK 91
Lycopersicon      APCLPYLQGRG----PLGGCCGGVKNLLGSAKTTADRKTACTCLKSAAN--AIKGIDLNK 88
Solanum           APCLPYLQGRG----PIGGCCGGIKGLLGAAKTPADRKTACTCLKSAAS--AIKGINVGK 88
Capsicum          TPCLPYLTGSG----PLGRCCGGVKGLLGAAKTPADRKTVCSCLKSAAG--SIGGINVRK 88
Nicotiana         APCLAYLRNTG----PLGRCCGGVKALVNSARTTEDRQIACTCLKSAAG--AISGINLGK 88
Atriplex          TPCMSYLTGGGS---PTPACCGGVKSLNSMASTPADRKAACGCLKSAAG--AMTNLNMGN 91
Gossypium         APCIGYLTGNGAGG-VPPGCCGGIKSLNSAAQTTPDRQAACKCIKSAAA--GISGINYGI 94
Vitis             SPCISYLQKGGA---VPAGCCSGIKSLNSAAKTTGDRQAACKCLKTFSS--SVSGINYGL 93
Helianthus        APCISYLTKGGA---VPPACCSGVKSLNSAAKTTPDRQAACGCLKSAYN--SISGVNAGN 90
Avicennia         APCIPYVTNRG--P-LG-GCCGGVKSLYGLARTTPDRQSVCGCLKSLAS--SY-NVNLGK 91
Prunus            TPCINYVANGG--A-LNPSCCTGVRSLYSLAQTTADRQSICNCLKQAVNGIPYTNANAGL 97
Pisum             TPCIPYGTLGGS---VLPLCCQGVHSLNAAYKNGDDRRLACHCVQDRAAL--IPLIDYTR 89
Pinus             TSCATYLTTGTPVPQEESSCCQGVQSLYGDATTTEEIQQICTCLKNEAIN---YNLNDRA 100
Ricinus           AACVGFATGKDSKP--SQACCTGLQQLAQTVKTVDDKKAICRCLKASSKS---LGIKDQF 66
Zinnia            ADCLSYVTAGSTVKKPEGTCCSGLKTVL-----KTDAECLCEAFKNSAQLG--VSLNITK 97
Picea             SSCAPYLNATTK---PDSSCCSALISVID-----KDSQCLCNLLNSDTVK--QLGVNVTQ 87
                    *  :             **  :  :        : .  *  .:

Arabidopsis       ASGLPGKCGVSIP----YPISMSTNCNNIK------------------------------ 115
Brassica          ASSLPGKCGVSIP----YPISMSTNCDTVK------------------------------ 112
Cicer             AAALPGKCVVNIP----YKISTSTNCATIRV---------------------------- 116
Medicago          AAGLPGKCGVNIP----YKIST------------------------------------- 82
Euphorbia         AESLPSKCKVNIP----YKISFSTNCNSIK------------------------------ 134
Vigna             AASLPGKCKVNVP----YKISTFTNCANIK------------------------------ 116
cv. Elstar        AAGLPGKCGVNVP----YKISTSTNCATVK------------------------------ 115
cv. Remo          AAGLPGKCGVNVP----YKISTSTNCATVK------------------------------ 115
Fragaria          AAGLPGKCGVSVP----YKISTTTNCAAVK------------------------------ 117
Hordeum           AAGIPSMCGVSVP----YAISASVDCSKIR------------------------------ 115
Triticum          AAGIPSKCGVSVP----YTISASVDCSKIH------------------------------ 115
Sorghum           AASIPSKCGVSVP----YTISTSTDCSRVS------------------------------ 118
Zea               AASIPSKCGVSIP----YTISTSTDCSRVN------------------------------ 121
Oryza             AASIPSKCGVSIP----YTISPSIDCSSVN------------------------------ 118
Citrus            AVGLPRACGVSIP----YKISISTDCSKVR------------------------------ 91
Daucus            AAGLPARCGVNIP----YKISPTTDCNRVV------------------------------ 120
Beta              AASLPRQCGVSVP----YAISPNTNCNAIH------------------------------ 117
Spinacia          AAGLPGMCGVHIP----YAISPSTNCNAVH------------------------------ 117
Lycopersicon      AAGIPSVCKVNIP----YKISPSTDCSTVQ------------------------------ 114
Solanum           AAGIPRLCGVNIP----YKISPSTDCSKVR------------------------------ 114
Capsicum          AAGLPNMCGVNIP----YQISPSTDCTKVQ------------------------------ 114
Nicotiana         AAGLPSTCGVNIP----YKISPSTDCSKVQ------------------------------ 114
Atriplex          AASLPGKCGISLP----YPISTSTDCSKVN------------------------------ 117
Gossypium         ASGLPGKCGVNIP----YKISPSTDCNSVK------------------------------ 120
Vitis             ASGLPGKCGVSVP----YKISPSTDCSKVT------------------------------ 119
Helianthus        AASFPGKCGVSIP----YKISPSTDCSKVQ------------------------------ 116
Avicennia         AAGLPGQCGVNIP----YKISPSTDCSKVH------------------------------ 117
Prunus            AAGLPGKCGVNIP----YKISPSTDCKSIK------------------------------ 123
Pisum             INQIGDLCGSKCP----FKVYPSTDCDKVK------------------------------ 115
Pinus             LQSLPSNCGLQLS----FTITRDIDCSSISL---------------------------- 127
Ricinus           LSKIPAACNIKVG----FPVSTNTNCETIH------------------------------ 92
Zinnia            ALALPSACHINAPSATNCGISPGTAVAPALAPIGVGMAPSGAAVAFGPSMAVAPTPASQT 157
Picea             AMKMPAECGKNVSATQCNKTATSGGSSVGKTPTSTPPPSSATPSTTTITKSNSNAAASVS 147
                     :   *

Arabidopsis       --------------------------
Brassica          --------------------------
Cicer             --------------------------
Medicago          --------------------------
Euphorbia         --------------------------
Vigna             --------------------------
cv. Elstar        --------------------------
cv. Remo          --------------------------
```

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Fragaria          -------------------------
Hordeum           -------------------------
Triticum          -------------------------
Sorghum           -------------------------
Zea               -------------------------
Oryza             -------------------------
Citrus            -------------------------
Daucus            -------------------------
Beta              -------------------------
Spinacia          -------------------------
Lycopersicon      -------------------------
Solanum           -------------------------
Capsicum          -------------------------
Nicotiana         -------------------------
Atriplex          -------------------------
Gossypium         -------------------------
Vitis             -------------------------
Helianthus        -------------------------
Avicennia         -------------------------
Prunus            -------------------------
Pisum             -------------------------
Pinus             -------------------------
Ricinus           -------------------------
Zinnia            SGSFALAMSTVSVFLSMLLSAYFYSC 183
Picea             VKMFPVAALVFVAVASVLGLKGPCLR 173
```

Figure (3.10): Multiple alignment of the amino acid sequence of nsLTP from different plant species**.** Asterisk means conservative residue; colon means conservative with small changes**.** *Arabidopsis thaliana* (AAM66088); *Atriplex nummularia* (BAC77694); *Avicennia marina* (AAK01293); *Beta vulgaris* (CAA63407); *Brassica napus* (AAD09107); *Capsicum annuum* (AAF23460); *Cicer arietinum* (CAA05771); *Citrus sinensis* (CAH03799); *Daucus carota* (P27631); *Euphorbia lagascae* (AAM00272); *Fragaria x ananassa* (CAC86258); *Gossypium barbadense* (AAN77147); *Helianthus annuus* (CAA63340); *Hordeum vulgare* (CAA85484); *Lycopersicon esculentum* (CAA39512); *Medicago sativa* (AAZ32875); *Nicotiana tabacum* (CAA44267); *Oryza sativa* (AAB70539);*Picea abies* (BAA23548); *Pinus radiata* (AAB80805); *Pisum sativum* (AAF61436); *Prunus dulcis* (CAA65477); *Ricinus communis* (S01796); *Solanum tuberosum* (AAM82607); *Sorghum bicolor* (CAA50660); *Spinacia oleracea* (P10976); *Triticum aestivum* (AAV28706); *Vigna radiata* (AAQ74628); *Vitis vinifera* (AAO33394); *Zea mays* (AAB06443); *Zinnia elegans* (BAD24657). All accession numbers belong to NCBI database.

The phylogenetic tree of different 33 plant species based on the amino acid sequence of nsLTP showed that these species are classified into three major groups. The first group includes *Arabidopsis thaliana*, *Brassica napus*, *Pisum sativum*, *Pinus radiate*, *Ricinus communis*, *Picea abies*, and *Zinnia elegans*. On the other hand the second group includes *Atriplex nummularia*, *Beta vulgaris*, *Spinacia oleracea*, *Nicotiana tabacum*, *Capsicum annuum*, *Lycopersicon esculentum*, *Solanum tuberosum*, *Helianthus annuus*, *Gossypium barbadense*, *Vitis vinifera*, *Avicennia marina*, and *Prunus dulcis*. The last group also includes *Cicer arietinum*, *Medicago sativa*, *Euphorbia lagascae*, *Vigna radiata*, *Fragaria x ananassa*, *M.domestica* cv. Elstar, *M.domestica* cv. Remo, *Hordeum vulgare*, *Triticum aestivum*, *Oryza sativa*, *Sorghum bicolor*, *Zea mays Citrus*, *sinensis*, and *Daucus carota*. In addition the two *M. domestica* cultivars Elstar and Remo were closely related to *Fragaria x ananassa*.

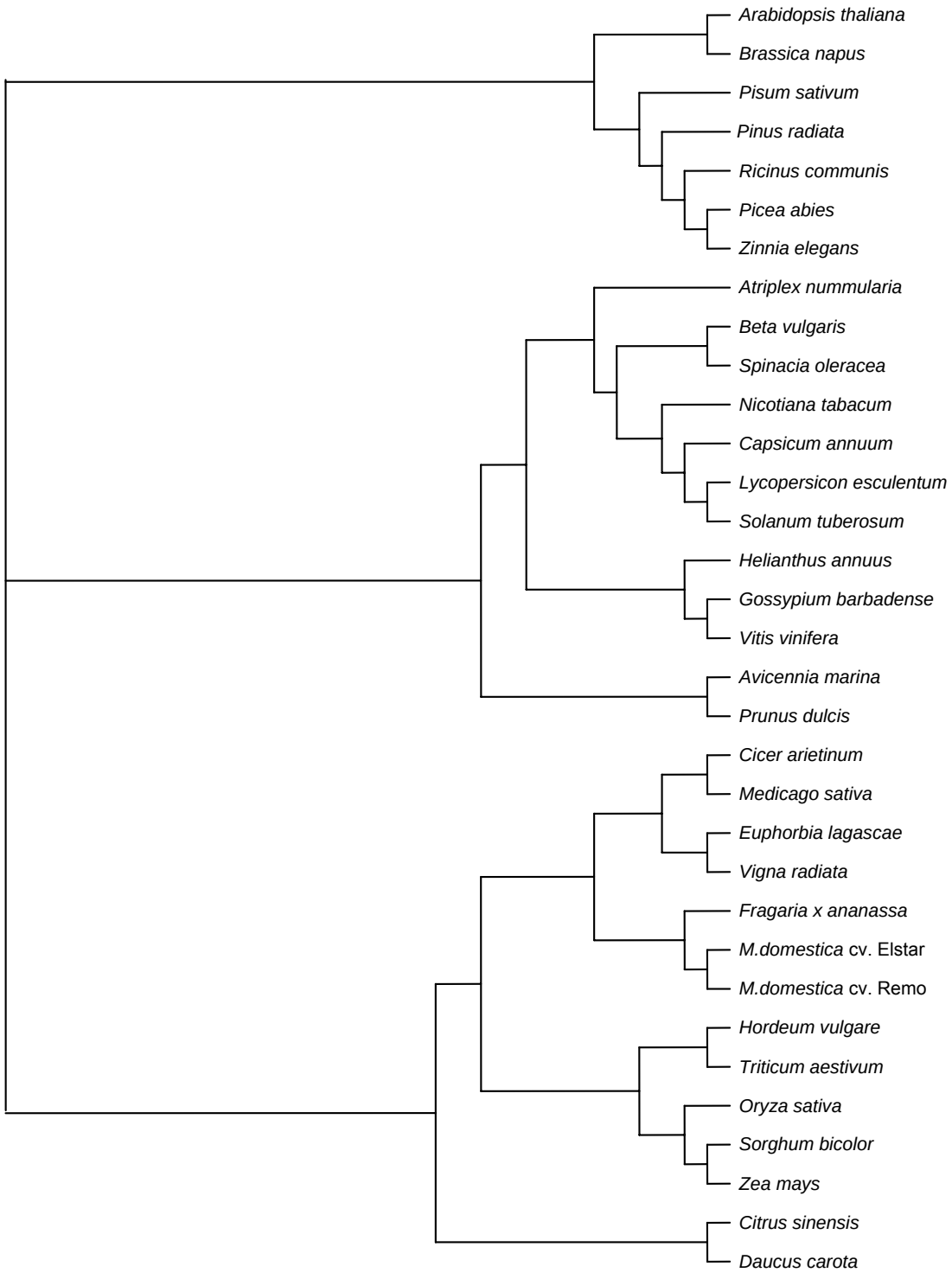

Figure (3.11): Phylogenetic tree of the amino acid sequences of nsLTP within different plant species, using ClustalW method (phylip)**.**

3.6 Southern blot analysis

3.6.1 DIG labeling confirmation

It was necessary to determine how many copies of ns*ltp* are distributed in apple genome therefore southern blot technique was used. After extraction of genomic DNA from Elstar, this pure DNA was used as a template for PCR to amplify the ns*ltp*. PCR products were diluted 1 to 10 and again used as a template for PCR by using the same primers and the U-DIG labeled nucleotide in the nucleotide mixture. It was necessary to confirm the DIG labeling process before the hybridization with the labeled probe during southern blot. Therefore, after performing the PCR reaction the PCR products were checked on 1.5 % agarose gel (Figure 3.12). From this figure one could confirm that the probe is already DIG labeled because it has a larger size than that nonlabeled due to the incorporation of the DIG-labeled nucleotide.

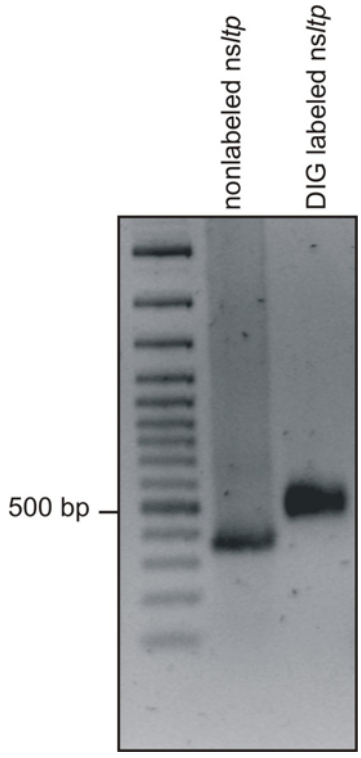

Figure (3.12): DIG labeling confirmation of ns*ltp* from *M. domestica* cv. Elstar before using it as a probe in the hybridization in southern blot on 1.5 % agarose gel.

3.6.2 Determination of the copy number of the ns*ltp*

Copy number of ns*ltp* gene in Elstar and Remo was determined by southern blot and hybridization.

Although the ns*ltp* is an intronless gene, different restriction enzymes were used for genomic DNA digestion. Three different enzymes *Bam*HI, *Eco*RI and *Hind*III were used in this study. The result presented in Figure 3.13 showed that at least 6 copies of ns*ltp* are located in the susceptible apple cultivar Elstar as well as in the resistant one Remo. Moreover in the case of *Bam*HI/*Eco*RI the pattern showed some differences between the susceptible cultivar Elstar and the resistant cultivar Remo.

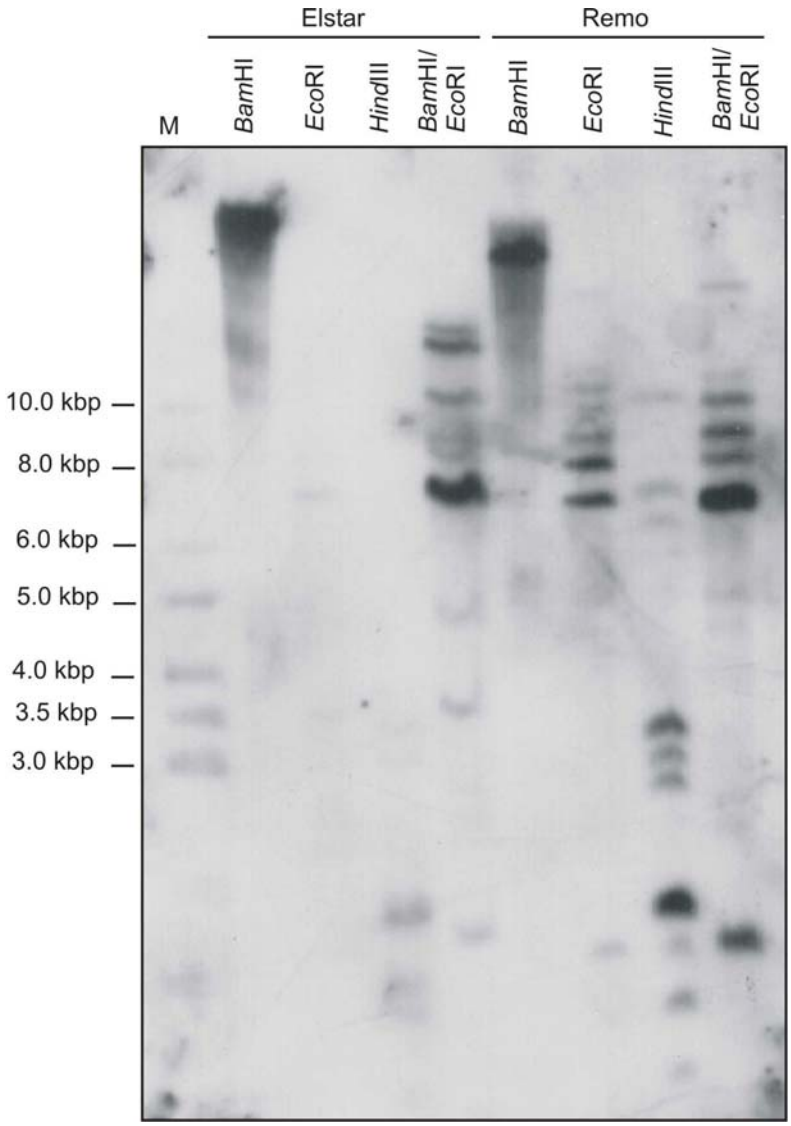

Figure (3.13): Determination of ns*ltp* copy number in *M. domestica* cv.Elstar and Remo by southern blot. Each lane was loaded by 50 µg of genomic DNA after digestion with restriction enzymes.

3.7 Gene expression of nsltp from Elstar during the infection *V. inaequalis*

It was shown in Figure 1.4 that the protein level of nsLTP decreased drastically to undetectable level in the apoplast after infection of apple leaves with *V. inaequalis*. It was necessary to investigate the plant response to the infection process in short intervals. Therefore a time curve experiment was performed by artificial infection of apple trees of the susceptible *M*. *domestica* cv. Elstar with conidial suspension of *V. inaequalis*. Total RNA was extracted from young leaves from the treated trees at five intervals (1, 3, 5, 7, and 14 days) and as well as from the control plant that was treated with water. Northern blot was done by using a radioactively labeled probe of the ns*ltp* with a size 349 bp. RNA analysis of apple leaves revealed that the transcript level of mRNA of ns*ltp* was dramatically declined to undetectable level after one day infection (Figure 3.14). The transcript level of ns*ltp* is still undetectable until two weeks after infection. However after five days a weak signal has been detected that might reflect the discrepancies among the individual responses to the infection process.

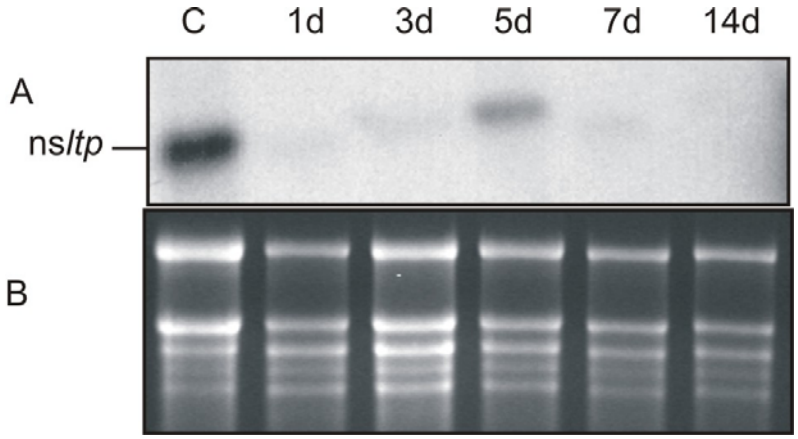

Figure (3.14): Detection of ns*ltp* by northern blot and radioactive labeled ns*ltp* probe C**:** Control uninfected Elstar; 1, 3, 5, 7 and 14d: days after infection with *V. inaequalis*. **A:** X-ray membrane; **B:** Ethidium bromide staining for RNA gel before blotting. Each lane was loaded by 10 µg total RNA.

3.8 Upstream region amplification by Genome walker kit

To understand more about the structure of the ns*ltp* gene in apple *M*. *domestica* cv. Elstar and Remo, the upstream sequence was amplified by using Genome walker kit according to the manufacture instructions. This was a primary step to analyze the upstream region sequence searching for the cis-acting regulatory elements and as well as to study the promoter activity by particle bombardment and transient expression of DsRed.

3.8.1 Library construction

The library was built by digesting the genomic DNA of both Elstar and Remo with four different restriction enzymes that produce blunt ends. DNA fragments were purified and checked on agarose gel before ligation with the adaptor (Figure 3.15).

