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CHARACTERISATION OF NON-HOST RESISTANCE in *Solanum nigrum* AGAINST *Phytophtora infestans*

Dissertation

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Submitted by

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Abstract

Late blight, caused by the oomycete *Phytophthora infestans* is the most devastating disease of potato worldwide. The pathogen is very variable and has overcome several specific resistance genes that were bred into cultivated potato within a few years. Therefore, there are no useful resistance genes against this pathogen in the currently grown potato varieties. The black nightshade (*Solanum nigrum*), a relative of potato, can grow as a weed in potato fields without being infected with *P. infestans*. *S. nigrum* was therefore considered a non-host for *P. infestans*. However, the present status on *S. nigrum* as a non-host has to be reconsidered because of the presence of field infections in the Netherlands and results presented in this thesis. Twenty *S. nigrum* accessions were screened for *P. infestans* infections using four isolates but no sporulation was found in any interaction. T04 was found more virulent and able to develop some hyphae but no sporangia were produced. The fact that the pathogen is stopped by a very fast hypersensitive response (HR) strongly indicates the involvement of a resistance gene. The test of these accessions with *P. infestans* elicitins did not result in necrotic cells, indicating that the HR is not due to recognition of elicitins. Since *S. nigrum* is a hexaploid, positional cloning or a mutant approach are very difficult. Therefore virus-induced gene silencing (VIGS) was used to characterise genes involved in this interaction. Vacuum infiltration was found to be the best technique to deliver the TRV vector carrying the genes of interest into *S. nigrum* plant cells through *Agrobacterium.* GV3101 was found as the most appropriate *Agrobacterium* strain because it did not cause any HR in these accessions but gave a good GUS expression in the infiltrated leaves. The TRV vector was able to replicate and spread systemically in *S. nigrum* plants and phytoene desaturase (*PDS*) was found as an effective visual indicator of VIGS efficiency in this species The genes of interest were amplified from *S. nigrum* genomic DNA and cDNA. The mRNA levels of *Eds1, Ndr1* and *Rar1* were not reduced after silencing but, surprisingly, there were some leaves with lesions in some pTRV-*Eds1*- and pTRV-*Rar1*- infiltrated plants after *P. infestans* infection although no sporulations were found. *S. nigrum,* being a hexaploid, might contain genes similar to *SnEds1* and *SnRar1* which were partly silenced but not detected in the RT-PCR.

Kurzfassung

Die Kraut- und Knollenfäule, verursacht durch *Phytophtora infestans*, ist eine der gefährlichsten Erkrankungen der Kartoffel weltweit. Der Krankheitserreger zeichnet sich durch hohe Anpassungsfähigkeit aus und konnte innerhalb weniger Jahre die Effektivität mehrer Resistenzgene brechen, wodurch zurzeit keine funktionierenden Resistenzgene in aktuellen Sorten vorhanden sind. Der Schwarze Nachtschatten (*Solanum nigrum*), ein Verwandter der Kartoffel, wächst als Unkraut in Kartoffelfeldern und wird nicht von *P. investans* befallen. *S. nigrum* wurde daher als Nicht-Wirt angesehen. Diese Ansicht muss auf Grund von Feldinfektionen in den Niederlanden und den Ergebnissen dieser Arbeit überdacht werden. Es wurden 20 *S. nigrum* Kultivare mit vier *P. infestans* Isolaten untersucht wobei in keinem Fall eine Sporenbildung beobachtet werden konnte. Das Isolat T04 hatte doe höchste Virulenz und konnte einige Hyphen produzieren. Die Tatsache, dass der Erreger sehr schnell durch eine Hypersensitive Reaktion (HR) der befallenen Zelle gestoppt wird, lässt auf die Wirkung eines Resistenzgenes schließen. *S. nigrum* Kultivare wurden mit *P. infestans* Elicitinen getestet und es konnte keine Nekrotisierung festgestellt werden. *S. nigrum*,ist eine hexaploide Pflanze was die Klonierung von Genen sehr erschwert. Deshalb wurde transientes Gen-Silencing (VIGS) verwendet, um Gene, zu charakterisieren. Der Agrobakteriumstamm GV3101 wurde verwendet, weil dieser von mehreren getesteten Stämmen keine HR induzierte. Die Bakterien mit den TRV Vektoren wurden mittels Vakuum Infiltration in die Pflanzenzellen von Blättern von *S. nigrum* gebracht. Der TRV Vektor konnte sich replizieren und sich systemisch in der Pflanze verbreiten. Die Phytoen-Desaturase stellte sich als effektiver visueller Indikator für die Effizienz des Gen-Silencing heraus. Die zu untersuchenden Gene wurden mittels PCR von genomischer DNA amplifiziert. Ein Effekt des Gene Silencing auf die Genexpression von *Eds1*, *Ndr1* und *Rar1* konnte nicht nachgewiesen werden, aber interessanter Weise konnten auf einigen Blättern von pTRV-*Eds1*- und pTRV-*Rar1*- infiltrierten Pflanzen Läsionen nach *P. infestans* Infektion beobachtet werden. Dies könnte darauf hindeuten, dass *S. nigrum* als hexaploide Pflanze, Gene enthält, die *SnEds1* and *SnRar1* ähneln und partiell reprimiert wurden, was aber nicht durch RT-PCR nachgewiesen werden konnte.

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1 Introduction and objectives

Potato (*Solanum tuberosum*) is one of the most important crop plants in industrial and developing countries. Late blight is one of the most destructive diseases of crop plants and a threat to potatoes worldwide. Late blight epidemics on potato crops were the cause of the great Irish famine in 1840s; up to one million people died and a similar number of people emigrated to the rest of Europe and the USA. Since that time, even many chemical companies and a lot of researchers are focussed on producing new fungicides or new resistance gene against it, this disease is still a major problem nowadays; it is estimated to cost growers US $ 5 billion per year (Judelson and Blanco, 2005). It is caused by the Oomycete *Phytophthora infestans* (Mont.) de Bary, formerly classified as a fungus but now classified as a relative of brown algae (Baldauf *et al*., 2000). The genus was named after the Greek words for “plant destroyer” (Judelson and Blanco, 2005). Cultivated potato is thought to have originated from the Andean region of South America whereas *P. infestans* is thought to have its origin in the central highland of Mexico (Grünwald and Flier, 2005). *P. infestans* germinates either by releasing zoospores or by producing a hyphal outgrowth and has two mating types, called A1 and A2. Spores are wind-born and can travel several kilometres (Judelson and Blanco, 2005). If both mating types are present in a population, sexual reproduction may occur.

Late blight disease causes enormous losses every year for potatoes but also infects tomatoes. Race specific resistance genes derived from related *Solanum* species, has been incorporated into potato, but have all eventually been defeated by *P. infestans.* Therefore the pathogen is very dangerous. In western countries the disease is controlled by several applications of fungicides during the growing season which could have significant environmental problems. However, in developing countries, these chemicals are not always affordable. In addition, *P. infestans* is developing resistance against several agrochemicals (Gisi and Cohen, 1996).

Race specific resistance, which is controlled by single specific plant resistance genes, can give complete resistance; however, in most cases, this resistance is not durable in the field. Many of these resistance genes have been cloned during the last years and many more are known from large-scale sequencing projects. According to their protein sequence, these genes have been grouped into different classes (Dangl and Jones, 2001). The largest class comprises the nucleotide-binding (NB)-leucine-rich-repeat (LRR) genes which can be further divided into those having either an N-terminal coiled-coil (CC) or TIR domain. Other classes include the LRR genes that are anchored in the plasma membrane such as the *Cf*-genes from tomato (Jones *et al.,* 1994), kinases such as *Pto* (Martin et al., 1993), LRR-kinase genes (Song et al., 1995), and anchored CC genes such as RPW8 from *Arabidopsis* (Xiao et al., 2005).

Quantitative resistance, which is controlled by several different genes, is considered as durable but gives only a limited degree of resistance (Colon et al., 1995; Grünwald *et al*, 2002). Non-host resistance, on the other hand, is also considered stable and gives total resistance (Freialdenhoven *et al.,* 2005; Mysore and Ryu, 2004). Thus, the majority of plants are resistant to the vast majority of possible pathogens. The nature of non-host resistance is poorly understood but could be of enormous practical importance if it would be possible to transfer it to crop plants.

Black nightshade (*Solanum nigrum)*, a weed related to potato, is considered a non-host for *P. infestans* (Kamoun et al., 1999; Vleeshouwers et al., 2000). *S. nigrum* is often growing as a weed in potato fields but, with very few exceptions (Flier *et al*., 2003), has never been found infected by *P. infestans*. It would therefore be interesting to transfer this resistance to the cultivated potato. However, these species cannot be crossed and somatic hybridisation between *S. nigrum* and potato is possible but difficult and the outcomes are plants that have lost most properties of cultivated potatoes (Szczerbakova *et al.,* 2002).

Closer inspection has revealed that *P. infestans* tries to invade epidermal cells of *S. nigrum* but is stopped by a hypersensitive reaction (HR) (Vleeshouwers *et al.*, 2000). The HR is usually involved in race-specific resistance against biotrophic pathogens governed by resistance genes in a gene-for-gene interaction (Hammond-Kosack and Jones, 1996; Ballvora *et al.,* 2002). This strongly suggests that this non-host resistance of *S. nigrum* against *P. infestans* is controlled by specific resistance genes. The resistance genes from other *Solanum* species and wild potatoes which have been cloned were found to belong to the NBS-LRR class (Ballvora et al., 2002; Song et al., 2003; van der Vossen et al., 2003; Sliwka, 2004; Paal et al., 2004; Huang et al., 2005). Furthermore, other resistance genes from potato against other pathogens (Bendahmane et al., 1999; van der Vossen et al., 2000) also belong to this class.

During the last years many resistance genes have been cloned from many different plant species mainly by positional cloning (Huang *et al.*, 2005). *S. nigrum* is unfortunately a difficult experimental system. It is hexaploid, which means, that a mutation strategy to discover the resistance gene as well as positional cloning would be very difficult and time consuming. Therefore, virus-induced gene silencing (VIGS) was used in an attempt to test if the resistance genes are responsible for the resistance of *S. nigrum* against *P. infestans*.

1.1 Phytophthora infestans

Anton de Bary (1876) determined that the fungus, described *Botritis infestans* by Montagne (1845), which he renamed *P. infestans* was the cause of potato late blight.

The main hosts of *P. infestans* are potato (*S. tuberosum*) (Kamoun *et al.,* 2001) and tomato (*Lycopersicon esculentum*) crops (Figure 1) (Judelson, 1997) but pear melon (*S. muricatum*, "pepino") and other *Solanaceae* species in the genus *Solanum* can be attacked as well (Türkensteen and Flier, 2003). Other non-*Solanum* genera in the *Solanaceae* such are *Petunia* and *Calibrachoa* have also been implicated as hosts (Becktell et al., 2006).

Pictures from David Shaw's page: http://biology.bangor.ac.uk/~bss081/

Figure 1: Potato (left) and tomato (right) blights

There are many questions that remain to be answered concerning the origins of the late blight pathogen. However, it is now apparent that the pathogen originated in Mexico (Grünwald and Flier, 2005).

P. infestans is taxonomically classified as Oomycetes, in the Stramenopila Kingdom, family of the Pythiaceae, and order of the Peronosporales (Turkensteen and Flier, 2003). Oomycetes are a diverse group of eukaryotic microorganisms. The genus *Phytophthora* consists of over 60 different species; all but three species are plant pathogens. Oomycetes had been initially placed in the fungal kingdom, but now are excluded from this because of their characteristics such as cell walls composed of cellulose and glycan rather than chitin, aseptate hyphae, food storage as starch and the presence of biflagellate swimming zoospores. The position of the oomycetes as a unique lineage of stramenopile eukaryotes, unrelated to true fungi, but closely related to heterokont (brown) algae has been well established using molecular phylogenies based on ribosomal RNA sequences, compiled

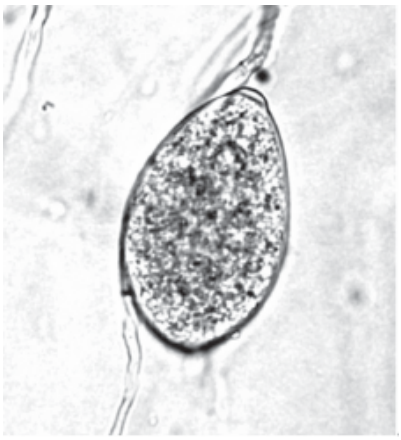
amino acid data for mitochondrial proteins, and four protein encoding chromosomal genes (Kamoun *et al*, 1999). One of the many features that distinguish oomycetes from true alguae is that oomycetes are diploid and lack a free haploid life stage. True fungi are typically haploid or dikaryotic (Judelson and Blanco, 2005). The key aspects of their structure, biology and pathology differ from true fungi. Their hyphal growth and variety of spores are morphologically and physiologically similar to fungi, for which they are occasionally mistaken, but their parasitic lifestyles have independent evolutionary origins (Nicholls, 2004).

P. infestans is an obligate biotroph. The thallus consists of coenocytic, diploid hyphae for most of its life cycle. *P. infestans* produces microscopic asexual spores called sporangia (Figure 2), which are either broken off from the filamentous hyphae to become airborne or remain attached and divided into swimming zoospores (Figure 3) following rain, and sexual spores named oospores (Figure 4) with characteristics that greatly contribute to their pathogenic success (Judelson and Blanco, 2005). This species is heterothallic, requiring presence of two different strains A1 and A2 for sexual reproduction to occur.

P. infestans has a huge genome size which is about 250 megabases, it is about twice the size of Arabidopsis genome (Nicholls, 2004).

1.1.1 Sporangia

Sporangia are formed at the terminal end of the sporangiophores. Sporangia are hyaline, ovoid, ellipsoid to limoniform, tapering at the base, caducous (pedicel <3µm), and semipapillate. The average size of sporangia ranges from 36 x 22 µm to 29 x 19 µm ((Mont.) De Bary, 1876). Sporangia are formed on specialized branches called sporangiophores. The branched sporangiophore, with swellings at the points where sporangia are attached are distinctive for *P. infestans* and useful for the identification of this pathogen.