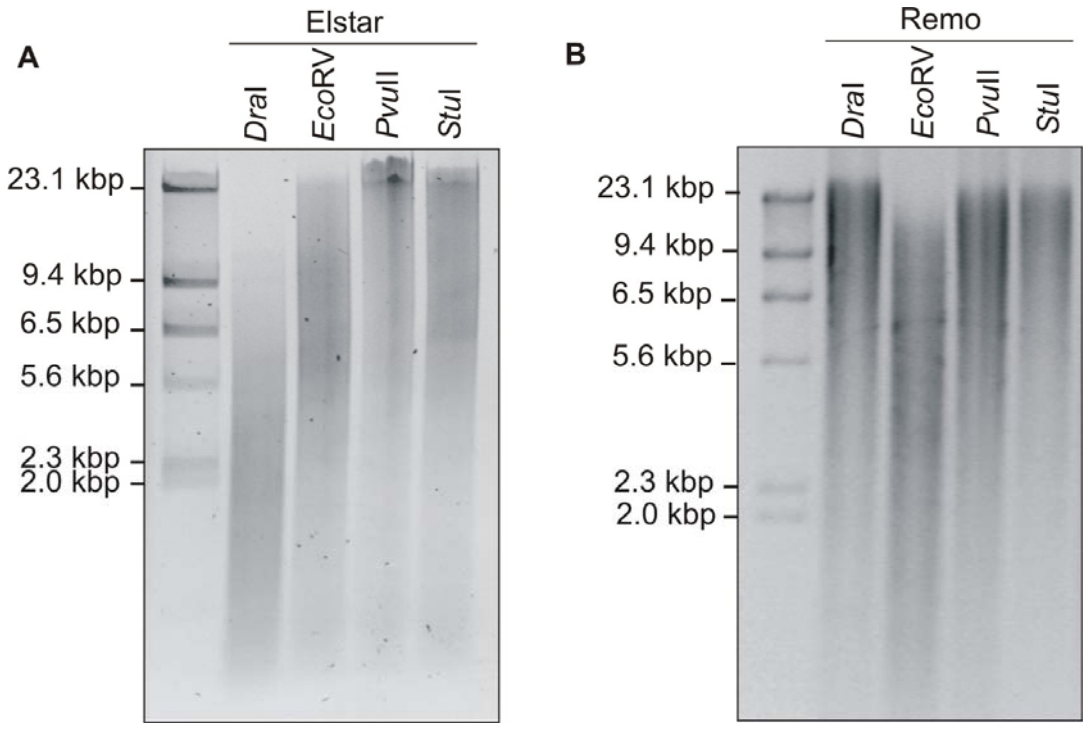

Figure (3.15): Digested apple genomic DNA by different restriction enzymes; **A:** *M. domestica* cv. Elstar digested genomic DNA and **B:** *M. domestica* Remo digested genomic DNA. 2 µl from each digestion assay were loaded in

3.8.2 First PCR products of the amplified upstream region

The first PCR was done by using 1 µl from each library as a template, adaptor primer 1 and gene specific primer 1. The amplified products that were obtained from the first PCR were tested on 1.5 % agarose. No clear bands have been detected in agarose gel only just a very weak smear (Figure 3.16).

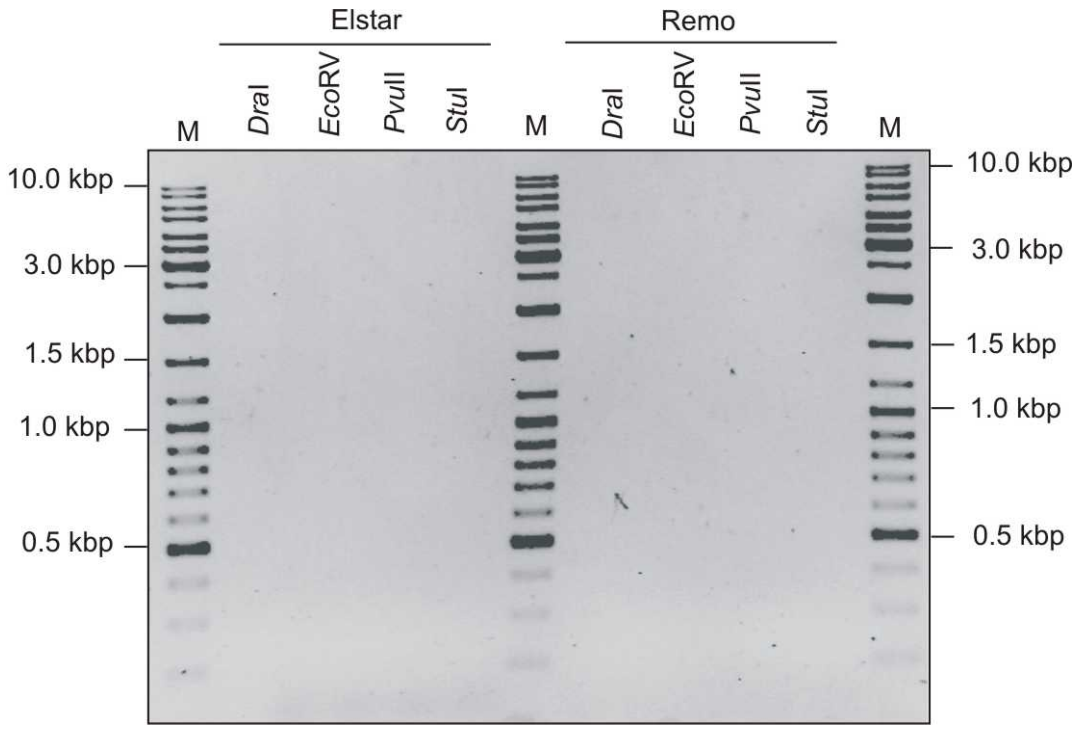

Figure (3.16): Analysis of the first PCR products of the amplified upstream regions in *M*. *domestica* cv. Elstar and Remo. 10 µl in were loaded in each lane on 1.5 % agarose gel.

3.8.3 Second PCR products of the amplified upstream region

The second PCR was performed by using the same PCR program that was used in the first PCR. Products were checked on 1.8 % agarose. One pure band has been observed in case of *Eco*RV and *Stu*I in both Elstar and Remo. The size of these fragments was almost the same, less than 500 bp (Figure 3.17).

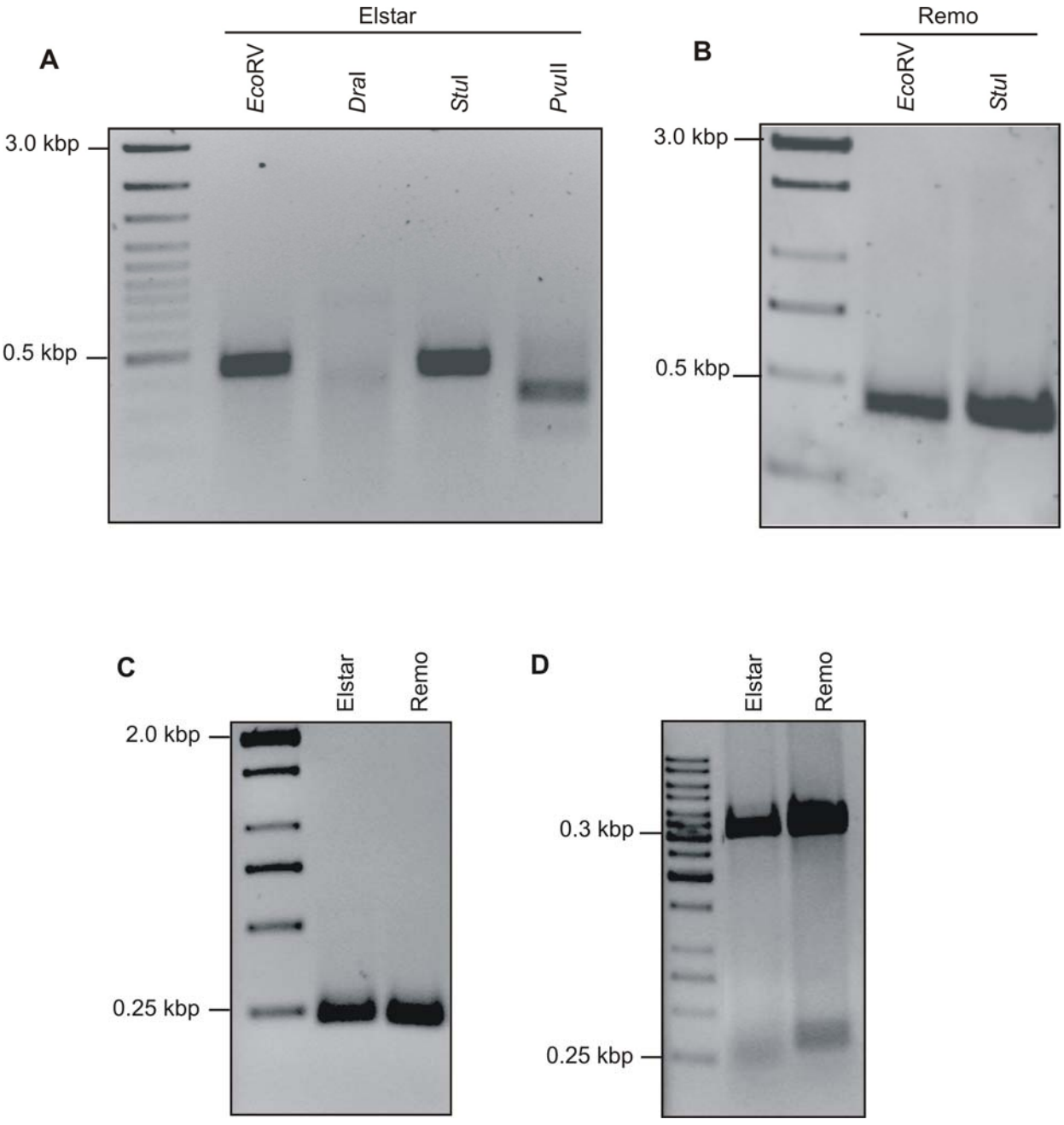

Figure (3.17): Analysis of the second PCR products of the amplified upstream (I) regions in *M*. *domestica* cv. **A:** Elstar; **B:** Remo; **C:** upstream (II) region in Elstar and Remo. 10 µl in were loaded in each lane on 1.8 % agarose gel; **D:** Cloning of the amplifed downstream regions from Elstar and Remo in pGEMT-vetor after digestion with *Nco*I and *Not*I.

To gain more information on the upstream region this procedure was repeated with only one restriction enzyme *Stu*I and the corresponding gene specific primers (see 2.11.4). Second PCR results showed that fragment with a size around 250 bp has been amplified in both Elstar and Remo as an additional sequence in the upstream region (Figure 3.17C). In another step the downstream regions in Elstar and Remo were amplified by using *Stu*I and the corresponding primers (2.11.4). Subsequently the amplified fragments in the second PCR (around 250 bp in Elstar and a slightly larger in case of Remo) were cloned in pGEMT-vector (3.17D) to send for sequencing.

3.8.4 Cloning of the upstream region of the ns*ltp* in pGEMT-vector

The obtained fragments from the second PCR were ligated in pGEMT vector and the ligation assay was used for *E. coli* transformation. Plasmid was isolated from the right colony and digested with two restriction enzymes from the MCS. To confirm the presence of the upstream region before send for sequencing, the digested plasmids were checked on agarose gel (Figure 3.18).

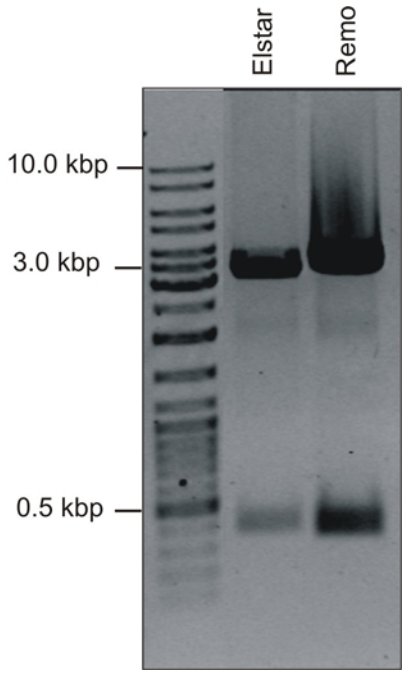

Figure (3.18): digested pGEMT with *Nco*I and *Not*I containing the upstream Regions in *M. domestica* cv. Elstar and Remo.

The adaptors and gene specific primers and as well as the vector contamination from were removed from the retrieved sequences. Results revealed that by this kit 416 bp could be amplified by two steps (362 bp and 54 bp) upstream the ATG of the ns*ltp* in apple *M*. *domestica* cv. Elstar. In the case of Remo also 525 bp could be amplified by two steps (369 bp and 156 bp) upstream the ATG of the ns*ltp*. In addition 352 bp could be amplified downstream of the ns*ltp* in *M*. *domestica* cv. Remo, but unfortunately in case of Elstar the quality of the retrieved sequence was very poor.

3.8.5 Gene structure of ns*ltp* in the susceptible apple cultivar Elstar

```
  1 gaatagtcga actaagggta ttgtggtcaa caacttttgt ggccacgttt
 51 acgtatccct tacattttaa aatagagtgt tttcttataa aaaaaagatc
101 atttttcact gtaaacaaat cataatttga tttatgttcc gacgagtttt
151 caataacttt aaaatgtaag gagttcaaat taataattca tggaaatgat
201 tgctgtaaca tgtaattaaa cacgtaaacg tggccacaaa agttgttgac
251 cacaataccc ttagttcgac tattctttct caatttttgt ctatataagc
301 accacaccat agtgccttta taactcacta gctactcgag ttttcaaatc
351 aagttctttc atatccatct ttcatacaca tatttggtaa tccacagcct
401 ttttaagtca ttaattatgg ctagctctgc agtgaccaag cttgctttgg
                      M   A   S   S   A   V   T   K   L   A   L

451 tggtggcctt gtgcatggcg gtgagcgttg ctcatgccat aacatgtggc
     V   V   A   L   C   M   A   V   S   V   A   H   A   I   T   C   G

501 caagtgacca gcagccttgc gccatgcatt ggctacgtga ggagtggcgg
      Q   V   T   S   S   L   A   P   C   I   G   Y   V   R   S   G

551 agctgtccct ccagcttgct gcaatggaat cagaaccatt aacggcttgg
    G   A   V   P   P   A   C   C   N   G   I   R   T   I   N   G   L

601 ccaggaccac cgctgaccgc cagactgctt gcaactgcct gaagaatctt
     A   R   T   T   A   D   R   Q   T   A   C   N   C   L   K   N   L

651 gccggcagca tcagtggtgt taaccctaac aatgcagcag ggcttcctgg
      A   G   S   I   S   G   V   N   P   N   N   A   A   G   L   P

701 aaagtgtgga gtcaacgtcc cctacaagat cagcacctcc accaactgcg
    G   K   C   G   V   N   V   P   Y   K   I   S   T   S   T   N   C

751 ccaccgtgaa gtaatcccgc ggcc
     A   T   V   K   -
```

Figure (3.19): Assembled sequence of the coding region of the ns*ltp* from *Malus domestica* cv. Elstar and its upstream region (blue coloured).

3.8.6 Gene structure of ns*ltp* in the resistant apple cultivar Remo

```
   1 taatagttag catcatttcc tgaattatta atttgaactc cttacatttt
  51 aaagttattg aaaactcgtc ggaacataaa tcaaattatg atttgtttac
 101 agtgaaaaat gatcttttt ttaaaagaaa acactctatt ttaaaatgta
 151 agggatgtgg tcgacggccc gggctggtcc tctaatccac gcaagtcccc
 201 tttagtccca tttaacttag tccctataca aaccaaacat gggactacag
 251 tctaattcaa tccagtccca gttaacgagg tcaaacaaac gccccttaa
 301 aatataagga gtacttcaag gttataattc atcgccatga ttgatgtaac
 351 aagtaattaa acacgtaaag cgttgccaca aatttttttg accactatac
 401 ccttcgttca cacgttttct ctcttaattt ttctctatat aagcaccacc
 451 atagtgcctt tacaactcac tagctacacg agtcttcaaa tcaagttctt
 501 tcacatccat tactcataca cacttatggc tagctctgca gtgaccaagc
                                 M   A  S  S  A   V  T  K
 551 ttgctttggt ggtggccttg tgcatggcgg tgagcgttgc tcatgccata
      L  A  L   V  V  A  L   C  M  A   V  S  V   A  H  A  I
 601 acatgtggcc aagtgaccag cagccttgcg ccatgcattg gctacgtgag
       T  C  G   Q  V  T   S  S  L  A   P  C  I   G  Y  V
 651 gaatggcgga gctgtccctc cagcttgctg caatggaatc agaaccatta
     R  N  G  G   A  V  P   P  A  C   C  N  G  I   R  T  I
 701 acagcttggc caggaccacc gctgaccgcc agactgcttg caactgcctg
      N  S  L   A  R  T  T   A  D  R   Q  T  A   C  N  C  L
 751 aagaatcttg ccggcagcat cagtggtgtt aaccctaaca atgcagcagg
       K  N  L   A  G  S   I  S  G  V   N  P  N   N  A  A
 801 gcttcctgga aagtgtggag tcaacgtccc ctacaagatc agcacctcca
     G  L  P  G   K  C  G   V  N  V   P  Y  K  I   S  T  S
 851 ccaactgcgc caccgtgaag taatcccgcg gccacccccg tttagccgaa
      T  N  C   A  T  V  K   -
 901 cgctgcgctt atcgggaact atctcttgag tcaaccggta aaacaactat
 951 tcccttgcac acccctgtaa aagatatcaa accgagattt tgggggcccc
1001 aaattttaaa gggggcccat ccggccccca aaaaacaatt tgtttccccc
1051 cctgaacccc tcctttaaaa aaaatttttt tttgtcaaaa accccggggg
1101 gggttgtttt ttccccaatc cccaaaaaaa aaaaaaaatt ttttttgggg
1151 gggcggggaa aaaaaagagg gtgtgggaaa aaaacacccc caaaaaaaaa
1201 aaaaaaaagc aagccccccc ccccccccccc cccccc
```

Figure (3.20): Assembled sequence of the coding region of the ns*ltp* from *Malus domestica* cv. Elstar, upstream region (blue coloured) and its downstream region (red coloured).

3.8.7. Sequence submission to NCBI database

A consensus of the full length sequence of the ns*ltp* and its upstream region was built by using computer program. Consequently 716 bp and 719 bp for *M. domestica* cv. Elstar and Remo respectively were submitted to the NCB database. The submitted sequences have taken the accession numbers; DQ295056 for Elstar and DQ295057 for Remo.

3.8.8 Alignment of the upstream regions

The nucleotide sequence of 359 bp for Elstar and 360 for Remo were submitted to the NCBI database for two sequences alignments. Results are presented below (Figure 3. 21) revealed that there are some nucleotides in the nucleotide sequence of Elstar upstream region which have been changed in Remo. At position 25 thyamine is changed to cytosine, position 26 thiamine to guanine, 75 thyamine to guanine, position 145 cytosine to adinine, position 195 adinine to guanine, position 223 cytosine to thyamine, position 290 adinine to cytosine, position 312 cytosine to thiamine and position 324 thiamine to guanine. One additional region in the sequence was not clear in Elstar from position 33 to 41.

```
Elstar: 1   cccttacattttaaaatagagtgtttcttatnnnnnnn-gatcatttttcactgtaaac 59
            |||||||||||||||||||||||  ||||||        ||||||||||||||||||||
Remo:   1   cccttacattttaaaatagagtgtcgtcttataaaaaaaagatcatttttcactgtaaac 60


Elstar: 60  aaatcataatttgatttatgttccgacgagttttcaataactttaaaatgtaaggagttc 119
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Remo:   61  aaatcataatttgagttatgttccgacgagttttcaataactttaaaatgtaaggagttc 120


Elstar: 120 aaattaataattcatggaaatgattgctgtaacatgtaattaaacacgtaaacgtggcca 179
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Remo:   121 taattaataattcatggaaatgattgatgtaacatgtaattaaacacgtaaacgtggcca 180


Elstar: 180 caaaagttgttgaccacaataccccttagttcaactattctttctcaatttttgtctatat 239
            ||||||||||||||||||||||||| ||||||||||||||| ||||||||||||||||
Remo:   181 caaaagttgttgaccacaataccccttggttcaactattctttcttaatttttgtctatat 240


Elstar: 240 aagcaccacaccatagtgcctttataactcactagctactcgagttttcaaatcaagttc 299
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Remo:   241 aagcaccacaccatagtgcctttataactcactagctactcgagttttcaactcaagttc 300


Elstar: 300 tttcatatccatctttcatacacatatttggtaatccacagccttttaagtcattaatt 359
            |||||||||||| |||||||||| ||||||||||||||||||||||||||||||||||
Remo:   301 tttcatatccatttttcatacacagatttggtaatccacagccttttaagtcattaatt 360
```

Figure (3.21): Alignment of the upstream region of the susceptible and resistant apple cultivars *M. domestica* cv. Elstar and Remo. Blue coloured nucleotides indicate the substitutions.

3.8.9 Analysis of the upstream regions

Adaptors and gene specific primers were removed and the obtained sequences of the upstream regions I and II were assembled to a contiguous nucleotide sequence of a 416 bp for Elstar and 525 bp for Remo. Afterwards the resulted nucleotide sequences were directly used for computer analysis.

Promoter prediction was done by using different computer programs, the first promoter prediction program that was available in http://www.fruitfly.org gave a score 0.99 for promoter prediction in both Elstar and Remo. In case of Elstar the predicted sequence started at 282 bp and ended at 332 bp in the upstream region with a putative transcription start site. The predicted sequence in Remo started at 426 bp and ended at 476 bp with a putative transcription start site. In the second part of the analysis the GENSCAN program available in http://genes.mit.edu was used for promoter prediction. The predicted promoter sequence in Elstar has been given by this program was 40 bp in length started at 286 bp and ended at 325 bp. In the upstream region of Remo, the predicted promoter sequence was also 40 bp in length started at 430 bp and ended at 469 bp.

To determine the cis-acting regulatory elements that were distributed in the upstream regions these sequences were analyzed by using the PlantCARE database available at http://intra.psb.ugent.be:8080/PlantCARE/. The predicted cis-acting regulatory elements are listed in table 3.2 and 3.3 for Elstar and Remo upstream regions respectively. Interestingly in table 3.3 nine motifs involved in light responsiveness were predicted in Elstar. Different single motifs were predicted and involved in responsiveness to abscisic acid, ethylene, gibberellin, fungal elicitor, and wound. In addition, one motif involved in anoxic specific inducibility was predicted. Two more motifs were predicted that are required for endosperm expression. Additionally the core promoter region also was predicted. In Remo upstream region the same kind of motifs were predicted, but in case of light responsive elements the number of motifs was 13 in Remo instead of 10 in Elstar. One additional motif was predicted and involved in heat stress responsiveness.

Table (3.2): "Cis-acting regulatory elements" in the upstream region of ns*ltp* of *M. domestica* cv. Elstar (PlantCARE, Lescot et al., 2002, accesed in 04.05.2005).

No.	Motif name	Organism	Position	"Core"	"Matrix"	Sequence	Function	References
1	ABRE	*Arabidopsis thaliana*	-219	1.000	1.000	tACGTg	Involved in the abscisic acid esponsiveness	Yamaguchi-Shinozaki and Shinozaki 1994
2	ACE	*Petroselinum crispum*	-40	1.000	0.969	gccACGTtta	Involved in light responsiveness	Feldbrugge et al., 1996
3	AT1	*Solanum tuberosum*	-95	1.000	0.859	gatcATTTttca	Part of a light responsive module	-
4	Box-W1	*Petroselinum crispum*	-23,-244	1.000	1.000	TTGAcc	Fungal elicitor responsive element	Rushton et al., 1996
5	CAAT-box	*Nicotiana tabacum*	-18	1.000	1.000	CAAT	In promoter and enhancer regions	Klotz and Lagrimini 1996
6	ERE	*Dianthus caryophyllus*	-62	1.000	0.875	ATTTtaaa	Ethylene-responsive element	Itzhaki et al., 1994
7	G-Box	*Antirrhinum majus*	-219	1.000	1.000	CACGta	Involved in light responsiveness	Arguello-Astorga and Herrera-Estrella 1996
8	G-box	*Brassica oleracea*	-217	1.000	1.000	aacACGTa	Involved in light responsiveness	Pastuglia et al., 1997
9	GA	*Helianthus annuus*	-92	1.000	0.875	AAAGatca	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
10	GAG	*Arabidopsis thaliana*	-72	1.000	0.875	AGAGtgt	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
11	GATA	*Arabidopsis thaliana*	-357	1.000	0.875	GATAtga	Part of a light responsive element	Arguello-Astorga and Herrera-Estrell, 1996
12	GC	*Zea mays*	-40	0.909	0.877	gcCACGt	Involved in anoxic specific inducibility	Arguello-Astorga and Herrera-Estrella 1996
13	GCN4	*Oryza sativa*	-402	1.000	0.934	taaGTCA	involved in endosperm expression	Washida et al., 1999
14	Gap-box	*Arabidopsis thaliana*	-276	1.000	0.889	AAATtgaga	Ppart of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
15	I-box	*Pisum sativum*	-290	1.000	1.000	tATATaa	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
16	LAMP	*Pisum sativum*	-273	1.000	0.875	CTTTctca	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
17	P-box	*Oryza sativa*	-396	1.000	0.857	CCTTttt	Gibberellin-responsive element	Kim et al., 1992; Washida et al., 1999
18	Skn-1	*Oryza sativa*	-405	1.000	1.000	GTCAt	Required for endosperm expression	Washida et al., 1999
19	TATA-box	*Oryza sativa*	-84	1.000	1.000	TATAaaa	Core promoter element around	Washida et al., 1999
20	TATC-box	*Oryza sativa*	-52	1.000	0.857	TATCcct	Iinvolved in gibberellin-responsiveness	Washida et al., 1999
21	WUN	*Brassica oleracea*	-189	1.000	1.000	tCATTtcca	wound-responsive element	Pastuglia et al., 1997

Table (3.3): "Cis-acting regulatory elements" in the upstream region of ns*ltp* of *M. domestica* cv. Remo (PlantCARE, Lescot et al., 2002, accesed in 04.05.2005).

No.	Motif name	Organism	Position	"Core"	"Matrix"	Sequence	Function	References
1	ABRE	*Arabidopsis thaliana*	-360	1.000	1.000	tACGTg	Involved in the abscisic acid esponsiveness	Yamaguchi-Shinozaki and Shinozaki 1994
2	ACE	*Petroselinum crispum*	-407	1.000	0.963	aaaACGTgtg	Involved in light responsiveness	Feldbrugge et al., 1996
3	AE-box	*Arabidopsis thaliana*	-425	1.000	0.852	AGAAaaat	Part of a module for light response	Park et al., 1996
4	AT1	*Solanum tuberosum*	-101	1.000	0.859	gatcATTTttca	Part of a light responsive module	-
5	ATC	*Arabidopsis thaliana*	-254	1.000	0.903	ttcaATCC	Part of a conserved DNA module involved in light responsiveness	Arguello-Astorga and Herrera-Estrella 1996
6	Box-W1	*Petroselinum crispum*	-277	1.000	1.000	TTGAcc	Fungal elicitor responsive element	Rushton et al., 1996
7	CAAT-box	*Nicotiana tabacum*	-338	1.000	1.000	CAAT	In promoter and enhancer regions	Klotz and Lagrimini1996
8	ERE	*Dianthus caryophyllus*	-44	1.000	0.875	ATTTtaaa	Ethylene-responsive element	Itzhaki et al., 1994
9	G-Box	*Antirrhinum majus*	-360	1.000	1.000	CACGta	Involved in light responsiveness	Arguello-Astorga and Herrera-Estrella 1996
10	G-box	*Brassica oleracea*	-368	1.000	1.000	aacACGTa	Involved in light responsiveness	Pastuglia et al., 1997
11	GA	*Helianthus annuus*	-108	1.000	0.875	AAAGatca	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
12	GAG	*Arabidopsis thaliana*	-129	1.000	0.857	AGAGtgt	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
13	GC	*Zea mays*	-184	0.909	0.855	tcCACGc	Involved in anoxic specific inducibility	Arguello-Astorga and Herrera-Estrella 1996
14	HSE	Brassica oleracea	-378	0.944	0.956	aAAAAattt	Involved in heat stress responsivenes	Pastuglia et al., 1997
15	I-box	*Pisum sativum*	-434	1.000	1.000	tATATaa	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
16	LAMP	*Pisum sativum*	-179	1.000	0.854	tctaATCCa	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
17	MRE	*Petroselinum crispum*	-229	1.000	0.857	AACCaaa	MYB binding site involved in light esponsiveness	Feldbrugge et al., 1996

Table (3.3): Continued "Cis-acting regulatory elements" in the upstream region of ns*ltp* of *M*. *domestica* cv. Remo (PlantCARE, Lescot et al., 2002, accesed in 04.05.2005).

No.	Motif name	Organism	Position	"Core"	"Matrix"	Sequence	Function	References
18	P-box	*Oryza sativa*	-197	1.000	0.857	CCTTtag	Gibberellin-responsive element	Kim et al., 1992; Washida et al., 1999
19	Prolamin-box	*Triticum aestivum*	-451	1.000	0.909	tgagttgtAAAGgcact	Involved in activation of zein gene endosperm development	Vicente-Carbajosa et al., 1997
20	Prolamin-box	*Oryza sativa*	-455	1.000	0.957	tgtAAAGg	Associated with GCN4	-
21	TATA-box	*Catharanthus roseus*	-321	1.000	1.000	TATA	Core promoter element	Pasquali et al., 1999
22	TCCC	*Spinacia oleracea*	-416	1.000	0.857	TCTCtct	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996
23	WUN	*Brassica oleracea*	-11	1.000	1.000	tCATTtcct	wound-responsive element	Pastuglia et al., 1997
24	chs-CMA2a	*Hordeum vulgare*	-189	1.000	0.875	GCAAgtcc	Part of a light responsive element	Arguello-Astorga and Herrera-Estrella 1996

3.9 Promoter activity analysis of the upstream region of ns*ltp*

3.9.1 Constructs

Computer analysis of the amplified upstream region by using different prediction programs revealed that this region has a predicted promoter activity close to 100%. The essential elements regions for promoter activity were amplified from *M. domestica* cv. Elstar and Remo 357 bp and 351bp respectively. The forward and reverse primers for PCR were designed containing *Bam*HI and *Eco*RI restriction sites. The amplified two fragments were inserted upstream of the DsRed gene and the constructs (Figure 3.22) were used for bombardment.

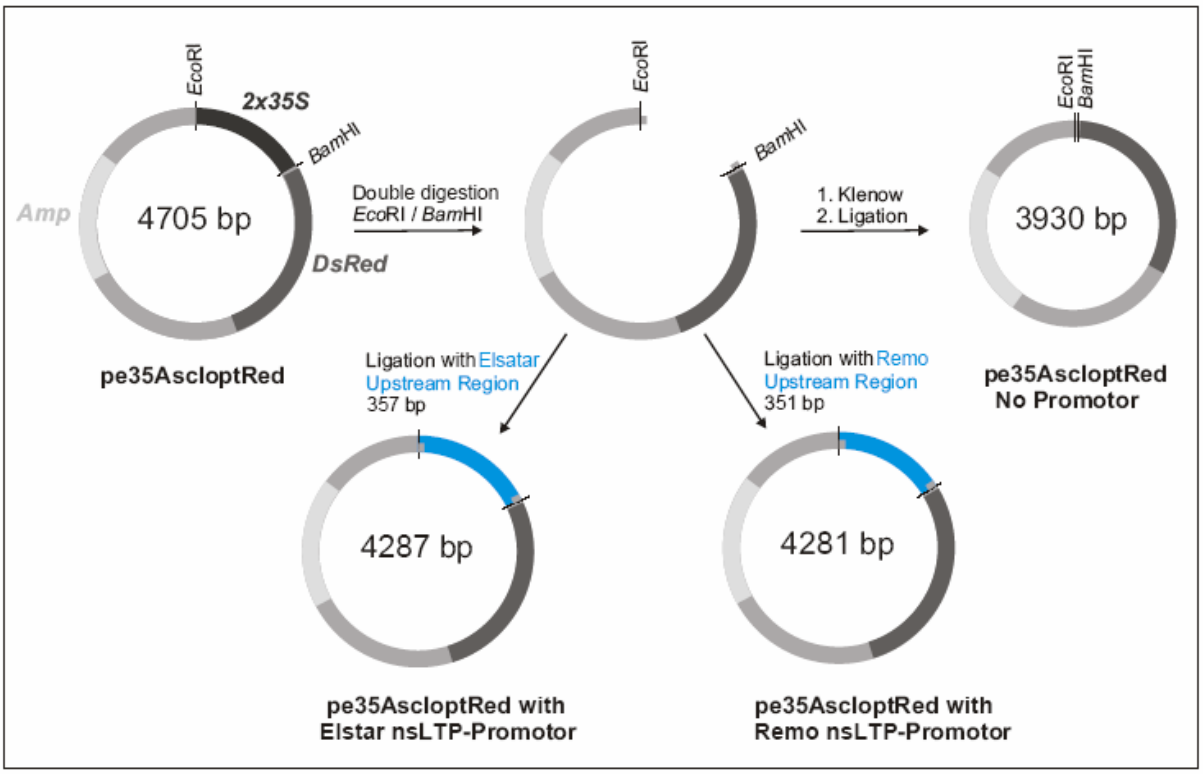

Figure (3.22): Schematic representation shows steps for cloning of the upstream regions in the pe35AscloptRed vector*; removing the double 35S promoter by double digestion with *Eco*RI and *Bam*HI; Klenow treatment and ligation to use it as a negative control without promoter; ligation of the upstream regions of *M. domestica* cv. Elstar and Remo instead of the double 35S promoter.

***** The plasmid pe35AscloptRed was kindly provided by Prof. Dr. Edgar Maiß.

For negative control preparation, the double 35S promoter was removed from the pe35AscloptRed vector by digestion with *Bam*HI and *Eco*RI.

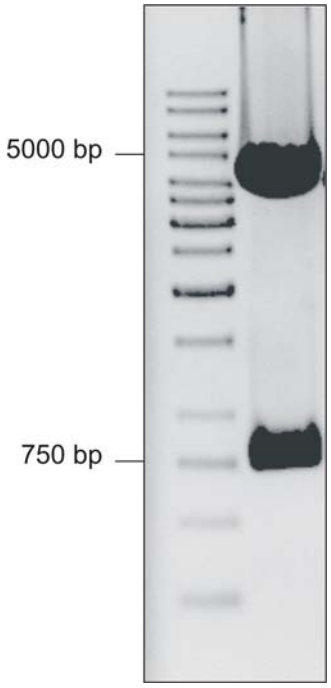

Figure (3.23): Deletion of the 2X35S promoter from the pe35AscloptRed vector.

3.9.2 Promoter activity in Elstar upstream region of ns*ltp*

After insertion of the upstream region in front of the DsRed, the plasmid was used in particle bombardment of apple leaves. Leaves from *M. domestica* cv. Gloster were used in bombardment because this cultivar showed a high frequency in transformation and transient expression. Bombarded tissues were incubated for 48 h in the growth chamber at approximately 24 °C and light-dark cycle about 12 h. During this period the plants were illuminated with fluorescent tubes (Radium white) at approximately 80 µmoles m^{-2} s^{-1} under a light/dark regime of 12 h. In the case of the negative control no signals have been detected in the bombarded tissues. On the other hand monitoring of the transformed cells by microscopy and DsRed filter when the Elstar upstream region was used showed the capability of promoter function in this upstream region (Figure 3.24).

Interestingly when the same experiment was performed but the bombarded tissues were covered in alumonium foil to keep it under dark conditions, no fluorescent signal has been detected in the bombarded tissues. These results indicate that the putative promoter of ns*ltp* in apple *Malus domestica* cv. Elstar is only active under light conditions.

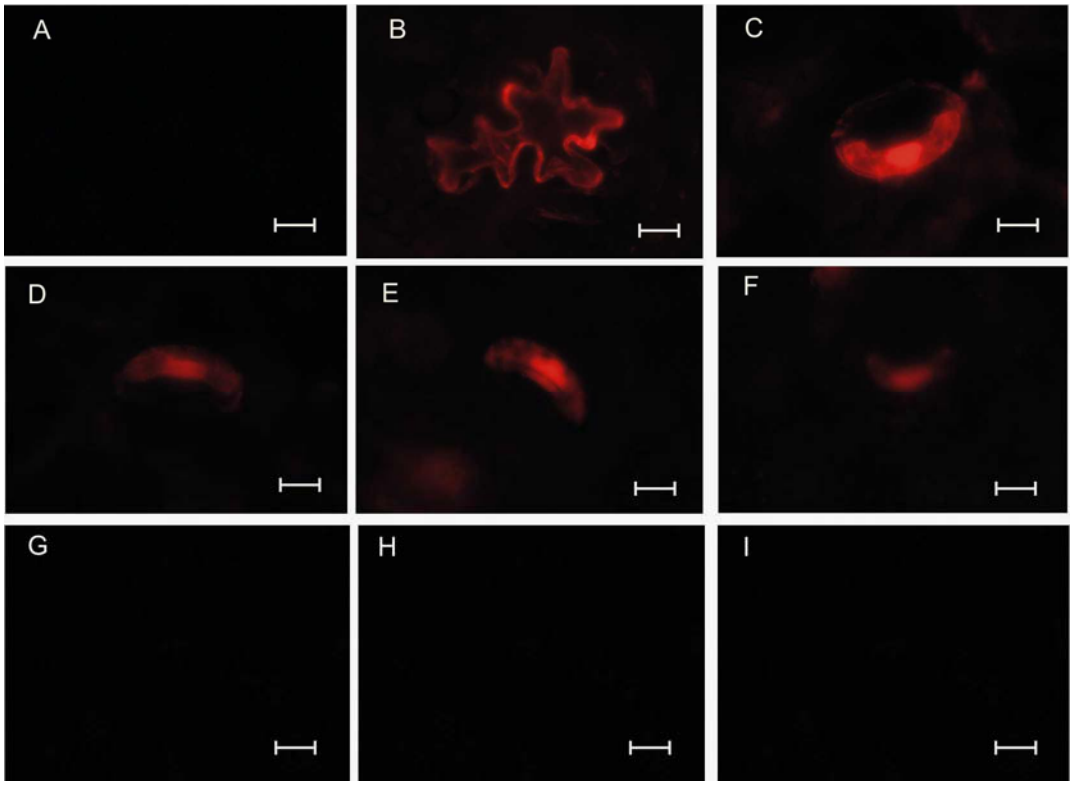

Figure (3.24): Fluorescence of DsRed in different guard and epidermal cells of apple *M. domestica* cv. Gloster after bombardment with pe35AscloptRed vector; **A:** pe35AscloptRed disarmed from the double 35S promoter as a negative control; **B** and **C:** epidermal and guard cells expressing the pe 35AscloptRed vector as a positive control; **D-F:** different guard cells expressing the Elstar upstream region- AscloptRed vector under light conditions; **G-I:**. Elstar upstream region- AscloptRed vector under dark conditions. Bar= 25 µm.

3.9.3 Promoter activity in Remo upstream region of ns*ltp*

The essential promoter elements of the ns*ltp* have been amplified from apple *Malus domestica* cv. Remo and consequently inserted in front of the DsRed in the vector pe35AscloptRed. Plasmid was used in particle bombardment of apple leaves. After shooting, leaves were incubated for 48 h in the growth chamber at approximately 24 °C and light-dark cycle about 12 h. During this period the plants were illuminated with fluorescent tubes (Radium white) at approximately 80 µmoles m^{-2} s^{-1} under a light/dark regime of 12 h. Monitoring the signal under microscope by using DsRed filter, showed that no signal in case of the negative control. On the other hand when the upstream region used, signals have been detected in the transformed cells.

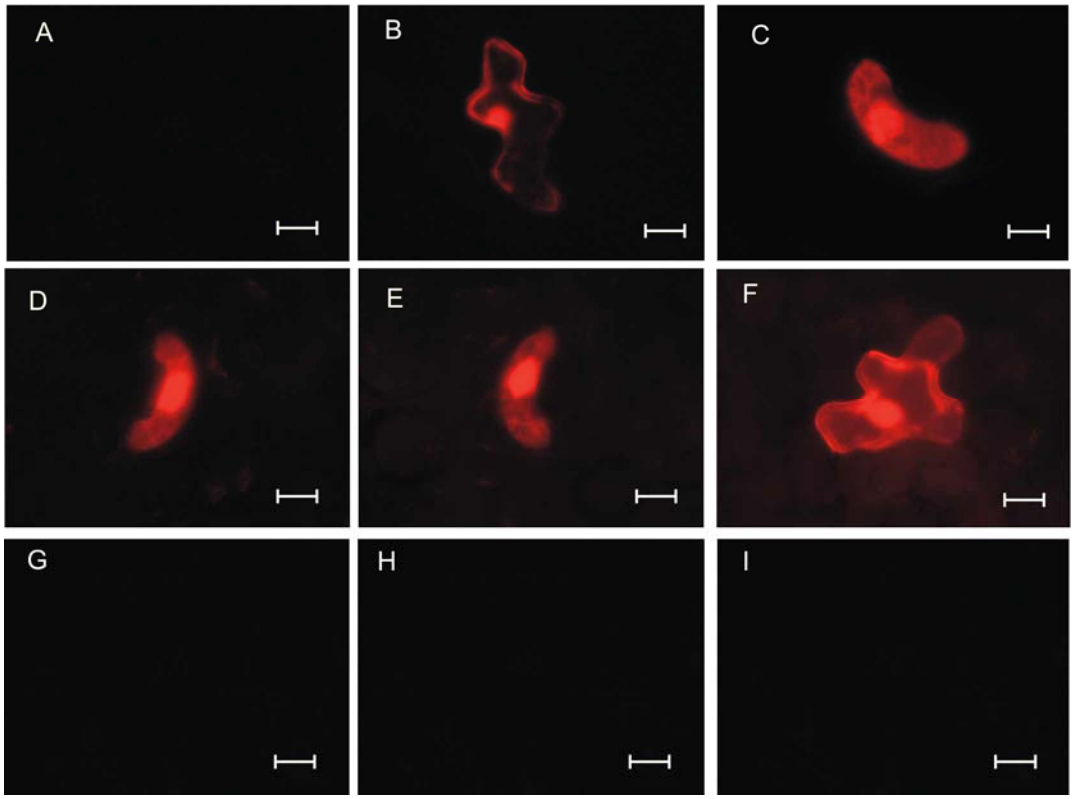

Figure (3.25): Fluorescence of DsRed in different guard and epidermal cells of *M. domestica* cv. Gloster after bombardment with pe35AscloptRed vector; **A:** pe35AscloptRed disarmed from the double 35S promoter as a negative control; **B** and **C:** epidermal and guard cells expressing the pe35AscloptRed vector as a positive control; **D-F:** different guard and epidermal cells expressing the Remo upstream region- AscloptRed vector under light conditions; **G-I:** Remo upstream region- AscloptRed vector under dark conditions. Bar= 25 µm.

However, the intensity of the fluorescence and the frequency of the transformed cells in case of Remo upstream region were higher than those of Elstar. Again when the same experiment was performed but the bombarded tissues were covered in alumonium foil to keep it under dark conditions, no fluorescent signal has been detected in the bombarded tissues. These results indicate that the putative promoter of ns*ltp* in apple *M*. *domestica* cv. Remo is only active under light conditions.

3.10 Localization of nsLTP in the plant cell

3.10.1 Overexpresion of ns*ltp* in *E. coli*

First, several attempts have done to purify the mature nsLTP from the apoplastic samples by SDS PAGE and biotrap and electroelution, but unfortunately none of them has succeeded. May be the small molecular mass of nsLTP caused the failure of the purification process.

Therefore, it was necessary to overexpress this protein in *E. coli*. In attempt to produce a recombinant nsLTP the cDNA was inserted in the expression vector pMAL-c2X downstream of the malE gene, which encodes for maltose binding protein (MBP). The Epicurian Coli® BL21-CodonPlus™ (DE3)-RIL was used as a host for this expression vector. The ns*ltp* was expressed in fusion to maltose binding protein in the cytocol. After rapture the bacterial cells by using Frensh press, affinity Chromatography and SDS PAGE (Figure 3.26), fusion protein has been treated by protease Factor-X. Unfortunately the overexpressed nsLTP could not be cleaved from the fusion protein. Therefore we used the whole fusion protein for further studies including polyclonal antibody production and as well as metal binding activity test.

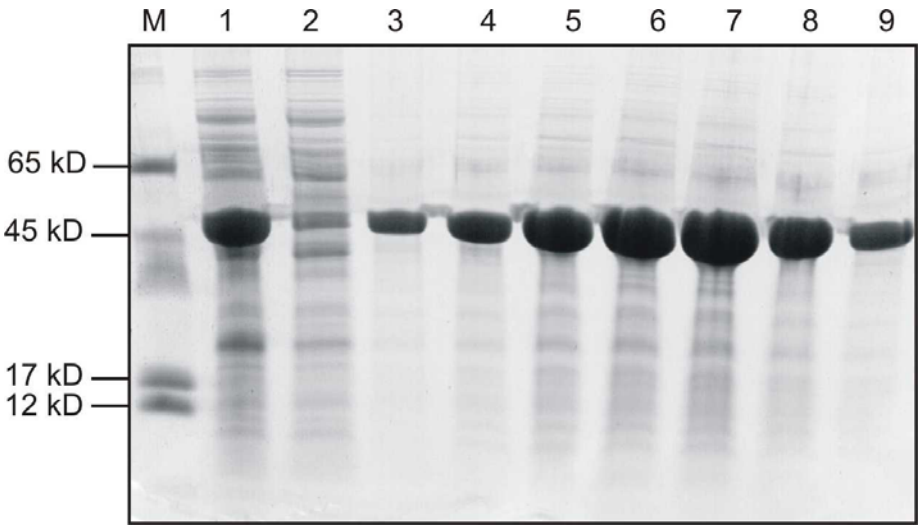

Figure (3.26): SDS PAGE of the over-expressed nsLTP in fusion with Maltose binding protein; **M:** protein marker; **1:** bacterial lysate after induction with IPTG; **2:** flow through; **3-9:** different fractions collected from the column. Each lane was loaded with 10 µl of protein sample.