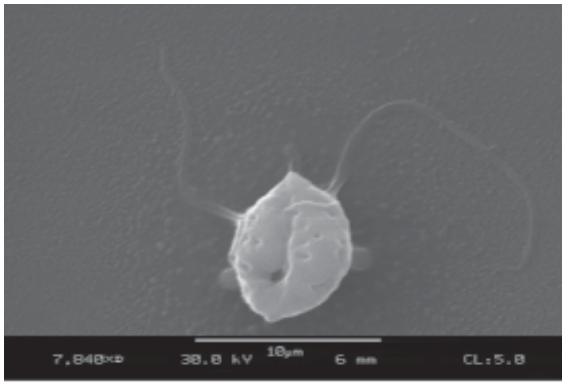


Source: Nicholls (2004)

Figure 2: Sporangia

When placed in water or a very high relative humidity, the cytoplasm in the sporangia divides and releases dozens of motile zoospores. A zoospore has two flagella (Figure 3). The zoospores eventually lose their flagella and encyst when they reach a wet potato leaf where they can germinate. In the absence of sufficient water or with temperatures above 24°C, no zoospores form. Sporangia can also germinate by producing germ tubes that penetrate the host.

Zoospores survived for 10 days, sporangia for 42 days, and mycelia for 28 days *in vitro* in nonsterile soil at 22°C. Survival depends on soil type and moisture content. Spores survived in surface water between 14 to 21 days. The plant canopy in a potato field would provide the shade that favours spore survival of up to three weeks in surface water. The presence of soil in the water significantly increased the length of spore survival and the number of spores surviving over extended periods. Shade and soil in water increased the duration and number of spores surviving (Poter and Johnson, 2004).



Source: Nicholls (2004)

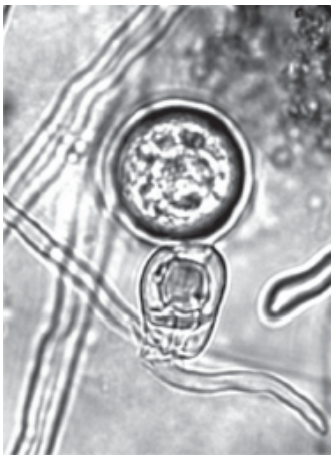
Figure 3: Zoospore

1.1.2 Sex organs

The sex organs, gametangia, form on two separate hyphae; therefore, *P. infestans* is heterothalic. Thalli of both compatibility types A1 and A2 need to be present for sexual reproduction to occur. These mating types are distinguished by the production of specific hormones that induce the formation of gametangia in the opposite mating type. Diploid vegetative mycelia differentiate to form either antheridium (male gametangia) or oogonium (female gametangia) in which meiosis occurs. The oogonium is larger than the antheridium. The female hypha penetrates the male hypha, forming a spherical oogonium with the antheridium as a collar. The union of gametes occurs when oogonia moves through a loop in antheridia. The nucleus of the antheridium enters the oogonium and fuses with its nucleus to produce the zygote. This fertilization leads to the development of an oospore. A single oospore is produced within a larger oogonium (mother cell) and the antheridium is at the

base. Oospores have thickened walls and can survive several years in the soil, free of host tissue. Therefore, oospores serve as survival structures and primary inoculum. During outcrossing, genetic recombination occurs in the oospore and leads to genetic variation in the subsequent population of the pathogen.

Most likely, formation of oospores results from the stimulation by hormonelike substances emitted by the opposite mating type. Oospores formed in plant leaves are 24 to 35 µm in diameter; in artificial culture they measure 24 to 56 µm in diameter (Flier et al., 2003).



Source: Nicholls (2004)

Figure 4: Oospore

Until the early 1980s, A1 was the predominant mating type detected in all regions of the world except central Mexico, where both A1 and A2 mating types coexisted in a 50:50 ratio. Since the report of Hohl and Islen (1984), A2 isolates are now found in nearly all potato-growing regions of the world, but usually with ratios less than 50:50. It is not understood why the A2 type did not spread throughout the world with the movement of potato as a food crop during the 1800s (Flier *et al*., 2003).

1.2 Disease symptoms

Symptoms on plants include black lesions on the stems and leaves and rapid wilting. (Kamoun *et al.,* 1999). A pungent odour usually becomes prevalent in potato fields before the more obvious symptoms of the late blight are apparent. The first signs of impending disaster usually appear on the leaves, initially as brownish or purple-black lesions at the tip or margins. These then spread over the rest of the blade. The stalk and stem turn to black slime. Sometimes, the infecting spores attack the tubers directly when zoospores or sporangia are washed from infected stems or leaves and come in contact with tubers. Tuber infection occurs through buds, lenticels, and wounds. When that occurs, damage appears

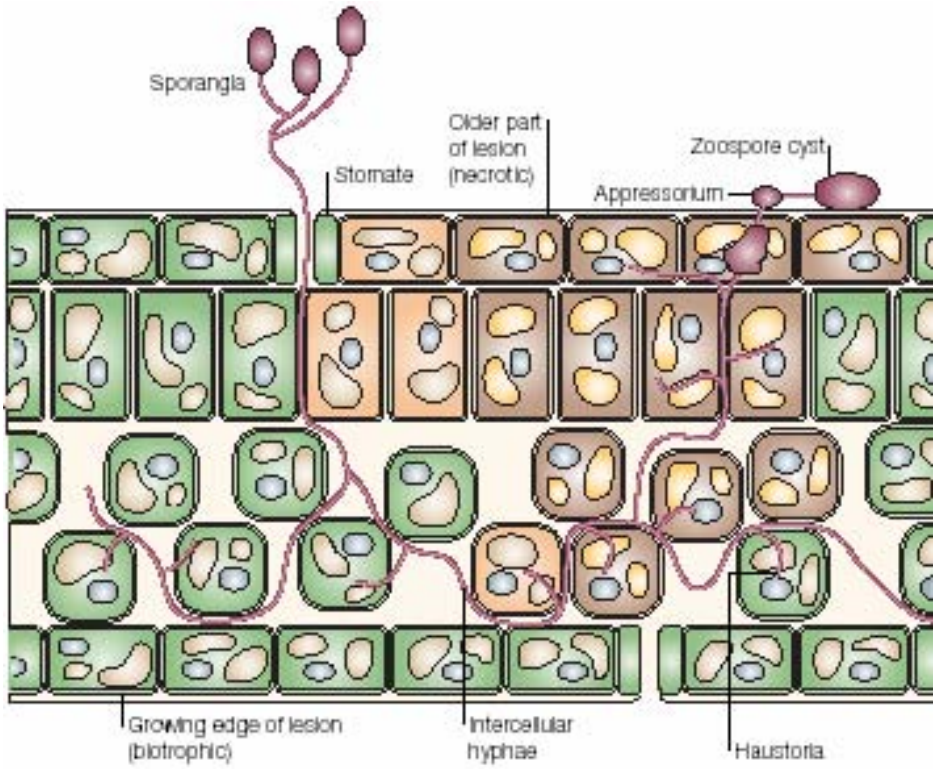
first as dark blotches on the skin of potato. As the incursion progresses, secondary invasions turn the weakened flesh to mush. The inner starchy tissue of infected tubers appears rusty red to dark brown, and initial lesion spread is most apparent just under the periderm of the tuber. In damp soils the disease progresses rapidly, and the tuber decays either before or after harvest. Within a week, an entire field can be wiped out (Porter and Johnson, 2004).