The recombinant nsLTP was purified by using two different column systems. Pure protein was checked in SDS PAGE (Figure 3.27) and subsequently sent for polyclonal antibody production from goat. The produced antibody was used to detect the protein level of nsLTP in the apoplastic fluid. Unfortunately, the specificity of this antibody was not perfect for the *in situ* localization analysis. A possible explanation for this low specificity it might be the proportion of the nsLTP to maltose binding protein (9:42 kD).

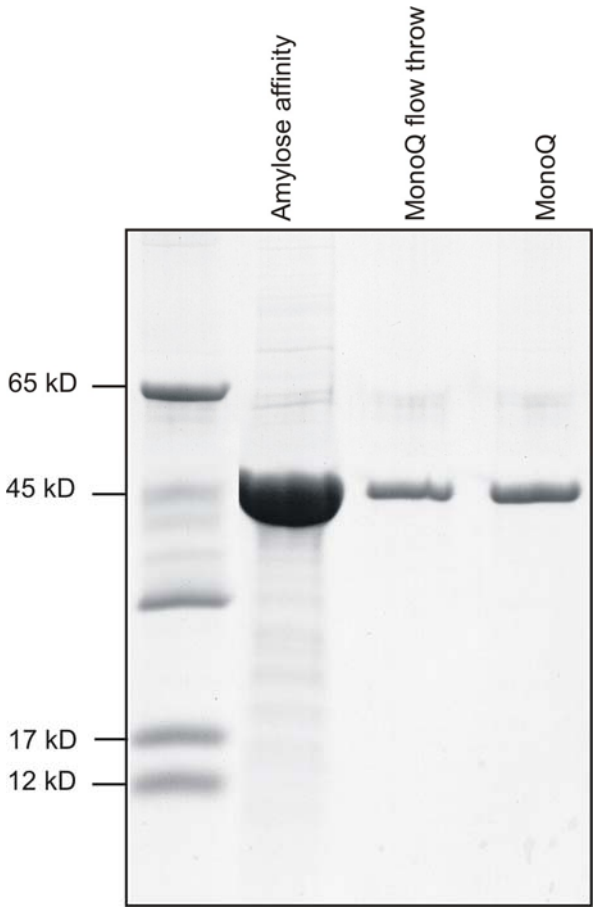

Figure (3.27): SDS PAGE of the column purification of the recombinant nsLTP.

3.10.1.1 Detection of the antibody specificity

To determine the degree of specificity of the antibody that has been raised against the recombinant protein (nsLTP in fusion with MBP), the produced antibody was used to detect the nsLTP in the apoplastic fluid that obtained from apple *M*. *domestica* leaves. Two different harvest of the antibody with a given numbers 134 and 135 were used in different dilutions. Since the nsLTP constitutes around 90 % of the in the apoplast of the young tissues, IWF was collected from young leaves of *M. domestica* cv. Elstar and as well as from the old leaves. Western blot was done as described in 2.11.1.8 Signals were detected by using CCD camera (Figure 3.28). The result presented below showed that this antibody could crosslinked with more than one protein in the apoplastic fluid. Moreover, the apparent molecular masses of the detected proteins are higher than that of the nsLTP. Therefore, based on this result, it can be concluded that the produced antibody seems to be nonspecific against nsLTP.

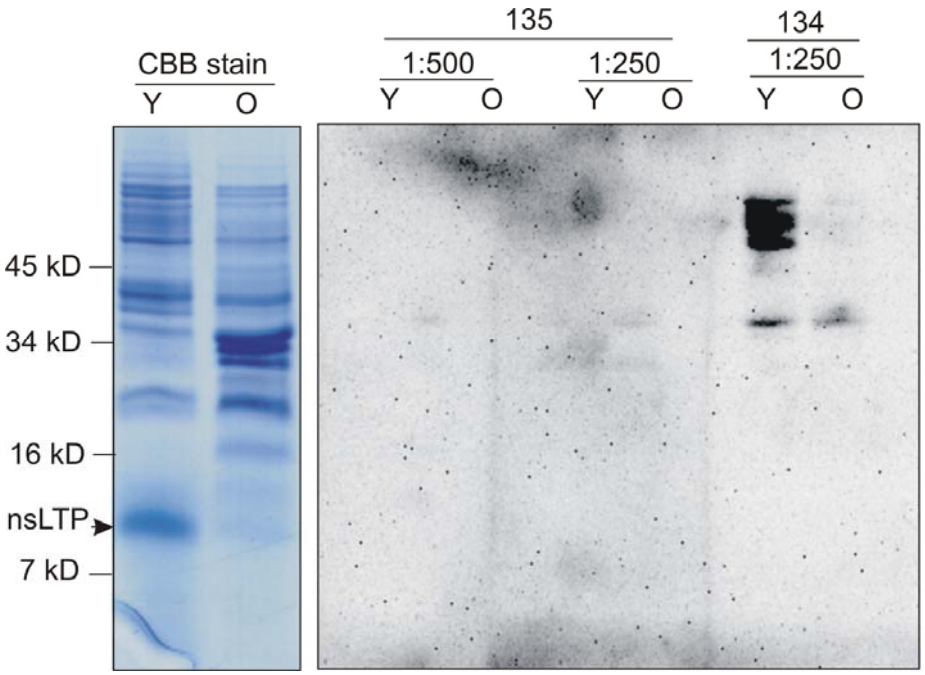

Figure (3.28): Immunoblot assay for nsLTP detection in the apoplastic fluid from *M*. *domestica* cv. Elstar **Y:** young leaves; **O:** old leaves. CBB: Coomassie brilliant blue. Each lane was loaded with 10 µg protein.

3.10.2 GFP based analysis and subcellular localization of nsLTP

3.10.2.1 Constructs design

In attempt to elucidate the possible localization and function of nsLTP different constructs (Figure 3.29) have been used in this study.

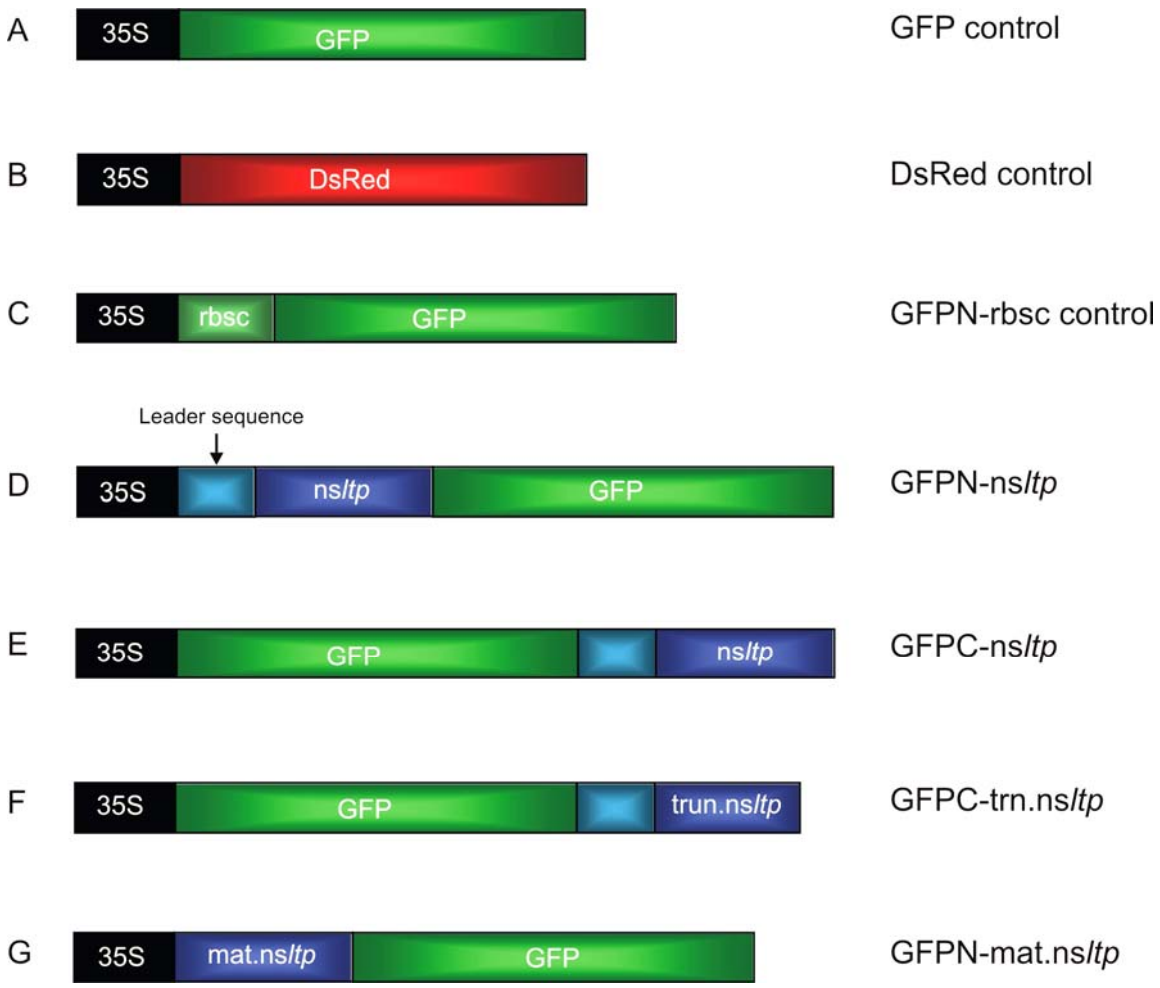

Figure (3.29): Plasmid constructs used for transient expression. Constructs are not drawn to scale. **A:** GFP under the control of 35S promoter as a positive control; **B:** DsRed under the control of 35S promoter as a second positive control; **C:** GFP in fusion with the transit peptide of the small subunit of RubisCO under control of 35 S promoter as a third positive control for chloroplast localization; **D:** N-terminal fusion of ns*ltp* with GFP; **E:** C-terminal fusion of *nsltp* with GFP; **F:** C-terminal fusion of the truncated ns*ltp* with GFP; **G:** N-terminal fusion of the mature ns*ltp* with GFP Abbreviations used: 35S: 540 bp fragment of the cauliflower mosaic virus (CaMV) 35S promoter; ns*ltp*: full length of nonspecific lipid transfer protein gene from *M*. *domestica* Elstar; GFP: green fluorescent protein gene from *Aequorea victoria*; rbsc: transit peptide of the small subunit of RubisCO; mat: mature (signal peptide of the nsLTP was deleted); trun: truncated (11 amino acids were deleted from the C-terminal domainof nsLTP).

3.10.2.2 Building the constructs

The full length, mature and truncated ns*ltp* were amplified by PCR and the corresponding primers which contained the suitable restriction sites for cloning in the pBSKGFP vector.

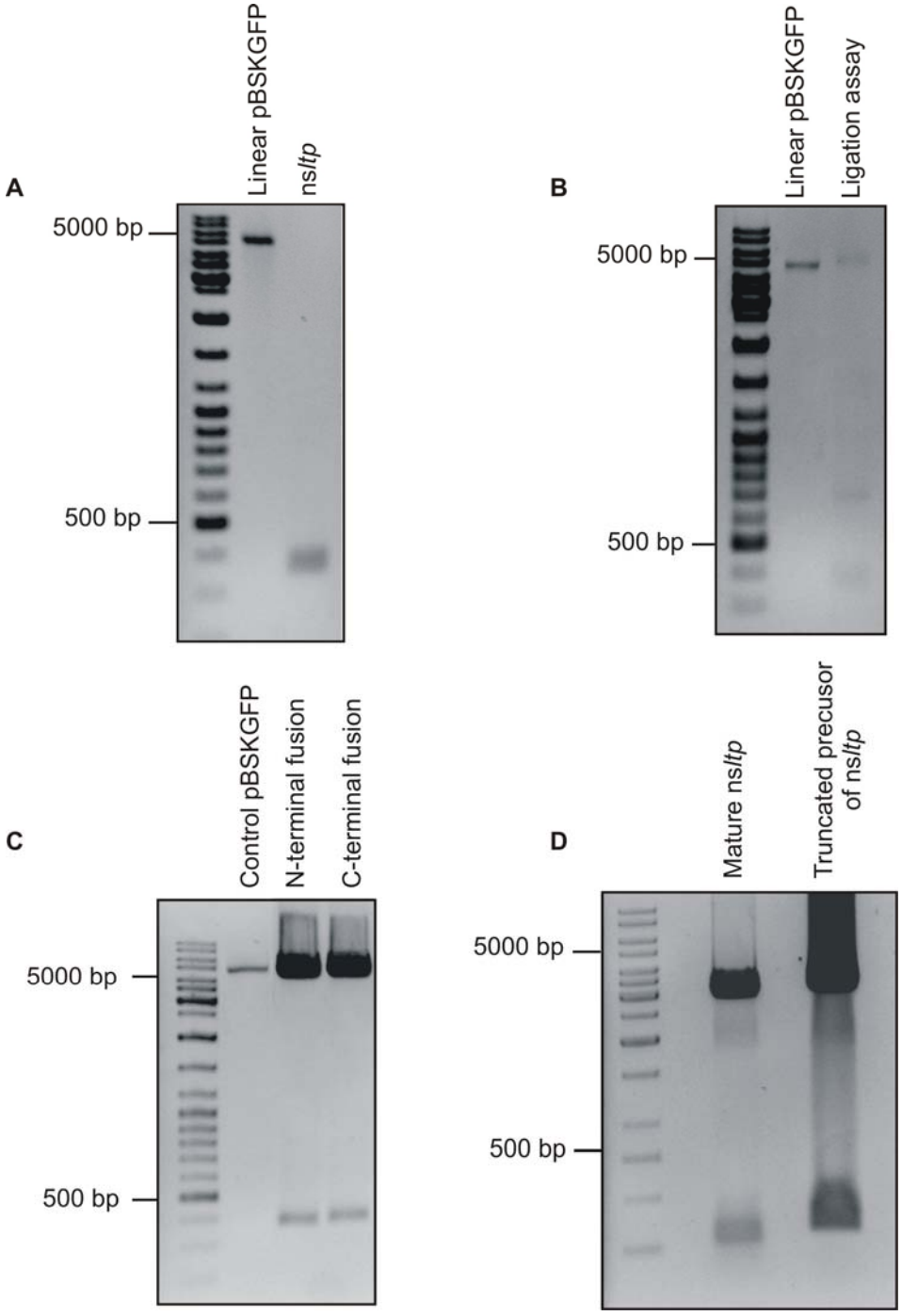

Figure (3.30): GFP constructs building **A:** Pure insert and linear backbone; **B:** ligation assay; **C:** N-terminal and C-terminal fusion of ns*ltp* with GFP constructs; **D:** mature and ligated ns*ltp* in pGEMT-vector.

3.10.2.3 Confirmation of the fusion chimera

It was necessary to confirm that there is no frame shift in the fusion chimera, therefore constructs have been sent for sequencing by using GFP specific primers and analyzed (see below).

Fusion of nsLTP in N-terminal of GFP

In this case it was necessary to avoid the stop codon of ns*ltp* to be in the same ORF with GFP and subsequently to allow perpetuation of the translation in fusion with GFP. Therefore, by using generated reverse primer and mutagenesis PCR, the stop codon has been changed to serine residue and the last positive amino acid lysine was also replaced by another positive one arginine. That was in attempt to keep the structure of nsLTP more or less similar to the wild type (see below).

```
atggctaact ctacaatgac caaccttact ttaatgatgg ccttgtacat gacgatgaac
  M  A  S   S  A  V   T  N  L  A   L  V  V   A  L  C   M  A  V  S

gttgctcatg ccataacatg tggccaagtg accagcagcc ttgcgccatg cattggctac
  V  A  H   A  I  T   C  G  Q  V   T  S  S   L  A  P   C  I  G  Y

gtgaggaagg gcggaggtgt ccctccaact tactacaatg gaatcagaac cattaacggc
  V  R  S   G  G  A   V  P  P  A   C  C  N   G  I  R   T  I  N  G

ttggccagga ccaccgctga ccgccagact gcttgcaact gcctgaagaa tcttgccggc
  L  A  R   T  T  A   D  R  Q  T   A  C  N   C  L  K   N  L  A  G

agcatcagtg gtgttaaccc taacaatgca gcagggcttc ctggaaagtg tggagtcaac
  S  I  S   G  V  N   P  N  N  A   A  G  L   P  G  K   C  G  V  N

gtcccctaca agatcagcac ctccaccaac tgcgccaccg tgagatctat ggtgagcaag
  V  P  Y   K  I  S   T  S  T  N   C  A  T   V  R  S   M  V  S  K

ggcgaggagc tgttcaccgg ggtggtgccc atcctggtcg a
  G  E  E   L  F  T   G  V  V  P   I  L  V
```

Figure (3.31): Deduced amino acid sequence of the retrieved nucleotide sequence of the full length of apple ns*ltp* in fusion with N-terminal domain of GFP. Underlined sequence indicates the fusion site and GFP N-terminal domain.

Fusion of nsLTP in C-terminal of GFP

```
cgcatcgcag acgacttaca gtctggaagcc atggctaggt ctgcagtgac caaccttgct
  R   I   A   D   D   L   Q   S   G   A   M   A   S   S   A   V   T   N   L   A

ttggtggtgg ccttgtgcat ggcggtgagc gttgctcatg ccataacatg tggccaagtg
  L   V   V   A   L   C   M   A   V   S   V   A   H   A   I   T   C   G   Q   V

accagcagcc ttgcgccatg cattggctac gtgaggagtg gcggagctgt ccctccaggt
  T   S   S   L   A   P   C   I   G   Y   V   R   S   G   G   A   V   P   P   A

tgctgcaatg ggatcaggaac cattaacggc ttggccaggg ccaccgctga ccgccaggct
  C   C   N   G   I   R   T   I   N   G   L   A   R   T   T   A   D   R   Q   T

gcttgcaact gcctgaaggaa tcttgccggc aggatcagtg gtgttaaccc taacaatgca
  A   C   N   C   L   K   N   L   A   G   S   I   S   G   V   N   P   N   N   A

gcagggcttc ctggaaagtg tggagtcaac gtcccctaca agatcagcac ctccaccaac
  A   G   L   P   G   K   C   G   V   N   V   P   Y   K   I   S   T   S   T   N

tgcgccaccg tgagatctta aa
  C   A   T   V   R   S   -
```

Figure (3.32): Deduced amino acid sequence of the retrieved nucleotide sequence of the full length of apple ns*ltp* in fusion with C-terminal domain of GFP. Underlined sequence indicates the fusion site and GFP C-terminal domain.

Fusion of truncated nsLTP in C-terminal of GFP

```
ttacaatcta gaaccatggc taactctaca gtaaccaaac ttactttgat gatggcctta
  L   Q   S   G   A   M   A   S   S   A   V   T   K   L   A   L   V   V   A   L

tgcatggcgg tgagcgttgc tcatgccata acatgtggcc aagtgaccag cagccttgcg
  C   M   A   V   S   V   A   H   A   I   T   C   G   Q   V   T   S   S   L   A

ccatgcattg gctacgtgag gagtggcgga gctgtccctc caacttgcta caatggaatc
  P   C   I   G   Y   V   R   S   G   G   A   V   P   P   A   C   C   N   G   I

aggaaccatta acggcttggc caggaccacc gctgaccgcc aggctgcttg caactgcctg
  R   T   I   N   G   L   A   R   T   T   A   D   R   Q   T   A   C   N   C   L

aagaatcttg ccggcagcat cagtggtgtt aaccctaaca atgcaggagg gcttcctgga
  K   N   L   A   G   S   I   S   G   V   N   P   N   N   A   A   G   L   P   G

aagtgtggag tcaacgtccc ctacaagtag
  K   C   G   V   N   V   P   Y   K   -
```

Figure (3.33): Deduced amino acid sequence of the retrieved nucleotide sequence of the truncated apple ns*ltp* in fusion with C-terminal domain of GFP. Underlined sequence indicates the fusion site and GFP C-terminal domain.

Mature nsLTP in in N-terminal of GFP

To avoid the stop codon of ns*ltp* the same procedure was followed as described in 3.8.2.1 (see below).

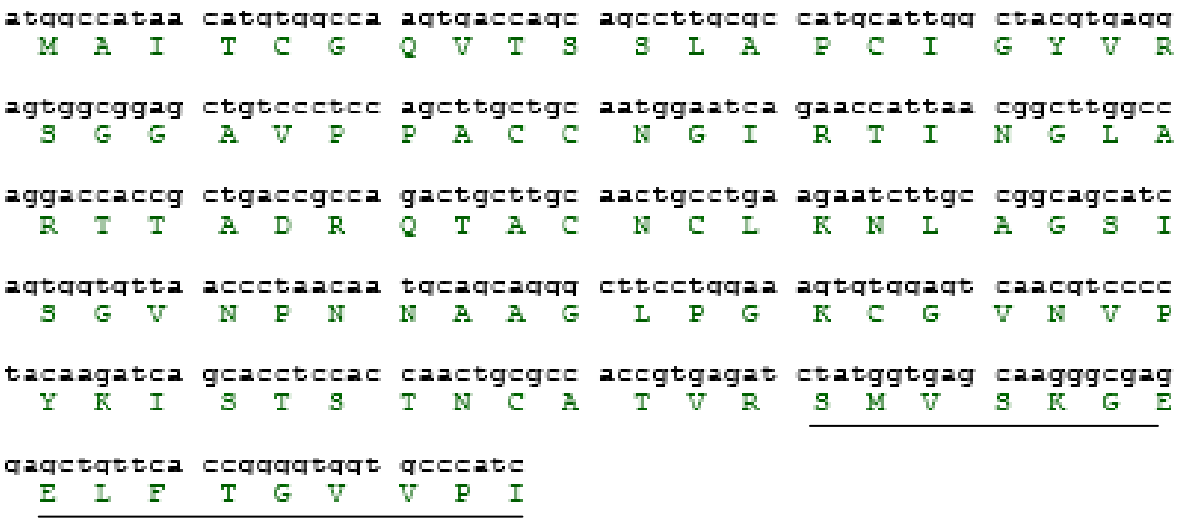

Figure (3.34): Deduced amino acid sequence of the retrieved nucleotide sequence of the full length of apple ns*ltp* in fusion with C-terminal domain of GFP. Underlined sequence indicates the fusion site and GFP N-terminal domain.

3.10.2.4 Particle bombardment and transient expression

3.10.2.4.1 N-terminal fusion under dark conditions

For functional localization of the nsLTP, particle bombardment and transient expression of different GFP-ns*ltp* constructs were performed. In all experiments GFP under the control of double 35S promoter was used as a first positive control and DsRed under the control of 35S promoter was used a second positive control. In these two cases the fluorescence detected under microscope was distributed through the whole cytosol with a higher density around the nucleus region (Figure 3.35).

On the other hand when the N-terminal fusion of GFP-ns*ltp* was used for shooting, leaves were wrapped in aluminum foile and to keep it in dark incubated for 48 h in the growth chamber at approximately 24 °C. The fluorescence pattern was completely different than in case of control. This pattern revealed that ns*ltp* was expressed and translocated through cytosol to chloroplast, and probably to other compartments including mitochondria, peroxisomes and bodies like organelles (Figure 3.36). Remarkably the transient expression of GFPN-ns*ltp* in the artificially infected leaves

showed that nsLTP is localized in structures propably the cytoskeletal filaments (3.36L), for details see (3.11).

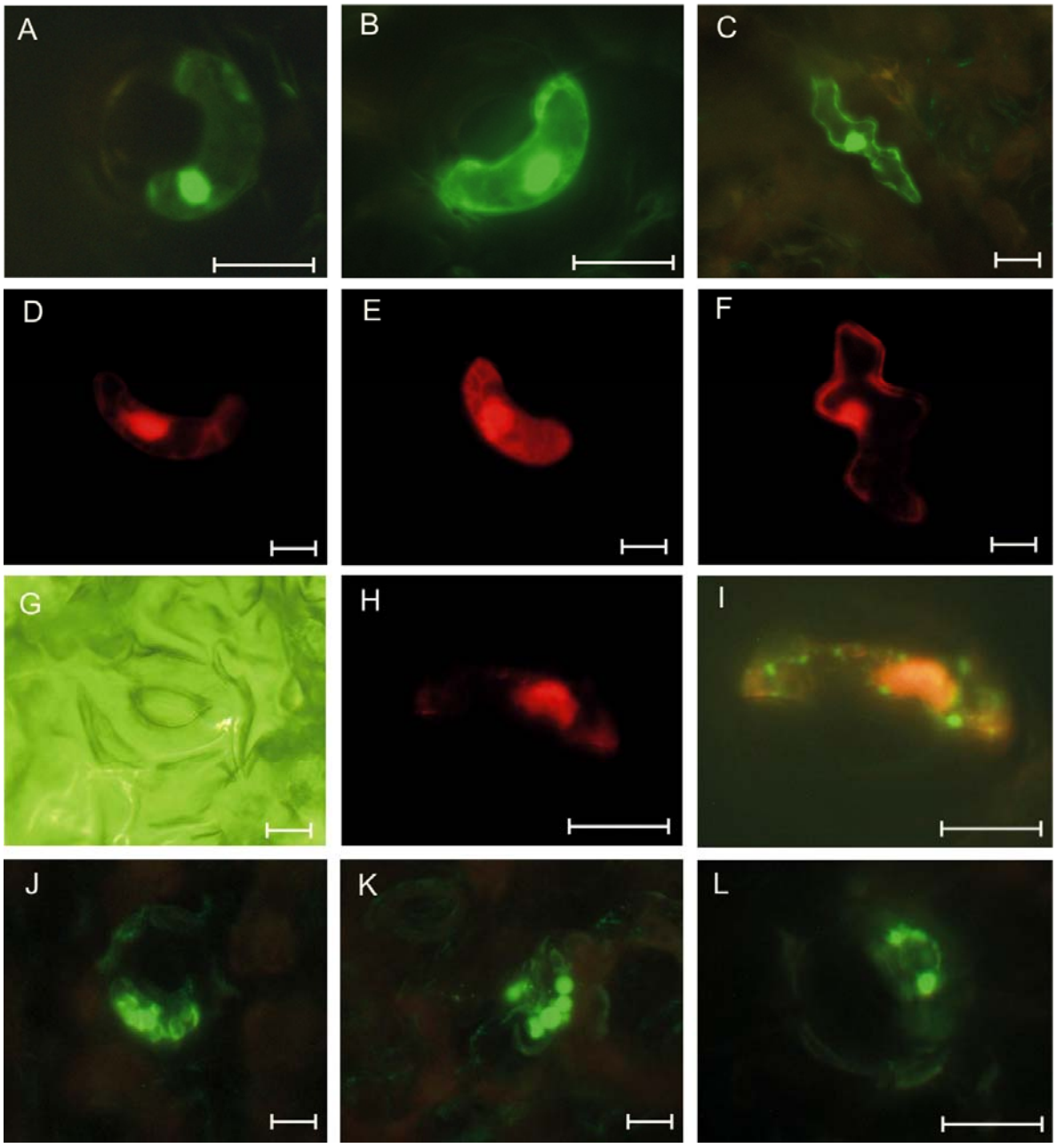

Figure (3.35): Transient expression of GFP and ns*ltp* in apple *M. domestica* cv. Gloster leaves tissues. **A-C:** fluorescence of GFP in different guard and epidermal cells after bombardment with pBSK carrying the GFP as a first control; **D-F:** fluorescence of DsRed in different guard and epidermal cells as a second control; **G-I:** Transformed guard cell bombarded with 1:1 mixture of control DsRed and pBSK carrying GFPN-ns*ltp* under dark conditions; **G:** bright field and high magnified image **H:** fluorescence o f DsRed; **I:** fluorescence of GFPN-ns*ltp*, indicating the localization of nsLTP in different cell compartments (mitochondria and peroxisomes); **J** and **K:** transformed guard cells bombarded with pBSK carrying GFPN-ns*ltp* under dark conditions, indicates the localization of ns*ltp* in the chloroplast and bodies like organelles; **L:** transformed guard cells expressing the GFPN-ns*ltp* under dark conditions in artificially infected leaves with *V. inaequalis*. Bar= 25 µm.

Moreover, the DsRed as a second positive control was combined with the N-terminus fusion of GFP-ns*ltp* in one experiment to build a clear conclusion. In this experiment the expressing DsRed control was localized in the cytosol with a higher density in the nucleus region forming a red background and again the fusion appeared in different compartments.

It is noteworthy that all of the above experiments were conducted under dark (leaves were covered with aluminum foil to keep it under dark conditions within the growth chamber.

3.10.2.4.2 N-terminus fusion under light conditions

To study the light influence, apple leaves were shot by using the N-terminus fusion GFPN-ns*ltp* and incubated for 72 h. It has been found that chloroplasts within the injured bombarded guard cell were highly swollen and the fusion protein in this case was localized in the chloroplasts within the guard cells (Figure 3.36). The fluorescence of GFP in the N-terminus fusion was slightly yellowish.

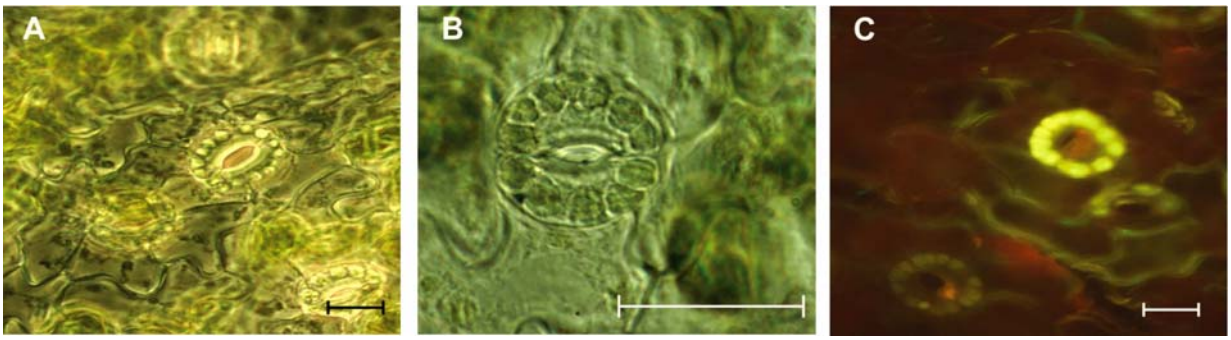

Figure (3.36): Transient expression of GFPN-ns*ltp* under light conditions in apple *M. domestica* cv. Gloster leaves tissues; **A:** bright field of different guard cells; **B:** bright field and high magnified image of one guard cell showing highly swollen chloroplasts; **C:** fluorescence of GFPN-ns*ltp*, indicating the localization of nsLTP in the swollen chloroplasts. Bombarded leaves were incubated in the growth chamber at approximately 24 °C and light-dark cycle about 12 h. During this period the plant leaves were illuminated with fluorescent tubes (Radium white) at approximately 80 µmoles m^{-2} s^{-1} under a light/dark regime of 12 h. Bar= 25 µm.

3.10.2.4.3 C-terminus fusion under light and dark conditions

In another experiment the ns*ltp* was fused in the C-terminal of GFP. The construct was used for bombardment and subsequently leaves were incubated for 48 h. Monitoring the fluorescent signals by microscope and GFP filter in the transformed cells revealed that the fusion protein has been expressed by cytoplasmic machinery and subsequently translocated to the chloroplast (Figure 3.37). On the other hand no signals could be detected when the same construct was used for particle bombardment under dark conditions. This experiment has been repeated several times.

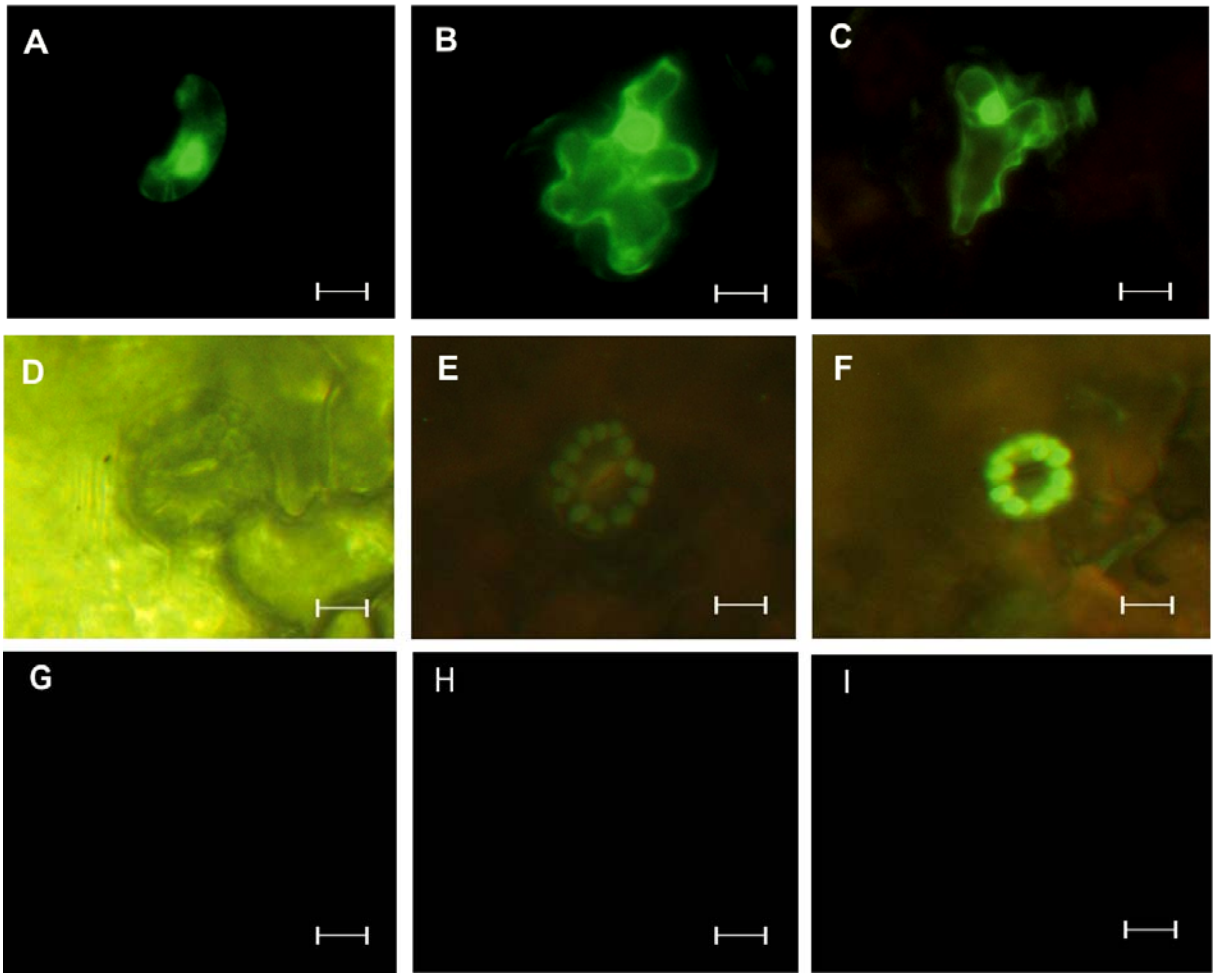

Figure (3.37): A-C: Fluorescence of GFP in different guard and epidermal cells of apple *M. domestica* cv. Elstar after bombardment with pBSK carrying the GFP as a control; **D** and **E:** two transformed guard cells expressing GFPC-ns*ltp* under light conditions; **D:** bright field; **E:** fluorescence of GFPC-*nsltp*, indicating the localization of ns*ltp* in the chloroplasts; **F:** additional two guard cells. G-I: GFPC-nsltp under dark conditions. Bombarded leaves were incubated in the growth chamber at approximately 24 °C and light-dark cycle about 12 h. During this period the plant leaves were illuminated with fluorescent tubes (Radium white) at approximately 80 µmoles m^{-2} s^{-1} under a light/dark regime of 12 h. For dark conditions plates were covered by aluminum foil. Bar= 25 µm.

3.10.2.4.4 Truncated ns*ltp* C-terminus fusion under light conditions

To determine whether the C-terminal domain in the nsLTP is involved in sorting process or not, 11 amino acids were deleted from this domain. The truncated ns*ltp* was fused with the C-terminal domain of GFP and the developed construct was used for particle bombardment. Transient expression of this fusion protein in apple leaves was detected after 48 h. Result showed that the fusion was localized in the chloroplasts in the guard cells (Figure 3.38).

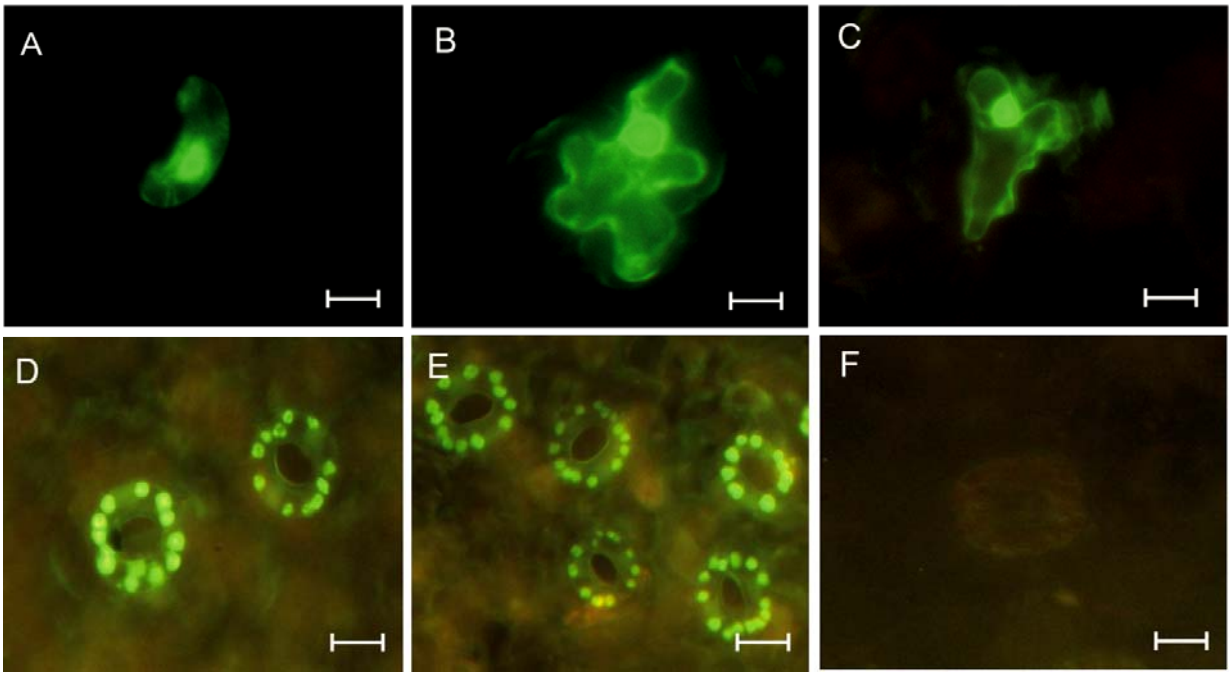

Figure (3.38): A-C: Fluorescence of GFP in different guard and epidermal cells of apple *M. domestica* cv. Elstar after bombardment with pBSK carrying the GFP as a control; **D:** and **E:** guard cells expressing GFPC-trun.ns*ltp* under light conditions, indicating the localization of the truncated nsLTP in the chloroplast; **F:** chlorophyll fluorescence in two guard cells as a negative control. Bombarded leaves were incubated in the growth chamber at approximately 24 °C and light-dark cycle about 12 h. During this period the plant leaves were illuminated with fluorescent tubes (Radium white) at approximately 80 µmoles m^{-2} s^{-1} under a light/dark regime of 12 h. Bar= 25 µm.

3.10.2.4.5 Truncated ns*ltp* C-terminus fusion under dark conditions

The truncated ns*ltp* was fused with the C-terminal domain of GFP and the developed construct was used for particle bombardment. After bombardment the leaves were covered with aluminum foil and incubated in the growth chamber at approximately 24 °C. Transient expression of this fusion protein in apple leaves was detected after 48 h after bombardment. Result showed that the fusion was localized also in the chloroplasts in the guard cells (Figure 3.39). No differences between light and dark conditions were detected with this construct.

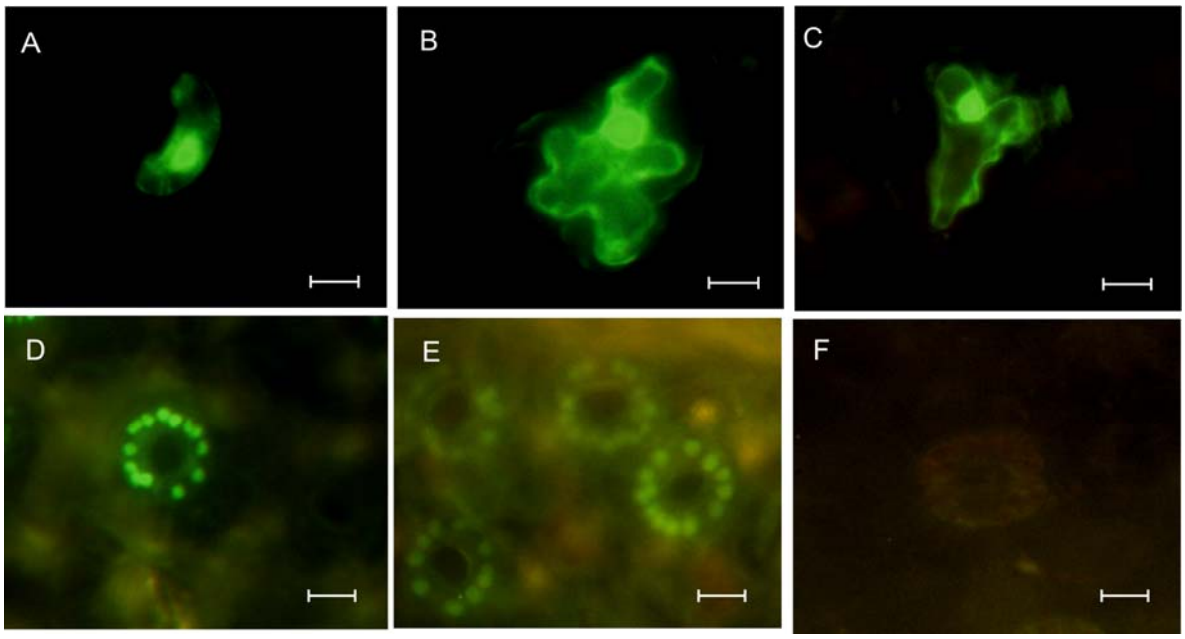

Figure (3.39): A-C: Fluorescence of GFP in different guard and epidermal cells of apple *M*. *domestica* cv. Elstar after bombardment with pBSK carrying the GFP as a control; **D:** and **E:** guard cells expressing GFPC-trun.ns*ltp* under dark conditions, indicating the localization of the truncated nsLTP in the chloroplast; **F:** chlorophyll fluorescence in two guard cells as a negative control. Bar= 25 µm.

3.10.2.4.6 Mature ns*ltp* and N-terminus fusion under light and dark conditions

A general consensus has been built for nsLTP that they have a putative leader sequence responsible for entering the secretory pathway. To verify this consensus the first 24 amino acids from the N-terminus domain were deleted by PCR. After insertion of the mature ns*ltp* in the N-terminal domain of the GFP plasmid was used for particle bombardment. Half of the bombarded samples were covered with aluminum foil to keep it under dark conditions in the same growth chamber. Transient

expression of mature ns*ltp* in fusion with N-terminal of GFP showed that the fusion protein failed to enter the secretory pathway and localized in the cytosol (Figure 3.40).

In addition no fluorescence could be detected in the apoplast when the precursor of nsLTP was used in fusion with GFP in both N and C-terminus fusion (GFPN-ns*ltp* and GFPC-ns*ltp*).