1.3 Disease mechanisms

Although a pathogen of a great economic importance, little is known about the molecular mechanisms that determine the outcome of interactions between *P. infestans* and plant hosts (Kamoun *et al.,* 1998).

In the presence of moisture and low temperature, zoospores are formed and released from sporangia. These zoospores swim in water and can migrate over the wet leaf surface. Coming in contact with the host tissue, they encyst and germinate (Kamoun *et al.,* 1998). If the temperature is over 15°C, however, then the sporangia will most likely germinate directly into a germ tube and penetrate the leaf tissues. Penetration can also be indirectly through stomatal openings. Germ tubes form an appressorium that directly penetrates the cuticle and epidermis layers of the plant tissue to form an intracellular infection vesicle just 16 hours after infection (hai). In leaf cells, the hyphae form haustoria that can uptake nutrients from the plant tissues (22 hai). The secondary hyphae continue to grow intercellularly, moving through the leaf mesophyll cells until they reach the abaxial side of the leaf. Later, from 46 hai, infected tissue starts to necrotize, and the mycelium develops sporangiophores that exit the leaf tissues through the stomata to produce numerous sporangia (Figure 5) (Vleeshouwers et al., 2000). Sporangia are produced rapidly on infected leaves at temperatures near 21°C when relative humidity is near 100%. Deciduous sporangia are readily splashed by water or spread by wind. Disease development is favoured by cool (16 to 21°C), cloudy, moist weather, during which new sporangia are continually being formed. The minimum temperature for growth is 4°C, optimum 20°C, and maximum 26°C.

Compared to infection by asexually produced zoosporangia and zoospores, oospores initiated infections are relatively rare. Infections through oospores are mainly through leaflets and young stem tissues. For oospores to germinate and to infect, a 24 hour period of puddles in the field appears to be necessary. However for a pathogen with an extremely rapid spreading capacity, this does not appear to be a major constraint (Turkensteen and Flier, 2003).



Source: Judelson and Blanco (2005)

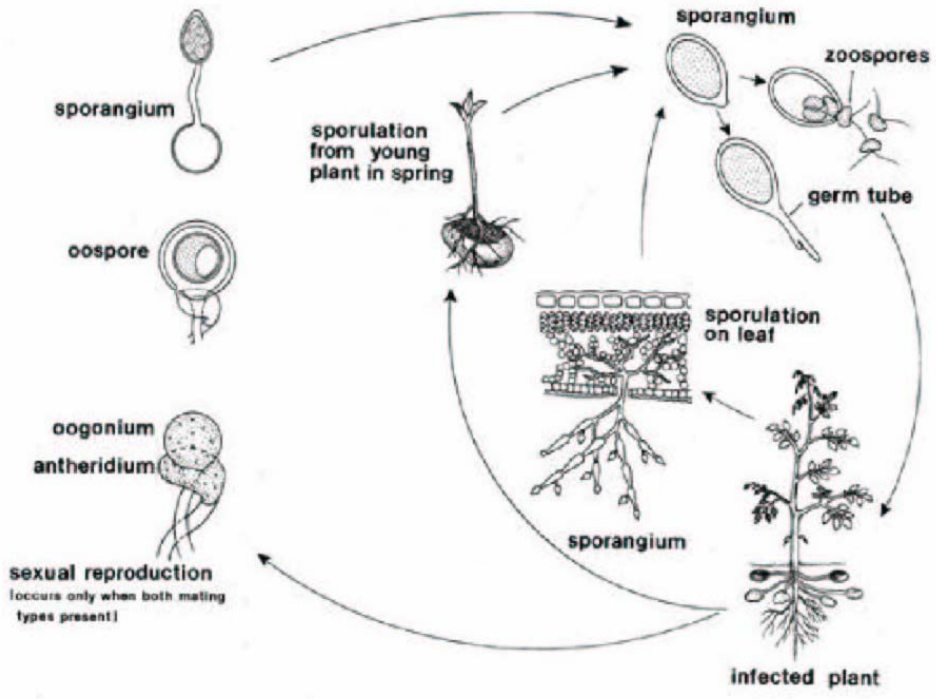
Figure 5: Disease mechanism

Typically wind-blown sporangia release zoospores on the plant surface, which encyst and germinate to form appressoria. These enable the host epidermis to be breached, after which hyphae spread throughout the plant. New sporangia usually appear near the boundancy between living and necrotic plant cells.

1.4 Disease cycle

It is believed that *P.infetans* reproduces predominantely by asexual reproduction. Sporangia on infected host tissue that either germinate directly or release zoospores are responsible of infection. The asexual cycle of the pathogen was thought to be the primary disease cycle in the most fields. *P. infestans* can be disseminated by airborne sporangia and spread for miles from point sources by water splash of sporangia from infected leaves. Sporangia land on healthy foliage and produce zoospores. The germ tubes formed by zoospores penetrate the leaf epidermis at multiple sites causing small spots. These small spots expand rapidly and leaf spots and blights develop. At close to 100% relative humidity, *Phytophthora* produces abundant amounts of sporangia on the surface of leaves. Sporangial masses are white on dark coloured leaf lesions. These are blown to healthy plants and continue the disease cycle. Thus, epidemics can result from the dispersal of inoculum from distant fields or from infected seed pieces when the environment is highly conducive for disease. Infection of new host plant may take place inside 2 hours and production of new sporangia within 3 to 5 days (Kamoun *et al.,* 1998). During rainy periods, sporangia or

zoospores can be washed down to the soil level from the leaves where they can infect tubers near the soil surface. Once in the tubers, the fungus can overwinter as mycelium until the following spring when new sporangiophores can develop. *P. infestans* survives from season to season as mycelium in infected potato tubers in storage, in potato cull piles and in tubers left on the field after harvest and debris when asexual cycle is predominant and thought to be the initial sources of inoculum in the following growing season (Türkensteen *et al.*, 2000). This inoculum source contributes to epidemic development on subsequent potato crops. Oospores are also considered to be a source of both inoculum and pathologic variability. The oospore is hardy and can overwinter in debris in the soil. In the spring, the oospore produces germ tube which yields either a sporangia or mycelia. Thus, oospores are thought to have two main functions in the life cycle of *P. infestans.* First, they provide a means of survival and could serve as primary inoculum for epidemic development; second, they provide a means of sexual recombination (Grünwald and Flier, 2005). Throughout their life cycle, *P. infestans* hyphae are diploid (Figure 6) but the only haploid stages occur in the gametangia that formed immediately prior to fertilization (Roetschi *et al*, 2001).