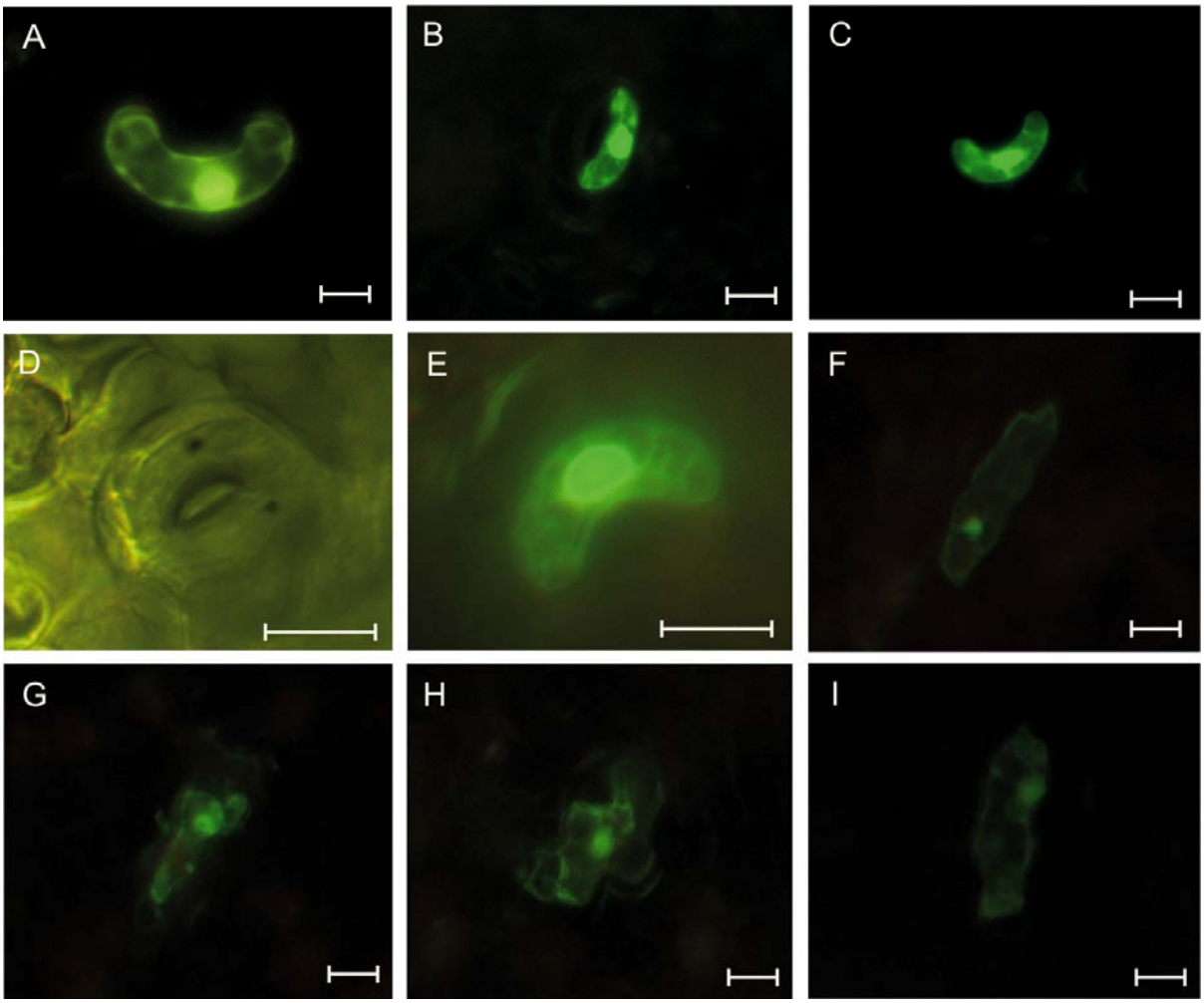

Figure (3.40): A-C: Fluorescence of GFP in different guard cells of apple *M. domestica* cv. Gloster after bombardment with pBSK carrying the GFP as a control; **D-E:** guard cell expressing the GFP-mat.ns*ltp* under light conditions; **D:** bright field and high magnified image; **E** and **F:** dark field image showing the failure of the mature protein to enter the secretory pathway in guard and epidermal; cells; **G-I:** epidermal cells expressing the GFP-mat.ns*ltp* under dark conditions. Bombarded leaves were incubated in the growth chamber at approximately 24 °C and light-dark cycle about 12 h. During this period the plant leaves were illuminated with fluorescent tubes (Radium white) at approximately 80 µmoles m^{-2} s^{-1} under a light/dark regime of 12 h. Bar= 25 µm.

3.11 Transient expression of the GFPN-ns*ltp* in response to the infection with *V. inaequalis* under dark conditions

In an attempt to elucidate the role and the behavior of *M. domestica* nsLTP toward the infection process, leaves of the susceptible apple cultivar Gloster were harvested and sprayed with conidial suspension of *V. inaequalis*. Subsequently these leaves were used for bombardment with a plasmid containing the GFPN-ns*ltp* chimera. Bombarded leaves were incubated in Petri dishes that were covered with aluminum foil for 48 h at 24 C°. The bright field image showed that *V. inaequalis* conidia started to germinate sending their germ tubes bypassing the stomatal pore to penetrate the cuticle and subsequently to invade the plant tissues (Figure 3.41A). On the other hand monitoring the GFP signals by microscopy and GFP filter revealed that the fluorescence are coming from structures possibly the cytoskeletal filaments within the transformed guard cell (Figure 3.41B).

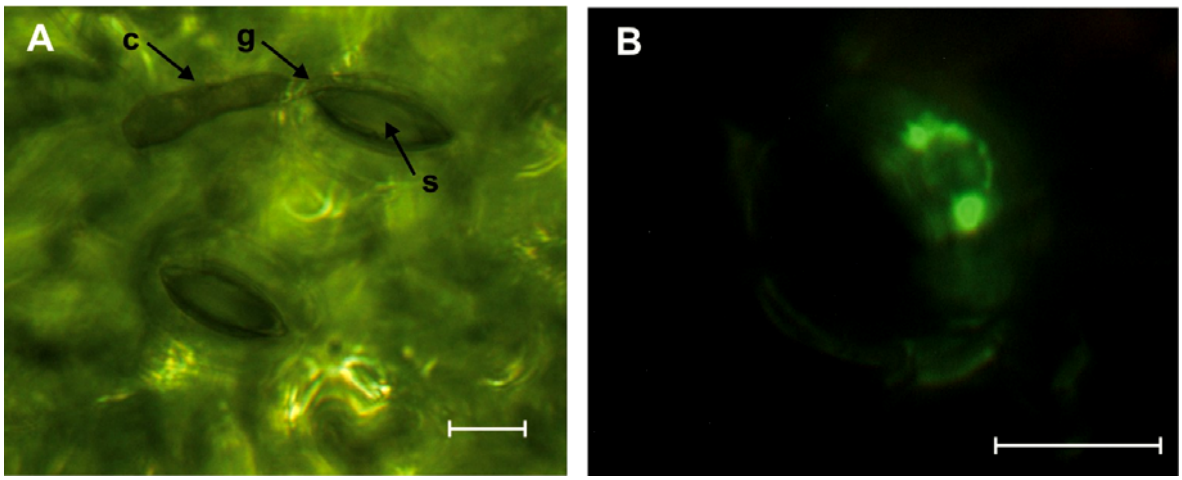

Figure (3.41): A: Bright field image shows the conidial germination on the lower leaf surface of *M. domestica* cv. Gloster; c: conidia; g: germ tube; s: stomatal aperture. **B:** One transformed guard cell expressing the GFPN-ns*ltp*. Bar= 25 µm.

3.12 Protein level of nsLTP and age

It has been reported that the fully expanded leaves are resistant to *Venturia inaequalis* in all members of the genus *Malus* (Valsangiacomo and Gessler 1988). One question has been arisen is there a possible correlation between nsLTP and the ontogenic resistance? To address this question it was necessary to determine whether the level of nsLTP in the apoplast is influenced by the age of the plant particularly in the susceptible cultivar, three different IWF samples (young, middle and old) from healthy *M*. *domestica* cv. Elstar were collected by using PBS buffer. Soluble protein contents of the apoplastic fluid were separated on SDS PAGE. It is noteworthy to mention that same amounts of proteins were used for comparison. Result presented in Figure 3.42 revealed that the level of the putative nsLTP with the same molecular mass 9 kD has declined by age to almost undetectable level in the old leaf tissues.

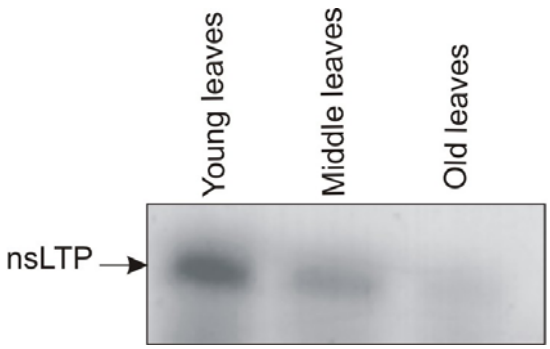

Figure (3.42): SDS PAGE for soluble protein in the IWF from leaves of *M*. *domestica* cv. Elstar with different age; young, middle, and old. Each lane was loaded by 50 µg protein (based on Bradford determination) and subsequently stained by CBB. The corresponding part for nsLTP bands was excised from gel by computer program.

3.13 Epigenetic information and gene expression

The finding that the declining of the protein level of ns*ltp* was accompanied by decrease of ns*ltp* transcript suggests a transcriptional or posttranscriptional control. To confirm this suggestion the DNA methylation has been studied as a key epigenetic parameter that can affect the transcription process. To perform this, the methylation sensitive restriction endonucleases and southern blot procedure was used.

3.13.1 Amplification of the full length of the ns*ltp* and DIG labeling

After extraction of genomic DNA from Elstar, this pure DNA was used as a template for PCR to amplify the full length of ns*ltp* and the upstream region using the corresponding primers (forward; 5'-CCC TTA CAT TTT AAA ATA GAG TG-3', reverse; 5'-TAC TTC ACG GTG GCG CAG TT-3'). PCR products were diluted 1 to 10 and again used as a template for PCR by using the same primers and the U-DIG labeled nucleotide in the nucleotide mixture. The PCR products were checked on 1.5 % agarose gel (Figure 3.43). From this figure it can observed that the probe is already DIG labeled because it has a larger size than that nonlabeled due to the incorporation of the DIG-labeled nucleotide.

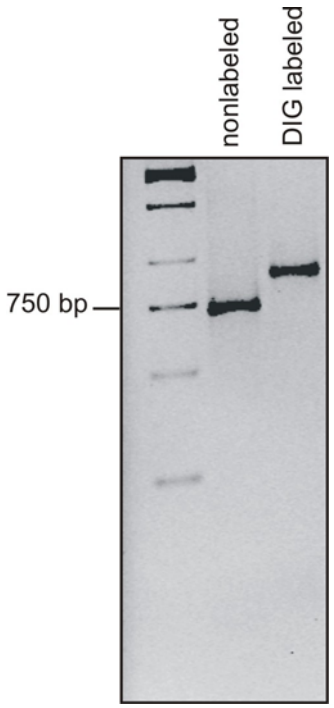

Figure (3.43): DIG labeling confirmation of the full length of ns*ltp* from *M. domestica* cv. Elstar before using it as a probe in the hybridization in southern blot on 1.5 % agarose gel.

3.13.2 Distribution of the methyl sensitive restriction enzyme sites in ns*ltp*

For hybridization the full length of ns*ltp* with 716 bp (the upstream region and as well as the transcribed region) was used as a probe. This amplified fragment was screened for the presence of the methylation sensitive restriction enzymes sites. Four different recognition sites were found distributed through out the probe (Figure 3.44).

Figure (3.44): Diagrammatic map shows sites for methyl sensitive endonucleases in the hybridization probe; **A:** *Ava*II at 20 and 557 bp; **H:** *Hpa*II; **M:** *Msp*I at 13 and 605 bp. Map is not drawn to scale.

The same amount of genomic DNA from young, old, young Elstar leaves infected with *V. inaequalis*, young and old Remo samples were used for this study. The methylation insensitive restriction enzyme *Msp*I and its isoschizmer methylation sensitive *Hpa*II and another methylation sensitive enzyme *Ava*II were used to digest genomic DNA (Figure 3.45).

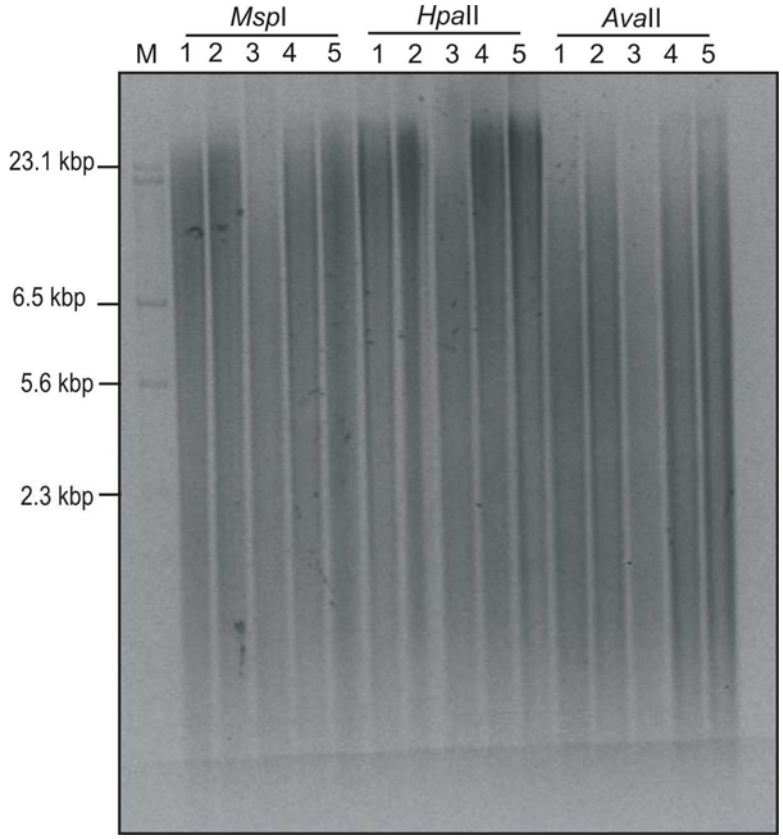

Figure (3.45): Overnight digested genomic DNA on 0.8 % agarose gel; **1:** Young Elstar leaves; **2:** Old Elstar leaves; **3:** Elstar leaves infected with *V. inaequalis*; **4:** Young Remo leaves; **5:** Old Remo leaves.

Firstly, methylation analysis presented in figure 3.46 confirmed the previous results for southern blot and copy number of ns*ltp* in apple genome. It has been easily detected at least more than 10 bands -in case of *Ava*II at least 14 bands- in both Elstar and Remo. Secondly and the most important part of this analysis is the confirmation of cytosine methylation through ns*ltp* and its upstream region.

In this experiment, different plant materials have used from Elstar (healthy young, old and young infected with *V. inaequalis* leaves) and from Remo young and old leaves. Interestingly in most of the cases, around six bands disappeared when the methylation sensitive isoschizmer was used. Results show that nsltp is highly methylated in Remo than in Elstar. More interestingly despite the same amounts of genomic DNA were used (Figure 3.45), in case of infected Elstar very few bands were detected in both methylation sensitive and nonsensitive restriction enzymes. The methylation pattern in case of *Ava*II was similar in healthy resistant cultivar Remo and susceptible infected cultivar Elstar.

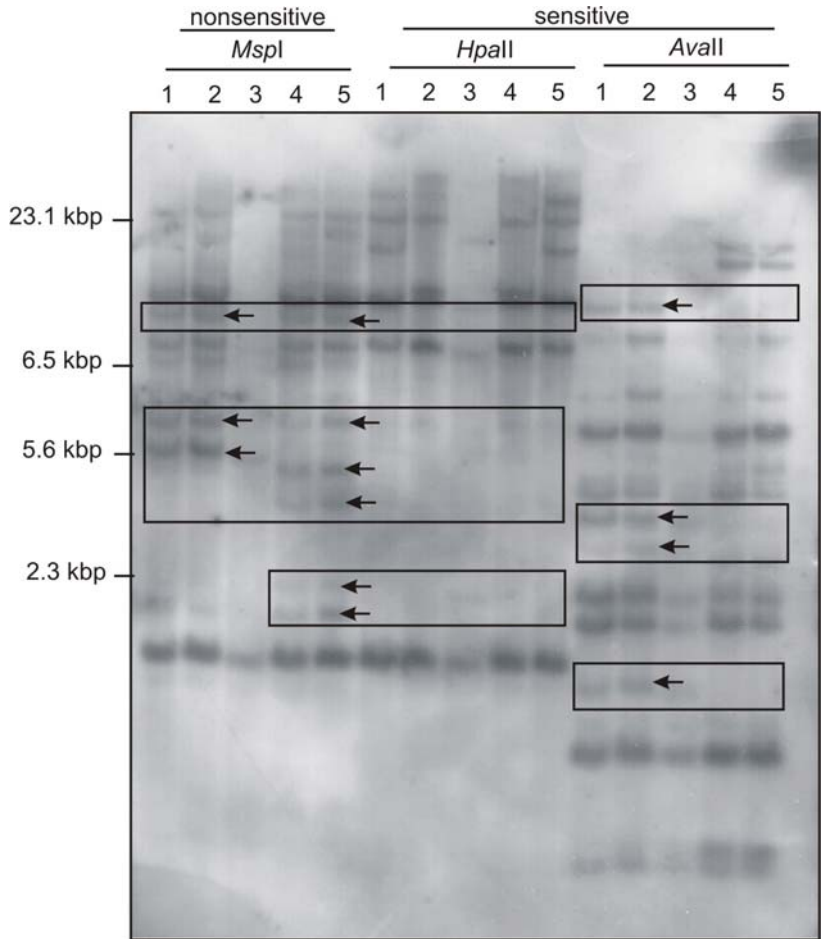

Figure (3.46): Determination of the methylation pattern in the upstream region and as well as the transcribed region of the ns*ltp*. **1:** Young Elstar leaves; **2:** Old Elstar leaves; **3:** Elstar leaves infected with *V. inaequalis*; **4:** Young Remo leaves; **5:** Old Remo leaves. Arrows indicate the putative methylated bands.

3.14 Posttranslational modification of nsLTP

One of the most important posttranslational characters for protein is the addition of phosphate group from S-adenosyl methionine (SAM) via protein kinase. This phosorylation process is very pivotal for protein function. Because the main aim is to explore the function of nsLTP therefore the retrieved DNA sequence was translated to the corresponding amino acids. In order to predict the possible phosphorylation sites, deduced amino acids sequence was submitted to the NetPhos 2.0 prediction server (Blom et al., 1999; http://www.cbs.dtu.dk/services/NetPhos/). Prediction analysis revealed that nsLTP has a possible phosphorylated tyrosine residue at position 40 with a score 0.937. This score is above the threshold close to the maximum score of 1.0. To confirm computer prediction western blot has been performed for the soluble protein contents in the apoplast and membranes were detected by antiphosphoserine, antiphosphotheronine and antiphosphtyrosine. In cases of serine and theronine no signals could be detected. On the other hand we found a high signal in case of tyrosine. Immunoblotting analysis revealed that nsLTP is tyrosine phosphorylated in healthy Elstar (Figure 3.47).

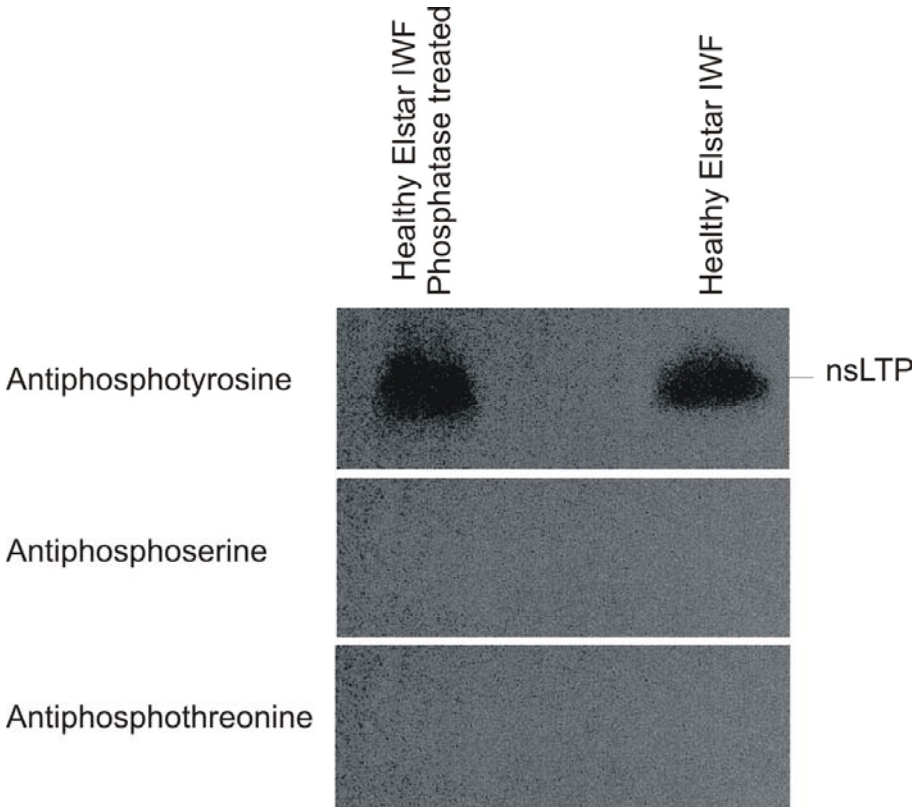

Figure (3.47): Immunoblot assay for phosphorylation sites detection in the apoplastic nsLTP of *M. domestica*. The first antibody dilution was 1:1000. In SDS PAGE each lane was loaded with 50 µg protein based on Bradford determination.

Furthermore when the protein contents in the apoplastic fluid were treated by phosphatase, signal in nsLTP still present. This might be attributed to the low specificity of the general phosphatase to dephosphate tyrosine residues.

Interestingly the signal of phosphtyrosine has been detected only in the healthy tissues of both susceptible and resistant apple cultivars (Figure 3.48). This result was comparable to SDS PAGE and CBB staining results.

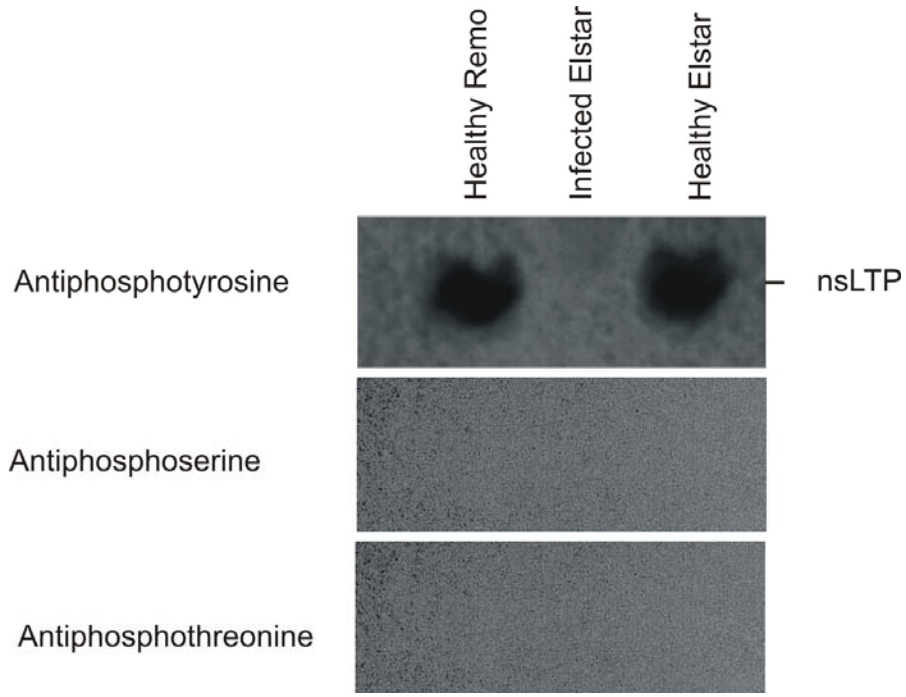

Figure (3.48): Immunoblot assay for phosphorylation sites detection in the apoplastic nsLTP of *M*. *domestica*. The first antibody dilution was 1:1000. In SDS PAGE each lane was loaded with 50 µg protein based on Bradford determination.

4 Discussion

4.1 The nsLTP and pathogenesis

The declining of ns*ltp* transcript level in the apple trees after infection with *Venturia inaequalis* after one day and still undetectable until two weeks as observed by northern blot analysis. This finding confirms the previous observation by Gau et al., 2004 on the protein level. The authors found out also the protein level of nsLTP in the apoplastic fluid of the leaves of the susceptible apple cultivar Elstar has declined to undetectable level after infection by *V. inaequalis*. These results were in the consistence with the finding that was given by Rep et al., (2003). They found out that protein level of *ltp* in tomato xylem sap has declined during *Fusarium* colonization. In another example, it has been reported that the transcript level on nsLTP in the inoculated *A*. *thaliana* leaves with avirulent strain of *Pseudomonas syringae* was reduced after infection (Maldonado et al., 2002).

Very recently it has been documented that the protein level of ns*ltp* of apple *M*. *domestica* cv. Holsteiner Cox has been declined in the apoplast after application of the nonpathogenic antagonist *P. fluorescence* Bk3 (Kürkcüoglu et al., 2004). This finding is in contrast with several previous *in vitro* studies that were suggesting antimicrobial activity for nsLTPs (Terras et. al., 1991; Molina et. al., 1993; Segura et. al., 1993; Dubreil et. al., 1998; Kristensen et. al., 2000; Regent and de la Canal 2000; Carvalho et. al., 2004; Velazhahan et. al., 2001). On the other hand most of these results have been based on *in vitro* studies. More recently it has been documented that plant nsLTP possess antifungal activity by involvement in membrane permeablization of the fungal cell (Regente et al., 2005).

Therefore, if this notion is adopted, it should be expected the increase of nsLTP at least on the transcript level. Based on this finding it could be suggested that *M*. *domestica* nsLTP does not exhibit direct antifungal activities *in vivo* against *V. inaequalis*.

In fact this suggestion has been confirmed by analysis of the soluble protein contents in the apoplast of the young susceptible and resistant apple cultivars. This analysis revealed a higher level of nsLTP in the apoplast of the susceptible apple cultivar Elstar than in the resistant cultivar Remo. Consequently, this finding supports the idea that apoplastic nsLTP in apple does not posses direct antifungal activity toward *V. inaequalis*, otherwise it should be more in the resistant cultivar than in the

susceptible one. More interestingly the nsLTP represent more than 90 % of the total protein in the apoplast in both cultivars in these young leaves.

4.2 Southern blot and copy number of the intronless apple ns*ltp*

The current results showed that ns*ltp* in four different apple cultivars; apple *M. domestica* cv. Elstar, Gloster, Holsteiner Cox and Remo is an intronless gene. This finding is in the agreement with the recently published data about ns*ltp* in another 10 different apple cultivars. Moreover, one of the *ltp* genes in both barley (White et al., 1994), and sorghum (Pelèse-Siebenbourg et al., 1994), were found to be intronless genes. On the other hand, almost all of the isolated *ltp*s members in plant contain an intron, particularly placed in the region corresponding to the C-terminal domain in the protein (Kader 1996). This intron varies in length from one gene to another, for example 89 bp in rice (Vignols et al., 1994), 114 bp in sorghum (Pelèse-Siebenbourg et al., 1994), 115 bp in *A. thaliana* (Thoma et al., 1994), 133 bp in barley (Skriver et al., 1992; Ma et al., 1995), 271 in broccoli (Pyee and Kolattukudy 1995), and 980 bp in tobacco (Fleming et al., 1992).

Southern blot analysis in this study revealed that apple *M. domestica* has at least 14 copies of ns*ltp* distributed through out the genome. This result increased the complexity that was confirmed suggesting the presence of several *ltp*: only one in carrot and spinach (Bernhard and Somerville 1989; Sterk et al., 1991); at least two copies in cotton, maize, *Gerbera hybrida*, and tomato (Tchang et al., 1988; Torres-Schumann et al., 1992; Kotilainen et al., 1994; Ma et al., 1995); three in rice (Vignols et al., 1994); four in broccoli (Pyee and Kolattukudy 1995); five in sorghum (Pelèse-Siebenbourg et al., 1994); and seven in barley (White et al., 1994). On the other hand the same copy number 14 for *ltp* was previously reported in loblolly pine (Kinlaw et al., 1994). In addition, it has been reported that *A. thaliana* genome has at least 15 *ltp* and at least six individual ns*ltp* have been found in three chromosomes (Arondel et al., 2000). More recently, the linkage map positions and allelic diversity of two ns*ltp* in *M. domestica* was determined. This study confirmed the current finding that apple *M. domestica* ns*ltp* is intronless. Moreover, the recent results revealed that the two ns*ltp* in cultivated apple *Mal d 3.01* and *Mal d 3.02* could be mapped on two homologous segments of linkage groups 12 and 4 respectively (Goa et al., 2005).

4.3 Promoter activity and light dependency

Prediction of the cis-acting regulatory elements revealed that these upstream regions of ns*ltp* in *M. domestica* cv. Elstar and Remo are very rich in motifs, particularly in the light responsive elements. Insertion of the PCR amplified ns*ltp* upstream regions from Elstar and Remo in front of the DsRed reporter gene, could switch on the expression machinery during transient expression of this marker gene in *M. domestica* cv. Gloster tissues. However the intensity and frequency of the detected fluorescence in the transformed cells were higher in case of Remo ns*ltp* upstream region than those of Elstar.

The expression of DsRed gene was exclusively dependent on light. These findings reflect close correlation between the light responsiveness motifs and the dependence of promoter activity on light in one side and the chloroplast localization in fusion with GFP on the other side. Therefore it can be concluded that *M. domestica* ns*ltp* is a light regulated gene.

Similar observation has been found in *Rhodopseudomonas sphaeroides*. A cytoplasmic lipid transfer protein was purified from this gram-negative bacterium with a molecular mass 27 kD and 5.2 isoelectric point (Cohen et al., 1979; Tai and Kaplan 1984). Whereas, a non-specific lipid transfer protein with a 56 kD was purified from the periplasm in *R. sphaeroides* (Tai and Kaplan 1985). The latter organism is a photoheterotrophic bacterium that forms a cytoplasmic membrane structure containing the photosynthetic apparatus (Kaplan 1981). The most interesting finding in this bacterium was the clear correlation between light intensity during growth and the level of lipid transfer activity.

In addition, it has been reported that no *ltp* transcript was detected in the root of different plants (Bernhard et. al., 1991; Wirtz 1991; Fleming et. al., 1992; Torres-Schumann et al., 1992; Weig and Komor 1992; Molina and Garcia-Olmedo 1993; Ma et. al., 1995; Pyee and Kolattukudy 1995), only in rice seedling a weak level has been detected (Vignols et. al., 1994). On the other hand *ltp* was actively expressed in the aerial parts of the plants including leaves, stem and shoot meristems (Bernhard et. al., 1991; Fleming et. al., 1992; Thoma et al., 1993; Molina and Garcia-Olmedo 1993; Vignols et. al., 1994; Ma et. al., 1995; Pyee and Kolattukudy 1995).

These observations can support our finding that the *M*. *domestica* ns*ltp* is a light dependent gene.

It has been reported that the release of ROS intermediates (superoxide radicals, hydrogen peroxide and hydroxyl radicals) was controlled by light, gibberellin and abscisic acid (Schopfer et. al., 2001). It is clear that chloroplast represents a very hot source for ROS especially under light condition.

These results could support the hypothesis for nsLTP that can protect membranes in general and particularly chloroplast envelope in this case against damage with free radicals. Regarding thylakoid membranes, it has been documented that cabbage cryoprotectin as a member of plant nsLTP from cold-acclimated leaves can protect nonacclimated spinach thylakoids from freeze-thaw damage (Hincha et. el., 2001).

4.4 Subcellular localization of nsLTP and possible function

Basically in most of the cases, protein localization is closely correlated with the function. This was the basic rationale to trace the nsLTP targeting in fusion with GFP. Based on the finding of a putative signal peptide in all members of LTP, Vignols et. al., (1994) suggested that LTP could be targeted to a specific intracellular compartment and/or secreted. The extracellular localization of LTP has ruled out its participation in lipid metabolism, including lipid transfer between intracellular membranes. This led to the suggestion for possible existence of at least two different populations of LTP. One of them is intracellular and the other is extracellular with different functions (Carvalho et al., 2004).

During the transient expression of nsLTP in the N-terminal fusion with GFP it has been observed that the chloroplast within the guard cell is swollen. Similar observation has been reported by Gray et al., (2002). He reported that the chloroplasts adjacent to the wound-induced *lls*1 (lethal leaf spot-1 in maize) are highly swollen and distorted before any other changes normally associated with dying cells. It has also been suggested for LLS1 that may act to prevent the formation of the reactive oxidative species or to remove cell death mediator to protect the chloroplast and to preclude cell death. Swelling the chloroplast was contributed to the loss of differential permeability of its envelope membranes that may result from changes within the chloroplast such as photooxidation, change in pH or loss of energy production (Wise and Cook 1998; Mostowska 1999).

The crucial episode in this scenario is the GFP based analysis which revealed that the subcellular localization of the nsLTP is restricted vastly to the swollen chloroplast particularly under light conditions and to other organelles in the cell probably including mitochondria and peroxisomes under dark conditions in case of N-terminal fusion. Since this fusion does not contain any motif or transit peptides that can direct the fusion toward the chloroplast or other organelles, one question has been arisen, why this fusion has been localized in this pattern? However, searching for DNA motifs in the ns*ltp* and the upstream region by using Omiga package, revealed the presence of 9 motifs that belong to chloroplast binding factor.

Intuitively, bombardment of plant leaves by gold particles triggers an oxidative stress producing reactive oxygen species (ROS), particularly H_2O_2 that can damage the cell. These ROS exacerbate the potential damage effect of the preexisting ROS within the cell. Membranes are highly prone to oxidative stress (Mittler et al., 2004). In general plants can protect their membranes enzymatically by the action of specific phosphlipid glutathione and nonenzymatically via the potent antioxidant α–tocopherol that can be reduced via reduced ascorbic acid (Porfirova et al., 2002; Müller-Moulé et al., 2003; Rodriguez Milla et al., 2003).

In an attempt to prevent or at least minimize H_2O_2 production in the apoplast after bombardment, the Diphenyleneiodonium chloride (DPI) has been used as a powerful reversible inhibitor for NADPH oxidase (O'Donnell et al., 1993) and in several studies of plant/pathogen interactions (Baker et al., 1998). Unfortunately, it was difficult to find the suitable condition for DPI application. On one hand when DPI concentration was high it was detrimental for plant tissues. On the other hand, when the DPI concentration was low it stimulates an extreme stomatal opening and consequently the bombarded tissues were occupied with endophytic bacteria and again this was detrimental for tissues (data not shown).

To address the question one assumption can be given that within the chloroplast of the injured guard cell, ROS resulted in a cascade of free radicals that initiated programmed cell death. The putative role of nsLTP in this scenario it might act as a transporter facilitating the replacement of the impaired membrane lipid or to recruit a lipid derived antioxidant molecules in attempt to quench free radicals and consequently prevent programmed cell death. A putative antioxidant candidate is α-tocopherol which is lipid derived potent membrane antioxidant.

An alternative interpretation can be given for localization of nsLTP in fusion with the GFP in the chloroplast of the guard cells, is the presence of lipid substrate for nsLTP. Generally, presence of transporter or carrier is correlated with the presence of cargo. Obviously leaf membrane lipids are synthesized as a co-operation between chloroplast and endoplasmic reticulum (Ohlrogge and Browse 1995). Moreover, fatty acids synthesis takes place predominantly in the chloroplast (Hellgren and Sandelius 2001). Therefore it seems a plausible localization for nsLTP in the chloroplast for docking and subsequently facilitates the transfer of lipid cargo to its destination, through the cytosol. It has been suggested that phospholipids are carried back from Golgi to the ER as individual molecules though the cytosol in order to maintain the integrity of the ER by LTPs (Wirtz 1991).

4.5 Evidence for presence of nsLTP in fusion with GFP in the chloroplast envelope

Previously it has been reported that no LTP was detected in the stroma of spinach chloroplast (Schwitzguebel and Siegenthaler 1985). In this study it has been found that the targeting of nsLTP to the chloroplasts in the guard cell started as a line girdled the chloroplast, and gradually this line covers the whole compartment (Figure 4.1A). This is a plausible evidence for the localization in the chloroplast envelope and not inside the chloroplast, because if it is localized inside the chloroplast a fluorescent signal coming from inside had to be detected as in case of the transit peptides of the small subunit of Ribulose-1,5-bisphosphat-Carboxylase-Oxygenase (RubisCO) in fusion with GFP (Figure 4.1B). Therefore it can be suggested that presumably the nsLTP might localize in the chloroplast envelope. Moreover, it has been reported that LTPs were used as a tool to modify the lipid composition of a membrane and to study the consequences of these changes on its properties (Kader 1996). This modification has been performed for chloroplast envelope membranes (Miquel et al., 1987).
Regarding lipid synthesis in the plant, the chloroplast envelope plays a key role in this process. It has been reported that the bulk of thylakoid lipids monogalactosyl-diacylglycerol (MGDG) and digalactocyldiacylglycerol (DGDG) are synthesized in the chloroplast envelope (Maréchal et al., 1997). It has been suggested that the precursor diacylglycerol (DAG) derives from phosphatidylcholine (PC) synthesized in the chloroplast envelope (Mongrand et al., 1997, 2000).

One possible explanation can be given for localization of nsLTP in the chloroplast envelope, that for docking by lipids that have been synthesized in the envelope and transfer it to other destinations within the cell.

In contrast to animal and fungi which produce fatty acids primarily in the cytosol (Ohlrogge and Browse 1995), plant mainly synthesizes fatty acids in the plastid stroma (Harwood 1996). These fatty acids are transported to the plastid envelope membrane and from there can be either exported to the cytosol or metabolized within the two envelope membranes (Maréchal et al., 1997). In addition, it has been also demonstrated that the chloroplast envelope membranes synthesize oxylipins from hydroperoxides of polyunsaturated fatty acids (Blée and Joyard 1996). Therefore the chloroplast envelope represents a hot area containing fatty acids that can easily oxidized by ROS. In animals it has been speculated that the mammalian sterol carier protein (SCP2) which is identical to mammalian nsLTP protects fatty acids from oxidation by ROS in the peroxisomes (Dansen et al., 2004). The same speculation could be valid for plant nsLTP in the chloroplast that represents the major source for ROS in plant particularly under light conditions. It has been shown that acyl-CoA thioesters synthesized on the outer membrane of the chloroplast envelope is avialible outside plastids for glycerolipid synthesis in the ER (Joyard and Douce 1977; Joyard and Stumpf 1981). Acyl-CoA thioesters have detergent properties that can damage the membrane integrity. It has been suggested that both nsLTP (Kader 1996) and specific acyl-CoA binding proteins (Hills et al., 1994) could be involved in the removal of acyl-CoA thioesters from the vicinity of the outer envelope membrane and transport it to the ER for rapid utilization.

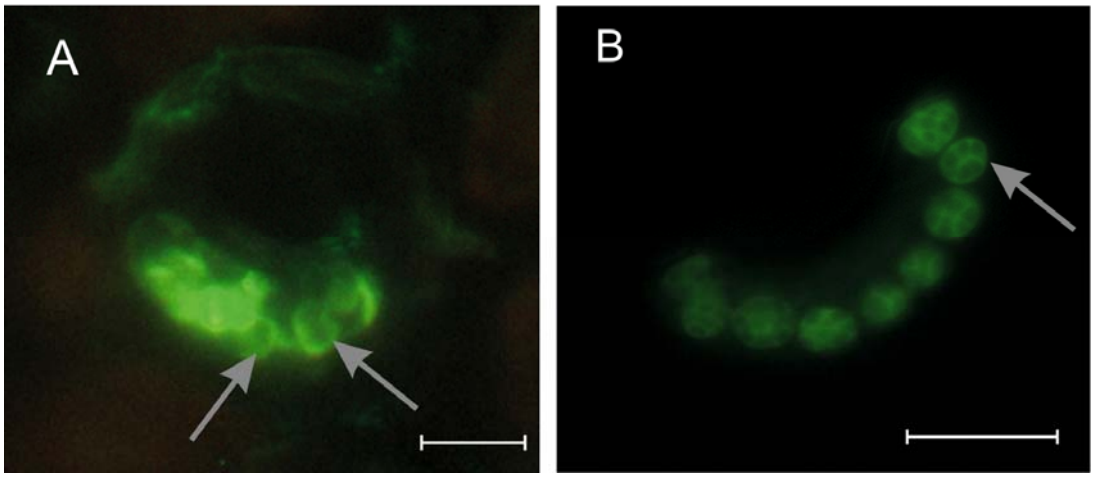

Figure (4.1): A: Targeting the nsLTP in N-terminus fusion with GFP to the chloroplast of the guard cells in *M. domestica* cv. Gloster; **B:** Targeting of GFP in fusion with the transit peptides of the small subunit of RubisCO to the chloroplast (under dark conditions) Bar= 25 µm.

4.6 The nsLTP of *M. domestica* cv. Elstar and its localization in the apoplast

Apparently the *in vivo* function of nsLTP is not restricted to the apoplast as one of the sources for ROS but it extends to the cytosol that harboring the other ROS sources including chloroplast, mitochondria and peroxisomes. Rationally, to avoid any hazards resulted from any leakage of ROS from cytosolic sources. It is noteworthy that the suggestion of localization of nsLTP in the peroxisomes is not the first record but it has been reported before in castor bean cotyledons glyoxysomes (Tsudoi et al., 1992). Recently, it has been found that LTP of *Vigna unguiculata* seeds localized in the lumen of organelles that has been suggested to be a protein storage vacuoles, as well as in vesicles similar to the lipid containing ones and in the extracellular space (Carvalho et al., 2004).

Paradoxically the absence of GFP fluorescent signals in the apoplast does not imply the absence of nsLTP from this compartment. It can be attributed to the change in pH toward acidity in the apoplast that might affect the GFP fluorescence exhibiting a weak undetectable fluorescence (Tsien 1998).

4.7 nsLTP of *M. domestica* cv. Elstar involved in vesicle trafficking and cytoskeleton dynamics

The transient expression of GFPN-ns*ltp* in the healthy apple leaves under dark conditions revealed that nsLTP is localized in some guard cells in bodies like vesicles distributed through the cytosol (Figure 4.2A). On the other hand when the artificially infected apple leaves were used for bombardment the localization pattern of GFPN-ns*ltp* was observed in structures that possibly seem to be the cytoskeletal filaments (Figure 4.2B). It is known that both plant-pathogens and symbiotic interactions implicated in modulation of cell polarity and cellular trafficking in plant cell and consequently are associated with the cytoskeleton reorganization (Wasteneys and Yang 2004). Regarding lipids it has been reported that lipids play a key role in vesicle trafficking, secretion, cytoskeletal rearrangement (Welti and Wang 2004). Recently it has been found that phosphatidylinositol 3- and 4-phosphate control the opening and closing of the stomatal pore in *Vicia faba* and *A. thaliana* (Jung et al., 2002), suggesting that the phosphoinositide system is involved in the actin filaments reorganization during stomatal movement. Phosphatidylinositol is considered to be a potential lipid substrate for the nsLTP.

Based on the current observations in this study it could be suggested that nsLTP can play a role in vesicle trafficking and cytoskeletal dynamics within the plant cell. These speculative functions can be achieved by nsLTP if it can be supposed that the *in vivo* function of nsLTP is to transfer lipids from their source of synthesis to their destinations. It seems a plausible interpretation due to the localization of nsLTP in fusion with GFP in the chloroplasts of the guard cells. Since the chloroplast in the plant cell represents the main source for lipid synthesis in cooperation with the ER.

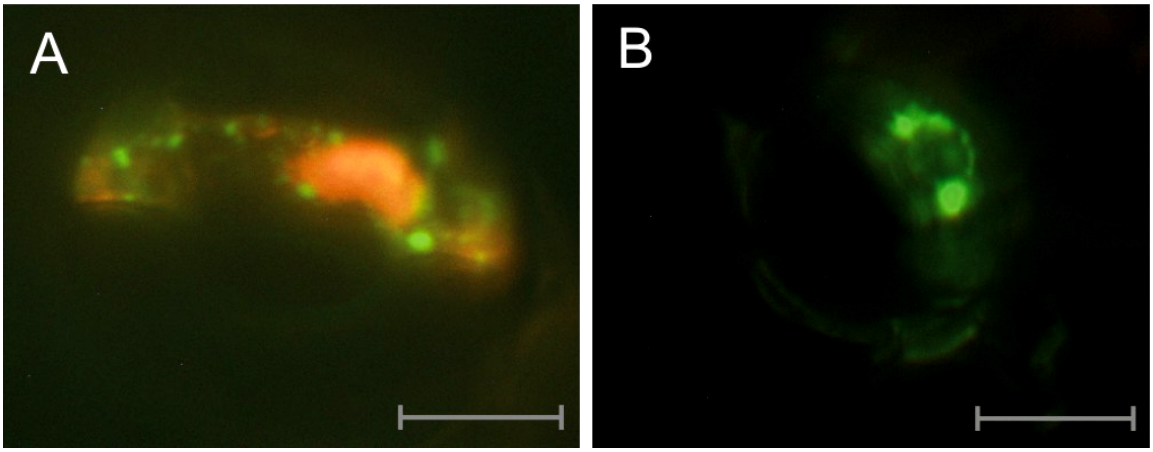

Figure (4.2): A: Localization of nsLTP in N-terminal fusion with GFP in bodies like vesicles in the cytosol of the guard cells of *M. domestica* cv. Gloster (under dark conditions); **B:** Possible localization of nsLTP in N-terminal fusion with GFP in the cytoskeletal filaments of the guard cells of *M. domestica* cv. Gloster infected leaves with *V. inaequalis* (under dark conditions). The red colour in A represents the expression of DsRed in the cytosol as a background positive control. Bar= 25 µm.

4.8 The leader sequence and C-terminal domain of nsLTP and its roles in protein targeting

The deletion of nucleotide sequence that encodes for the first 24 amino acids from the ns*ltp* precursor resulted in failure of mature protein in fusion with GFP to enter the secretory pathway or to be localized in any compartment within the cell. Subsequently the fusion protein was localized in a similar manner like the control GFP, through the whole cytosol with a higher density around the nucleus. This finding is in the agreement with the general consensus in the literatures on nsLTPs. It is known that LTPs have an N-terminal leader sequence responsible for the insertion of the polypeptide into the lumen of the ER *in vitro*, and subsequently the secretory pathway (Bernhard et al., 1991; Madrid 1991). Based on this observation, it has been

suggested that LTPs take part in the lipid movements within the lumen of the ER (Madrid 1991). Interestingly, the immature nsLTP had a calculated molecular mass around 12 kD, but the isolated apoplastic nsLTP has only 9 kD. This observation indicates that the N-terminal leader sequence in nsLTP has been deleted from protein and subsequently the nsLTP is localized in the apoplast as a mature protein.
On the other hand when the nucleotide sequence corresponding to the last 11 amino acids in the C-terminal domain was deleted, the resultant protein in fusion with the C-terminus domain of GFP was localized in the chloroplasts in the guard cell under light and as well as dark conditions. Since almost the same observation has been recorded in the case of N-terminal fusion, it might be concluded that the 11 amino acids in the C-terminal domain in the apple nsLTP are not necessary for protein targeting to its destinations.