Source: http://www.faculty.virginia.edu/evolutionlabs/Speciation_Lab_One.html

Figure 6: Disease cycle

The oomycetes have a fungus-like life cycle. The propagules of many soil-borne fungi do not increase over the course of a growing season (single-cycle pathogens). In contrast, *P. infestans* has a devastating potential because it is multi-cyclic and can produce continuously after the initial infection, as long as conditions remain favourable.

1.5 Resistance mechanisms

Plants are constantly exposed to various pathogens in nature. Resistance responses to pathogens are traditionally classified as race-specific, race-nonspecific, and non-host resistance.

1.5.1 Race-specific resistance (qualitative, vertical resistance)

Race-specific resistance, also called qualitative or vertical resistance, is based on the presence of major resistance *R*-genes which is simply inherited in accordance with Mendel's laws (Sliwka 2004). Race-specific resistance is only effective against certain strains of the pathogen, and is easily overcome by rapid evolution of the pathogen, resulting in a lack of durability in the field (Vleeshouwers, 2000).

1.5.2 Race-nonspecific resistance (quantitative, horizontal resistance)

Race-nonspecific resistance is thought to be based on many genes, or a combination of single genes (quantitative trait loci, QTL) conferring quantitative resistance (Grünwald *et al.,* 2002) but their number, individual contribution, and chromosomal linkage is unknown (Black 1945). It is also referred to as rate-reducing, general, field, polygenic, minor gene, partial, and horizontal resistance (Grünwald *et al.,* 2002). The durability of the race-nonspecific resistance is believed to be caused by the additive effect of polygenes (Umaerus, 1970) and is correlated with late maturity (Trognitz et al 2001).

By contrast to the race specific resistance, race-nonspecific resistance is effective against all known strains or races of a pathogen and is generally of a partial nature (Vleeshouwers, 2000). This resistance manifests itself by a reduction in the apparent infection rate, herein referred to as rate-reducing resistance and not (yet) determined to be based on a gene-for-gene interaction (Grünwald *et al.,* 2002). Race-nonspecific resistance may be due to intrinsic properties of the plant or may be induced by nonspecific elicitors produced by all races of the pathogen.

1.5.3 Non-host resistance

Non-host resistance is the most common, durable and non-specific type of resistance observed in plant-pathogen interactions, making this type of resistance of great interest for agriculture (Heath, 2000). Non-host resistance is defined as a full resistance at the species or genus level (Kamoun *et al.,* 1999b). It is thought to be genetically complex, involving preformed and inducible defences (Freialdenhoven *et al*., 2005).

Non-host resistance is considered to be durable in the field (Vleeshouwers et al., 2000). Kamoun and associates (1998) reported that durable and stable resistance responses may

have evolved in non-host plants through the accumulation of an arsenal of *R*-genes. Non-host resistance is presumed to be a complex, multi-component form of resistance, including both constitutive and inducible defences. Non-host resistance may also result from pathogen species being poorly adapted to the basic physiology or growth habit of the plant species (Kamoun *et al*., 1999). A major component of non-host resistance is penetration resistance (Zimmerli *et al.,* 2004).

Notably, the non-host resistance in *Arabidopsis* to the wheat powdery mildew appears to consist primarily of *EDS1* (Enhanced Disease Susceptibility 1) (Yun *et al*., 2003; Zimmerli *et al*., 2004). The salysilic acid (SA) signal transduction pathway plays an important role in defense responses initiated by *R*-genes (Glazebrook, 2001); however, its contribution to non-host resistance is less clear (Parker *et al*., 1996).

1.6 Molecular basis of the resistance mechanisms

An important component of the plant defence is a gene-for-gene resistance response. The structurally and functionally conserved resistance (*R*) proteins, directly or indirectly, recognize specific molecules (usually proteins) produced by pathogen avirulence (*Avr*) genes and activate the different signal transduction chains which are functionally connected to a number of transcription factors regulating the activity of defence gene promoters (Thurow *et al*., 2005). The precise mechanisms controlling *R-avr* gene-specified resistance are poorly understood, although a requirement for SA, a phenolic compound derivative, has been demonstrated in several plant-pathogen interactions (Falk *et al*., 1999). This recognition event often leads to the hypersensitive response (HR), a form of programmed cell death, at the site of infection. The HR proceeds by a rapid oxidative burst, leading to generation of reactive oxygen intermediates (ROI) such as O_2^-, H_2O_2, and OH, various physiological events such as changes in ion flux (Ca^{2+} and H^+), cell wall strengthening and callose deposition, protein phosphorylation, accumulation of SA, and activation of various defence genes. Pathways involving SA are effective against biotrophs (Wiermer, 2005). In addition to SA, the signalling molecules jasmonic acid (JA), ethylene, and nitric oxide (NO) have been implicated in mediating plant defence (Liu *et al*., 2004). The HR is thought to confine the pathogen by stopping its spreading from the site of attempted infection, and is likely to involve active plant metabolism (Zhang *et al*., 2004). Other components involved in the expression of resistance have also been identified, such as pathogen-related PR proteins (e.g. PR1 to 5), enzymes required for phytoalexin biosynthesis (e.g. *PAD3*), signal transduction factors (e.g. *NPR1, EDS1*, and *PAD4*), and possible rate-limiting factors of defence (e.g. *RAR1* and *SGT1*). This suit of responses at the site of infection directs the plant to prime uninfected tissues against subsequent attack in a process called systemic acquired resistance (SAR) (Wiermer, 2005).

If pathogens breach this last line of defence, they cause disease (Liu *et al.,* 2004).

1.7 Resistance against *Phytophthora infestans*

P. infestans has the remarkable ability to manipulate biochemical, physiological and morphological processes in its host plants through a diverse array of virulence and avirulence molecules. It has a high degree of variability that permits it to originate more aggressive forms (Abad *et al.*, 1995). This problem has been challenging plant pathologists, breeders, molecular biologists, historians, and other professionals more than at any other time (Abad *et al.*, 1995).

In response to the burgeoning risk, scientists in 1996 established the Global Initiative on Late Blight (GILB), an undertaking of some 700 researchers in 76 countries to conduct and coordinate research into the potato and the pathogen. The same year, in recognition of the special vulnerability of Russia and Eastern Europe, a group of plant pathologists at Cornell undertook to organize CEEM (Cornell-Eastern Europe-Mexico Project). Such efforts are beginning to pay off as researchers uncover potential vulnerabilities in the pathogen and outline better defensive strategies (Garelik, 2002).

Frequent fungicide applications during the growing season have held the disease at bay even though imperfect, expensive and hardly environment friendly. However, fungicides are out of reach in developing countries because of their high cost and difficulties in distribution. For most countries, fungicides have never been an option. In addition, even in rich countries, although metalaxyl and other phenylamide fungicides are the most highly effective, the development of new fungicide-resistant races within populations of *P. infestans* has become a limiting factor in the use of fungicides. No fungicide has ever been found to which *P. infestans* could not ultimately adjust and potato growing is under great pressure to minimize pesticide inputs because of concern about environment and health aspects (Garelik, 2002). Therefore, to overcome this problem and to limit chemical control, the most promising and sustainable solutions will involve not new fungicides but genomics: breeding potato to incorporate durable forms of genetic resistance. Durable resistance was defined by Johnson as resistance that remains effective in a cultivar that is widely grown for a long period of time in an environment favorable to the disease (Grünwald *et al*., 2002). Resistance is a basic part of control strategies for many pathogens.