4.9 Epigenetic information and ns*ltp* regulation in susceptible and resistant apple cultivars of *M. domestica* cv. Elstar and Remo

After infection of apple trees with the fungus *V. inaequalis*, protein and as well as the transcript levels of apple ns*ltp* have been declined drastically. Based on this fact one question has been arisen, is ns*ltp* regulated transcriptionaly or posttranscriptionaly? DNA methylation analysis with methylation sensitive restriction enzymes (*Hpa*II and *Ava*II) and southern blot revealed that cytosine methylation in the upstream region and the structural ns*ltp* play a pivotal role in the regulation process. Reduction of number of detected bands when the methylation sensitive isoschizomer *Hpa*II was used, give a clear evidence that some sites in the target sequence were methylated and subsequently the methylation sensitive restriction enzymes failed to cleave these sites.

Results revealed that the methylation pattern of ns*ltp* was similar in healthy resistant cultivar Remo and susceptible infected cultivar Elstar. This finding is in the consistence with the previous finding by Gau et al., 2004 on the protein level. The authors found out that the protein pattern in the apoplast was similar in both healthy Remo and infected Elstar. This might reflect that the resistant *M. domestica* cv. Remo is always on alert against the potential infection with *V. inaequalis*.
It is noteworthy that this is the first time for investigation the role of DNA methylation on *M. domestica* ns*ltp* regulation pattern. Interestingly, our finding is in the agreement

with previously demonstrated results, that in the flowering plants, the light regulated genes are regulated mainly at the transcriptional level (Silverthorne and Tobin 1984; Tobin and Kehoe 1994; Terzaghi and Cashmore 1995). As discussed before it can be suggested that ns*ltp* is a light regulated gene, therefore it could be expected that ns*ltp* is transcriptionally regulated. Regarding infection and pathogenesis, it has been reported that DNA methylation can be altered and subsequently changes specific gene activity of the host cell by fungal pathogenic action on plants (Guseinov and Vanyushin 1975). Moreover, it has been documented that the amount of methylcytosine in stem DNA of alfalfa infected plants is approximately 25 % higher than that of the healthy plant DNA (Vanyushin et al., 1979). On the other hand regarding to age, it has been shown that DNA methylation level in young seedlings in both tomato and *A. thaliana*, were approximately 20 % lower than in mature leaves (Messeguer et al., 1991; Finnegan et al., 1998). It is known that there are several genes in plants that are transcriptionally inactive and methylated in the promoter and/or coding sequences (Finnegan et al., 1998). It was shown that coding region methylation inhibits gene expression in animal cells (Keshet et al., 1985). In 1996 it has been suggested that similar mechanisms operate in plant cells (Hohn et al., 1996).

4.10 nsLTP is developmentally regulated

The current results showed that the protein level of nsLTP has declined by age in the apoplastic fluid. Previously it has been demonstrated that the transcript of *ltp*1 has declined with age of the tissue in tobacco (Fleming et al., 1992), in barley (Molina and Garcia-Olmedo 1993) and sugar beet (Nielsen 1996). More recently, a higher level of nsLTP in the apoplast of the young apple leaves than in older one was documented (Koutb 2003). It has been reported that a high *ltp*1 promoter activity occurs in tobacco plant parts that are vulnerable to physical disruption and thus to pathogen invasion (Canevascini et al., 1996). Inductively it can be assumed that the down regulation of nsLTP in old leaves might implicate in the ontogenic resistance in apple. During the flower development; genes of *ltp* were expressed at early stages (Kader 1996). Interestingly, during the development in legume plants by the symbiotic bacterium *Rhizobium*, the transcript level of LT-like protein has elevated in root hairs after four days inoculation. On the other hand the transcript level was absent in the

differentiated nodules, indicating that the *ltp* was transiently expressed (Krause et al., 1994). High transcript levels of *ltp* were observed in young developing inflorescences in carrot (Sterk et al., 1991), and *A. thaliana* (Thoma et al., 1994), in the sepals of unpended flowers of tobacco (Fleming et al., 1992), in flower buds of broccoli (Pyee and Kolattukudy 1995), in microspores of rapeseed (Foster et al., 1992), and in corolla and carpel of *Gerbera hybrida* (Kotilainen et al., 1994).

Even in animals, one member on nsLTP was purified from rat hepatoma (Dyatlovitskaya et al., 1978), with a molecular weight 11 kD and isoelectric point 5.2. Interestingly, this protein has been found in several carcinoma cell lines as well as in fetal rat liver, but it has not been found in adult rat liver (Dyatlovitskaya et al., 1982). Based on this result it has been suggested that hepatoma nsLTP may belongs to the carcinoembryonic protein family (Wirtz 1991).

Within the inevitable equation of plant life cycle, plants must keep the balance between youth and senescence. The latter is one form of PCD that correlated with oxidative stress. Furthermore none of nsLTP has been reported to be induced by senescence (Yoshida et al., 2001). Therefore the expression of ns*ltp* must be thriftily regulated.

4.11 Tyrosine phosphorylation site and its possible impact on nsLTP

The predicted tyrosine phosphorylation site that has been done by computer program and confirmed via western blot with antibody against phosphorylated tyrosine residue indicates that nsLTP has a key element that enables it to play a pivotal role in the plant cell. It is well known that addition or removal of phosphate groups to serine, threonine, or tyrosine residues of protein can cause allosteric modifications. These modifications result in conformational modulations in protein leading to its activation or inactivation (Naz and Rajesh 2004). In general, protein phosphorylation plays an essential role in signal transduction from out side to the inside of a cell, and it regulates different cellular functions including growth, metabolism, proliferation, motility and differentiation (Bonenfant et al., 2003). Particularly tyrosine phosphorylation has a paramount importance in animals but in plants it has been neglected, because a typical tyrosine kinase was not found in plants. However, the activities of protein kinase have been reported in some plant species such as pea (Torruella et al., 1986), alfalfa (Duerr et al., 1993), tobacco

(Zhang et al., 1996), maize (Trojanek et al., 1996), and coconut (Islas-Flores et al., 1998). On other hand several protein tyrosine phosphatases (PTPs) have been characterized in *A. thaliana* and other species (Xu et al., 1998; Gupta et al., 1998; Fordham-Skelton et al., 1999).

It has been suggested that tyrosine phosphorylation in plants is strongly involved in the regulation of plant embryogenesis and tissue differentiation (Barizza et al., 1999). In addition by using several specific PTP inhibitors, it has been demonstrated that PTP activity is essential for stomatal closure induced by four different factors including ABA, external calcium, darkness and H_2O_2 (MacRobbie 2002). Furthermore, it has been suggested that tyrosine phosphorylation in the transition to light state 2 may be an important factor in regulation of photosynthesis (Forsberg and Allen 2001).

For nsLTPs it has been built a general consensus in the literatures that they are characterized by their ability to transfer phospholipids between membranes *in vitro*. Regarding to the binding activity of nsLTP, one can suggest that tyrosine phosphorylation can modulate this activity. As it has been reported in case of calmodulin, that tyrosine phosphorylation modulates the interaction of calmodulin with its target proteins (Corti et al., 1999). Moreover it has been observed that lipid transfer like proteins from wheat, barley, pine, and Petunia were found to be the substrates with various phosphorylation sites for Ca^{2+}-dependet protein kinases (Polya et al., 1992; Neumann et al., 1993; Neumann et al., 1995). Furthermore after labeling of petunia petals with ^{35}P phosphate, the major labeled protein has an apparent molecular mass close to that of LTPs (Neumann et al., 1995).

Recently, it has been suggested that H_2O_2 activates protein tyrosine phosphorylation (Rivlin et al., 2004). A lipid derived potent antioxidant α–tocopherol is known to modulate tyrosine phosphorylation (Chan et al., 2001). It has been observed that tocopherol synthesis takes place in photosynthetic organisms; plants, algae, and some cyanobacteria (Sattler et al., 2003). In plants α–tocopherol synthesis takes place in the inner membrane of the chloroplast (Soll et al., 1980).

Accordingly, it can be hypothesized that the ROS released by bombardment or any related situations *e.g.* pathogen attack act as an alert that activate apoplastic ns LTP via tyrosine phosphorylation. The phosphorylated form of nsLTP is responsible for impaired phospholipids replacement or recruiting α–tocopherol to protect

membranes in the influenced area. In the case of non producing α–tocopherol influenced tissues, nsLTP must travel for long distance through the plant.

In support of this hypothesis, Maldonado et al., (2002) suggested that an apoplastic lipid transfer protein interacts with lipid-derived molecules and operates as a mobile signal for a long distance in *A. thaliana* apoplast. Moreover, it has been reported that 90 % of the synthesized fatty acids in the nonphotosynthetic tissues and developing seeds of all plants are imported from the plastids (Browse et al., 1993).

4.12 Why are plant nsLPTs free of tryptophan?

Another support to this hypothesis is the lack of tryptophan (Trp) residue from all known plant nsLTPs (Kader 1997). A plausible explanation can be given as a precautionary strategy for the plant. It has been reported that Trp and tyrosine (Tyr) are susceptible to radicalization preferably in the lipophilic environments (Moosmann and Behl 2000). Moreover, Trp-derived peroxides were more efficient than Tyr-derived peroxides in inactivation of thiol-dependant enzymes (Hampton et al., 2002). One of these thiol-dependent enzymes is the glutathione reductase that represents a key enzyme in the ROS scavenging system. Mammalian nsLTP lacks Tyr residue (Dansen et al., 2004). Moreover, it has been speculated that the mammalian sterol carrier protein (SCP2) which is identical to mammalian nsLTP protects fatty acids from oxidation by ROS in the peroxisomes (Dansen et al., 2004).
In comparison with mammals plant kept Tyr in their nsLTPs that might be attributed to the production of α–tocopherol only by plant and may reflect the cross talk between α–tocopherol and Tyr phosphorylation.
Regarding plants if nsLTPs have Trp residue, any modifications in this amino acid will not only affect the function of nsLTP but also on the Trp pool and consequently on the synthesis of this protein.
In addition, allergy analysis provides a peciuliar character for nsLTP supporting our hypothesis is the insensitivity of nsLTP as allergen to oxidative attack that destroys the allergenicity of birch pollen-related fruit allergens (van Ree 2002).
Based on our results it can also be suggested that the reported phosphorylation of the Tyr residue in *M. domestica* nsLTP can possibly prevent the oxidation of this susceptible residue and consequently circumvent the oxidative damage.

4.13 Direct correlation of nsLTP with PCD

During the development of tracheary elements (TE) in zinnia, secretion of a TED4 protein that encoded for *ltp* into the medium, inhibit proteasome activity to protect the neighboring cells. The depletion of TED4 protein from the culture medium results in an increase in mortality of other living cells (Endo et al., 2001).
Recently, *ltp* in *Euphorbia lagascae* seedling were found with a high amount in the inner region close to the cotyledon, and less amount in the outer region of the endosperm (that must undergo programmed cell death). It has been proposed that *ltp* are involved in the recycling of endosperm lipids, or protecting the growing cotyledons from proteases released during PCD (Eklund and Edqvist 2003).

Several evidences have been accumulated regarding the localization of nsLTP in different plant species, around the leaf veins (Sossountzov et al., 1991), in the vessel cell wall (Tsuboi et al., 1992), in phloem (Ivashikina et al., 2003), in root xylem (Rep et al., 2003) and in the highly lignified area at the tip of the cotyledon (Thoma et al., 1994). From these observations one can conclude that the localization of *ltp* is mainly close to xylem vessels and close to the cell wall. The common feature in these niches for *ltp* is the presence of lignin. The latter is a major part of the apoplast and its synthesis is mediated by free radicals.

Another character for nsLTP in support of the hypothesis is the insensitivity to oxidative attack that destroys the allergenicity of birch pollen-related fruit allergens (van Ree 2002). Interestingly, it has been reported that the transgenic plants tobacco and *A. thaliana* expressing barley LTP2 showed a great reduction in the necrotic lesions after inoculation with virulent strains of *P. syringae* (Molina and Garcia-Olmedo 1997). This result was assessed as a defensive role for LTP2, but from our point of view LTP overexpression resulted in prevention of PCD and consequently necrotic lesion production. The knockout of *A. thaliana* acceleratd-cell-death11, that is encoding a sphingosine (lipid) transfer protein, resulted in PCD activation (Brodersen et al., 2002).
If the nsLTP can interfere and prevent PCD in the plant cell, it can play a vital role in the life cycle of *V. inaequalis* particularly after penetrating cuticle layer. During this critical period nsLTP can delay PCD until the fungus switch on its own mechanism by release melanoprotein to the apoplast. Melanoprotein revealed ability to bind iron and

therefore interferes with the oxidative stress (Singh et al., 2005). Consequently nsLTP can rank as a susceptibility factor especially in the case of biotrophic pathogens like *V. inaequalis*.

To confirm our hypothesis the current strategy is to transform protoplast or cell culture of tobacco or *A. thaliana* by sense and antisense apple ns*ltp* via *Agrobacterium* gene mediated transfer procedure. Subsequently, the transformed cells will undergo PCD induction by application of H_2O_2.

4.14 Putative lipid replacement therapy in the plant by nsLTP

In this current study the hypothesis for the *in vivo* function of at least the intracellular population of nsLTP is to protect membranes from the oxidative stress damage directly by replacement the impaired phospholipids and/or glycolipids, or indirectly by recruiting the membrane antioxidant α–tocopherol from the chloroplast. In support of our hypothesis, it has been suggested that a phospholipids-LTP complex in mammalian cells interacts with the membrane and replaces its bound phospholipids by another molecule from the membrane (Wirtz 1991). Comparable events have been proposed for plant LTP (Kader et al., 1982; 1984). More recently analysis of the human tear fluid revealed the presence of phospholipid transfer protein in this fluid. This finding led to the suggestion that LTP may play a role in the formation of the tear film by facilitating phospholipids transfer (Jauhiainen et al., 2005).

One fundamental question is still to be addressed; whether nsLTP can disturb the membrane integrity or not? Interestingly, it has been documented that bovine nsLTP is surface active and it is not necessary to penetrate the membrane to commence transfer activity (van Amerongen et al., 1989).

Obviously, the top target for the research activities is the human being. Therefore once this hypothesis is true, it would be a great milestone in the human nutrition interest, because the nsLTP will be considered as a dagger, on one side it can prevent or at least delay programmed cell death but on the other side it can enhance cancer.

Interestingly, plant non-specific lipid transfer proteins have been studied for their capability to act as a drug carrier and facilitating the delivery system (Pato et al., 2001).

5 Conclusion

In spite of the general consensus about the lipid transfer activity of plant nsLTP *in vitro*, it is still a very strong debate around the possible *in vivo* function/s of this group of proteins in the literature. For this reason this current study was conducted in an attempt to understand the biological role of this puzzling protein in the plant cell.

Very recently it has been found that the protein level of *M. domestica* cv. Elstar nsLTP has declined to undetectable level in the apoplastic fluid of the susceptible apple cultivar *Malus domestica* cv. Elstar after infection with *Venturia inaequalis*. In this current study, the transcript level of ns*ltp* has been found to be drastically declined to undetectable level in apple tissues after infection with *V. inaequalis* by one day. These findings indicate that nsLTP can play an important role in the infection process. The same protein has been detected in the apoplast of the healthy resistant cultivar *M. domestica* cv. Remo however, the level of the detectable protein based on the purification method was lower than that of susceptible cultivar Elstar. This observation indicates that nsLTP might be involved in the susceptibility to diseases directly or indirectly. Moreover apple nsLTP was found to decline by age of the leaves and it is known that leaves by age gain ontogenic resistance against diseases. Based on these results it could be suggested that apple nsLTP might be implicated in the ontogenic resistance in apple.

This study documented for the first time by using western blot analysis, that apple nsLTP has a phosphorylation site located presumably on the tyrosine residue number 40 in the immature protein. We have also found that aplple ns*ltp* is an intronless gene and represented by several copies, at least 10 copies have been found to be distributed through apple genome of the susceptible and resistant cultivars.

Interestingly, this is the first record for plant nsLTP to be localized in fusion with GFP in the chloroplast envelope (the main source for lipid production in the plant cell), especially under light conditions. Furthermore, nsLTP has found to be localized in small vesicles distributed through the cytoplasm. More interestingly, it was observed that nsLTP in fusion with GFP has localized in structures probably belong to the cytoskeleton. These observations can support the hypothesis that *M. domestica* nsLTP can play an important role in lipid transfer *in vivo*.

The upstream regions in both susceptible and resistant *M. domestica* cultivars have shown to be very rich in light responsive elements. Remarkably, the promoter activity

studies on these upstream regions revealed that the apple ns*ltp* is a light dependent gene. This finding reflects the strong cross talk between the light responsive elements within the promoter region and the protein targeting to the chloroplast envelope. Investigation on the role of epigenetic information particularly methylation pattern of the promoter region and as well as the encoding region on the regulation process of ns*ltp* showed that apple ns*ltp* is transriptionally regulated.

From this study different pieces of information could be gathered that can help us to suggest that *M. domestica* nsLTP can also facilitate the lipid transfer *in vivo* from their source of production to their destinations. Therefore, apple nsLTP must be involved in all biological functions in those lipids thought to be important including photosynthesis, signal transduction, vesicle trafficking, secretion, cytoskeletal rearrangement, growth and development, seed germination, organ differentiation, pollination, responses to biotic and abiotic stresses and PCD. Interestingly, most of these functions have been documented for plant LTPs in the literature, but the problem is mostly the researcher focuses only on each function separately.

Again to focus and specify one function regarding to the first finding that the protein and as well as the transcript level of ns*ltp* has declined drastically after the infection, one can hypothesize that nsLTP can delay PCD via phospholipids transfer and membrane repair and/or by recruiting lipid derived antioxidants.

Ultimately, if this notion is true, nsLTP can play a vital role in the life cycle of *V. inaequalis* particularly after penetrating cuticle layer. During this critical period nsLTP can delay PCD until the fungus switch on its own mechanism by release melanoprotein to the apoplast. Melanoprotein revealed ability to bind iron and therefore interferes with the oxidative stress (Singh et al., 2005). Consequently nsLTP can rank as a susceptibility factor especially in the case of biotrophic pathogens like *V. inaequalis*.

For further study it will be very useful to perform radioactive pulse chase experiment in order to confirm the lipid transfer activity *in vivo*. Since the apple ns*ltp* belongs to a multigene family represented by at least ten copies distributed through apple genome, the RNA interference technique seems to be promising approach to investigate the *in vivo* function of this protein.

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Gau, A.E., **Koutb, M**., and Kloppstech, K. (2002): Pathogen induced changes in the apoplast protein composition of *Malus domestica* 15. Tagung Molekularbiologie der Pflanzen vom 26.02–01.03.2002. In Dabringhausen, Germany. Abstract and poster presentation

Koutb, M., Piotrowski, M., Kloppstech, K., and Gau, A.E. **(**2004): Constitutive expression of pathogenesis-related genes in the resistant apple cultivar (*Malus domestica* cv. Remo) and accumulation of PR proteins in the apoplast of a susceptible apple cultivar Elstar after infection with *Venturia inaequalis*. Botanikertagung 05. bis 10. September 2004 in Braunschweig, Germany

Gau, A.E., **Koutb**, **M.,** Piotrowski, M., and Kloppstech, K. (2004): Accumulation of pathogenesis-related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo (Eur J Plant Pathol, 110, 703-711)

Degenhardt, J., **Koutb**, M., Piotrowski, M., Schönherr, J., Szankowski, I., Kloppstech, K., and Gau, A.E. (2004): Proteome and transcriptome analyses of resistant and susceptible apple cultivars. 17. Tagung "Molekularbiologie der Pflanzen" vom 09.03.-12.03.2004 in Dabringhausen; Abstract and poster presentation.

8 Erklärung

Die vorliegende Arbeit habe ich selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel verfaßt.

Mostafa Koutb

Hannover, November 2005

9 Acknowledgments

First I would like to thank our Almighty Allah who helped me to achieve this dream of mine successfully. I remember my late mother for her unequivocal sacrifices. I also would like to thank my father for his struggle to ascertain better education for me.

In deed words fail to express my sincerely grateful and deeply indebtedness to my supervisor PD. Dr. Achim Gau for his continuous guidance, patience, and encouragement during the entire study period. His teaching strategy helped me to build a self confidence beside the knowledge. Really his supervision was a profound inspiration and he was an excellent representative for the German scientists. Therefore I am so indebted to him there is no way to repay it.

My honest thanks and gratitude must go to Prof. Dr. Hans-Jörg Jacobsen for his delightfully acceptance to be the co-referee and as well as for giving me the opportunity to use freely the PDS-1000/He Biolistic®, Particle Delivery System during my study.

Special thanks must go to Prof. Dr. Klaus Kloppstech firstly for accepting me as a M.Sc. student in his group and secondly for his enthusiasm to solve all problems that I faced during my study.

Many thanks to Dr. Markus Piotrowski for performance mass spectrometry analysis.

Thanks to Prof. Dr. Edgar Maiß for his kindly providing the DsRed vector and to PD. Dr. Jutta Papenbrock for providing the GFP vectors.

I also wish to thank Dr. Iris Szankowski for providing the *in vitro* apple cultures and to Dr. Katia Schulze for providing *Venturia inaequalis* isolate.

I am very grateful to Mrs. Julia Lensing and Mrs. Gabi Kühne for their excellent technical assistance especially with the cultivation and propagation of the plant materials.

Thanks for all my colleagues of PD. Dr. Achim Gau laboratory for their unequivocal support and cooperation during the whole period of study.

Many thanks for my home institution, Botany department, Faculty of science, Assuit University, Assuit, Egypt for securing my sabbatical leave to complete my PhD study.

I am gratefully appreciating the financial support of this work that was awarded to me by the Egyptian government as a long-term scholarship.

Last but not least I would like to thank my beloved family; my devoted wife Afaf and our God`s gift Hagar, Ahmed and Hala without their love, patience, encouragement and support, this work would never been done. They sacrificed a better life so that I could follow this dream of mine.

10 Curriculum vitae

PERSONAL

FAMILY NAME	**KOUTB**
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EDUCATION

M. Sc. (Hannover, Germany)	2001 - 2003 Apoplastic protein analysis of apple leaves during infection with *Venturia inaequalis* the causal agent of apple scab. (Summa cum laude)
Preparation for M. Sc.	1995-1996 Microbiology Assuit University, Assuit, Egypt Set of advanced courses in microbiology (Very good)
B. Sc. (Honours)	1990 – 1994 Botany Assuit University, Assuit, Egypt Degree obtained in 1994 (Very good with honours)
LANGUAGE	Arabic - Mother tongue English - Excellent German - Fair