Avr genes have been isolated. General accounts of the genetics of *Phytophthora* are available (Shwa 1983; 1988) and methods are collected in “A sourcebook of the genus *Phytophthora*” (Ribeiro, 1978). The goal of genetic studies is to identify all genes in the pathogen and the host that are involved in the interaction. Researchers then hope to use that knowledge to breed, find, or engineer resistant varieties (2002).

Resistance breeding against late blight in potatoes started in the twenties of the last century with the introduction of *R*-genes which in a short period were neutralized by new virulent strains. The increase of aggressiveness of the pathogen requires higher levels of resistance to counterbalance the increased risk of attack. Unfortunately, moderate levels of resistance may imply also increased oospores production. In the *P. infestans* - potato interaction, the most commonly studied type of resistance is race-specific resistance, which is governed by single dominant resistant genes (*R*-genes). In the beginning of the 20th century, 11 *R*-genes conferring resistance to *P. infestans* were discovered in *Solanum demissum,* a wild potato species indigenous to Mexico (Sliwka 2004). *R1* was the first gene for resistance to late blight to be cloned from potato and belongs to the CC-NB-LRR class of *R*-genes. It is located in the resistance hot spot on potato chromosome V. *R1* is race-specific and confers resistance to races of *P. infestans* carrying *Avr1* (Ballvora *et al*., 2002). *R1* was located on the potato map in 1992 by Leonards-Schippers. Since then, nine R-genes (*R2, R3a, R3b, R6, R7, RB, Rpi-blb1*, R_{ber}*, Rpi1*) have been mapped, and four of them have been cloned: *RB* and *Rber* are two major resistance R-genes from two other wild species *Solanum bulbocastanum* and *Solanum berthaultii* respectively. *P. infestans* isolates overcoming the *RB* gene newly introduced to the potato gene pool have not been detected so far, which indicates that this gene may be very useful for breeding purposes (Sliwka 2004). Grünwald *et al*. (2002), however, have found isolates of *P. infestans* in central Mexico that have a corresponding avirulence gene overcoming the *S. berthaultii R*-gene, putting in question the durability of this *R*-gene (*Rber*) once it is released. The coding sequence of *Rpi-blb1*, which has also recently been cloned (Song et al., 2003), is identical to that of the *RB* gene. *Rpi-blb1* and *RB* are thus identical genes (Grünwald and Flier, 2005). The cloning of *Rpi-blb1* has paved the way to develop late blight-resistant potato and tomato varieties through a transgene approach (Sliwka 2004). Both *R1* and *RB/Rpi-blb1* have been verified by complementation analysis in potato, and *Rpi-blb1* has also been shown to function in tomato (Sliwka 2004). *R3* has also been introgressed from *S. demissum*. The *R3* locus is composed of two functional *R*-genes (*R3a* and *R3b*) with distinct specificities and they displayed a differential reaction to some well-defined *P. infestans* isolates (Huang *et al.,* 2004). Major genes for *P. infestans* resistance have also been found in *Solanum microdontum* (Sandbrink) and *Solanum stoloniferum* (Vleeshouwers *et al.,* 2000).

The gene *Rpi1* discovered in *Solanum pinnasectum* (a diploid species with Endosperm Balance Number 1 (1 EBN) has not been transferred to the *S. tuberosum* genome due to the crossing barrier between these two species; nevertheless, this gene can also be effective against late blight, since no compatible isolate of *P. infestans* has yet been described (Sliwka 2004). The diploid *Solanum phureja* has been known as a potential source of resistance to

late blight (De Maine *et al*., 1993), but early attempts to transfer its resistance to the common potato failed (Trognitz *et al*., 2001).

The *R*-genes which originated from *S. demissum* (*R1, R2, R3a, R3b, R6* and *R7*) have been applied in potato breeding programmes, and *P. infestans* races compatible to those genes have been identified. Moreover, isolates able to overcome the resistance conferred by those genes are commonly present in *P. infestans* populations world-wide. The inefficiency of these genes was demonstrated as early as the late 1940s in Central Mexico (Sliwka 2004). Breeding for race-specific resistance to late blight was once considered an efficacious approach to control but has since proved to be of only limited use because many pathologic races of *P. infestans* have the ability to attack new cultivars with single-gene resistance. A century and a half of research has failed to control the highly adaptable organism (Judelson and Blanco, 2005). Race-specific *R*-genes do not provide lasting resistance in the field because of the frequent occurrence of compatible races of *P. infestans* (Trognitz, 1998). Once newly bred potato cultivars are grown on large scale in commercial fields, new virulences emerge in *P. infestans*, which render the pathogen able to overcome the introgressed resistance to late blight (Van der Vossen *et al*., 2003).

The alternative is considered quantitative or multigenic resistance, which relies on getting plants to express several resistance genes at once, each of which makes a small contribution to the plant's overall resistance (Nicholls, 2004). Soon after it became clear that gene-for-gene resistance based on single major *R*-genes was not durable, some potato breeders concentrated on developing cultivars with race-nonspecific resistance. This kind of resistance involves lowering the effectiveness of infection, slowing down the rate of colonisation of host tissue and hampering the sporulation of *P. infestans*. Field resistance is being given increasing importance in breeding programs; this has stimulated research on the genetic nature of this kind of resistance. Nowadays, it is possible to detect QTLs by analysis of molecular markers and linking molecular markers to phenotypic values. The QTLs for resistance to *P. infestans* have been mapped in several diploid potato populations and two tetraploid populations (Sliwka 2004). The strongest QTLs for late blight resistance were detected on chromosome XII, explaining 43% of variance, on chromosome V-41% or 20.8% or 15.8% or 8.8%, on chromosome III-36% or 25% or 19.1%, on chromosome VIII-31.6%, on chromosome X-31%, on chromosome IV-30%, on chromosome II-20.7% (Sliwka, 2004).

Many factors conferring resistance to late blight which have been found and mapped on almost every potato chromosome confirmed that several genes are responsible for rate-reducing resistance (Grünwald *et al.,* 2002). However, the genetics of this resistance is still unknown (Colon *et al.*, 1995a; Trognitz *et al.,* 2001). Sliwka (2004) has also reported that genes underlying quantitative resistance to *P. infestans* in the potato plant are not known, although regions linked to this trait have been mapped in several mapping studies.

Quantitative field resistance to late blight has also been identified in wild potato species (Ross, 1986) and several wild *Solanum* species (Vleeshouwers, 2000).

Vleeshouwers (2000) has cytologically analysed *Solanum* species inoculated with *P. infestans*, and found that defence responses were always associated with the hypersensitive response. Also, based on her results, she suggested that constitutive expression of *PR* genes may contribute to non-specific resistance to *P. infestans* in *Solanum* (Vleeshouwers, 2000).

Trognitz *et al*. (2001), have hybridised a late blight susceptible potato dihaploid with diploid *Solanum phureja* as a source of quantitative resistance. Several hybrids possessed high levels of resistance in the field in the Andes of South America. Reduced disease level in the field and not under any other conditions, as well as the increase of heritability after some development of an epidemic, would imply that the factor “field” caused the potato plants to develop an unknown response which, in turn, resulted in reduced disease. It may therefore not be justified to describe the reduced disease in the field as true resistance, it might be better described as the result of unknown factors occurring in the field that, as a side effect, conditioned the plants physiology to reduce the disease development (Trognitz *et al*., 2000).

At present, the most stable and durable type of resistance against *P. infestans* is the quantitative resistance due to its race non-specific and polygenic nature. However, this type of resistance is difficult to transfer into potato cultivars through crossing and phenotypic selection (Van der Vossen *et al.*, 2003).

In wild *Solanum* species, in potato cultivars carrying known resistance *R* genes and in non-hosts the major defence reaction appeared to be the hypersensitive response (HR). In fully resistant *Solanum* species and non-hosts, the HR was fast and occurred within 22h. Other responses associated with the defence reaction were deposition of callose and extracellular globules containing phenolic compounds. These globules were deposited near cells showing HR, and may function in cell wall strengthening (Vleeshouwers *et al*., 2000). Non-hosts *Solanum nigrum* and parsley display a typical localised HR at all infection sites (Kamoun *et al*, 1998). Some of the most effective barriers to disease are expressed at the plant cell wall and plasma membrane, preventing pathogen penetration and accounting for the majority of aborted infections in non-host (species-level) resistance (Wiermer *et al*., 2005).

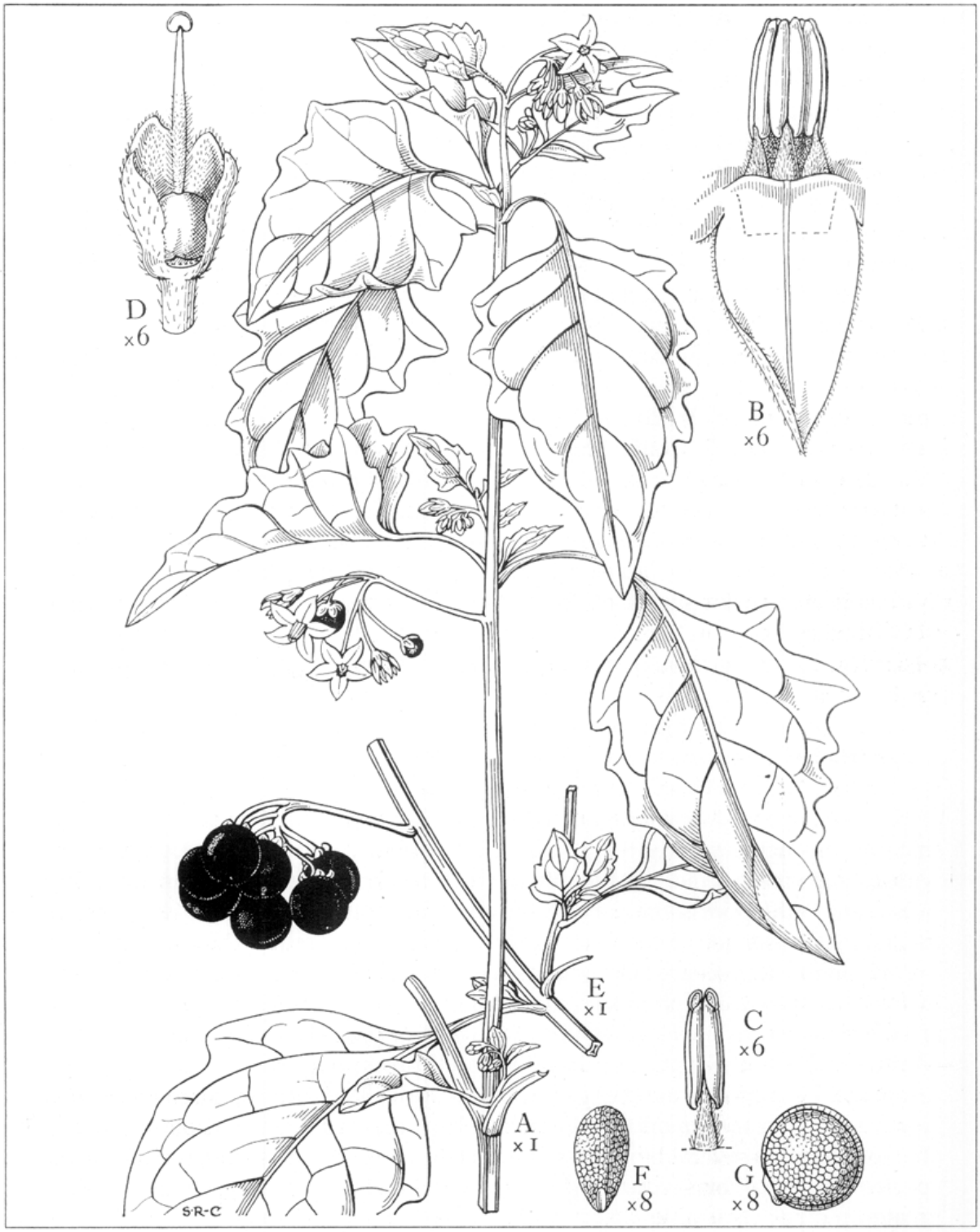
Unexceptionally, HR was found to be associated with all forms of resistance to *Phytophthora infestans*, including race- or cultivar-specific resistance, partial resistance, and non-host resistance, indicating that recognition through specific resistance genes occurs independently of the type of resistance (Kamoun *et al*., 1999; Vleeshouwers *et al.,* 2000; Huang *et al.,* 2004).

1.8 *Solanum nigrum* (Black nightshade)

The black nightshades are worldwide weeds of arable land, gardens, rubbish tips, soils rich in nitrogen and phosphorus, in moderately light and warm situations. In most parts of the world, particularly in Europe and North America, *S. nigrum* is considered to be troublesome weeds of agriculture, however, in many developing countries, the leaves and tender shoots of the species constitute a minor food crop. Moreover, it is not only used as vegetable but also for various medicinal and local uses (Edmonds and Chweya, 1997).

S. nigrum plants are also frequently associated with a broad spectrum of potentially destructive nematodes and microorganisms, serving as alternative hosts and potential disease vectors. *S.nigrum* is an especially valuable source of resistance to potato late blight caused by *P. infestans.* Colon et al. 1993 reported that although the black nightshade was present as very common weed in late blight field trials, no trace of infection was ever found on these plants despite heavy infection of potato plants in the same fields. This species is considered to be completely resistant to *P. infestans*, in view of absence of spreading lesions or sporulation in a number of experiments. Indeed, *S. nigrum* is apparently often considered to be a non-host to this pathogen.

S. nigrum plants are generally found in disturbed habitats, such as roadsides, often on arable land especially the edges of cultivated fields and plantations, in hedgerows, on railway cuttings, quaysides and rubbish tips, in areas around buildings and houses, under trees, on forest and grassland margins, as garden weeds, on shingle beaches, riverbanks and in gullies. Indeed the intensification of agriculture, particularly when associated with the extension of irrigation systems has been largely responsible for rapid spread of *S. nigrum* species (Edmonds and Chweya, 1997).



Source: Edmonds and Chweya (1997)

Figure 7: Morphology of *S. nigrum* L.

(A)- upper part of flowering stem, (B)- part of corolla and androecium with length of corolla tube shown by dotted line, (C)- stamen, (D)- calyx and gynoecium, (E)- part of fruiting stem, (F and G)- seed in two views.

The plants are subglabrous (subspecies *nigrum*) to villous (subspecies *schultesii*) annuals up to 70 cm high, covered with simple multicellular hairs with glandular or eglandular heads. Leaves (Figure 8) are ovate, ovate-lanceolate, ovate-rhombic to lanceolate, 2.5-7.0 cm long x 2.0 to 4.5 (6.0) cm broad, with entire to sinuate-dentate margins. The inflorescence (Figure 8) is simple, lax and often extended cymes, 5 to 10-flowered; peduncles 14 to 28 mm fruiting when usually erecto-patent; pedicels much shorter, curved in fruit. Berries (figure 6) are usually broadly ovoid, dull purple to blackish or yellowish-green, 6-10 mm broad, remaining on plants or falling from calyces when ripe. The seeds (Figure 8G) (1.7-2.4 mm long, 26 to 60 per burry) can be dispersed by water, birds and animals (Edmonds and Chweya, 1997).

The species belongs to the section *Solanum* in the *Solanaceae* family. It is hexaploid (2n = 6x = 72) and predominantly self-pollinating or autogamous though natural out- and cross-breeding does occur (Edmonds and Chweya, 1997).

Though its precise origin remains unknown, it is generally considered to be native to Eurasia; it is extremely well-adapted to the Mediterranean climate, and could have originated in the Middle East or even India. It is possibly also native to Africa where it is thought to be widely distributed (Edmonds and Chweya, 1997).

S. nigrum is mainly a non-host for *P. infestans* and non-host resistance is considered to be durable and stable even under field conditions. However, the nature of non-host resistance is poorly understood. This work aimed to analyse the characteristic of the resistance in *S. nigrum* against *P. infestans*, to uncover whether it is genetic dependent and which kind of gene for R-gene signal transduction is involved during this incompatible interaction. Virus Induced Gene Silencing (VIGS) technique was used to achieve this goal because mutation strategy and positional cloning would be very difficult and time consuming as *S. nigrum* is hexaploid. Based on the presence of field infections and results obtained in detached leaf inoculation studies by Flier et al. (2003), the present status of *S. nigrum* as a non-host plant for *P. infestans* needs to be reconsidered. Therefore, in the first part of this study, I have screened twenty *S. nigrum* accessions for *P. infestans* infection using four aggressive isolates. Then, I have tested whether the resistance in these *S. nigrum* accession is due to *P. infestans* elicitins. The third part consisted of the establishment of agroinfiltration and VIGS techniques, the construction of new pENTR11 vector, the cloning of genes for *R*-gene signal transduction from *S. nigrum*, the subcloning of these genes into pTRV-RNA2 gateway, the transformation into *Agrobacterium*, and the infiltration of the TRV vector carrying the gene of interests. The last part concerned about the infection of the infiltrated *S. nigrum* plants with *P. infesta*ns to check whether they had become susceptible.

2 Material and Methods

2.1 Plant materials

2.1.1 *Solanum nigrum* (Black nightshade)

In this study, I have examined 20 accessions of *S. nigrum* which are listed in Table 1. Plants were grown from seeds in individual pots under controlled conditions in climate chambers with a 16h/8h day/night regime at 20°C.

Table 1: List of *Solanum nigrum* accessions

Accessions	Origins	Number
Solanum nigrum atriplicifolium	Genbank Gatersleben 04/2004	SOL 50 / 77
Solanum nigrum atriplicifolium	Genbank Gatersleben 04/2004	SOL 169 / 01
Solanum nigrum nigrum f. humile	Genbank Gatersleben 04/2004	SOL 23 / 02
Solanum nigrum nigrum f. Humile	Genbank Gatersleben 04/2004	SOL 40 / 01
Solanum nigrum f. nigrum	Genbank Gatersleben 04/2004	SOL 33 / 78
Solanum nigrum f. nigrum	Genbank Gatersleben 04/2004	SOL 42 / 01
Solanum nigrum f. nigrum	Genbank Gatersleben 04/2004	SOL 55 / 77
Solanum nigrum	Wilbert Flier Renkum, The Netherlands (2000) 2004	Plant 2
Solanum nigrum	Wilbert Flier Renkum, The Netherlands (2000) 2004	Plant 3
Solanum nigrum	Wilbert Flier Renkum, The Netherlands (2000) 2004	Plant 4
Solanum nigrum	Wilbert Flier Renkum, The Netherlands (2000) 2004	Plant 5
Solanum nigrum schultesii f. luridum	Genbank Gatersleben 04/2004	SOL 35 / 79
Solanum nigrum schultesii schultesii	Genbank Gatersleben 04/2004	SOL 20 / 22
Solanum nigrum schultesii schultesii	Genbank Gatersleben 04/2004	SOL 25 / 78
Solanum nigrum schultesii schultesii	Genbank Gatersleben 04/2004	SOL 38 / 01
Solanum nigrum schultesii schultesii	Genbank Gatersleben 04/2004	SOL 44 / 77
Solanum nigrum nodiflorum	Madagascar	
Solanum nigrum	Prof. Glauninger , Austria 2003	2003
Solanum nigrum	Greenhouse Boku 2004	2004
Solanum nigrum nigrum	Hall, Gelderland (1990)	SN 18

Figure 8: *S. nigrum* (A)- plant, (B)- inflorescence, (C)- berries

2.1.2 *Solanum tuberosum* (Potato) culture

Potato var. desirée, which is susceptible to *P. infestans*, was maintained *in vitro* on modified MS medium supplemented with 20% sucrose (Murashige and Skoog, 1962) at 25°C. After allowing 1-2 weeks for root formation, plants were transferred into sterilized soil and grown in a growth chamber in a 16/8hours day/night light regime.

2.2 *Phytophthora infestans* culture

Four different isolates of *P. infestans* (listed in Table 2) were plated routinely on rye agar medium supplemented with 2% sucrose (Caten and Jinks, 1968) at 18°C in the dark with high humidity.

Table 2: list of Phytophthora infestans isolates

Isolates	Host of origin	Year of collection	Location of origin	Virulence to R-genes	Mating type	mtDNA haplotype	Provided by
APiO2	Hermes (Potato)	2000	Lower Austria	1.3.4.6.7.8.10	A1	1a	Bodo Trognitz (ARC)
LL8	Walisa (Potato)	2002	Upper Austria	1.3.4.7.8.10.11	A1	1a	Bodo Trognitz (ARC)
T04	Laura (potato)	2002	Tyrol Austria	1.2.3.4.6.7.10.11	A1	1a	Bodo Trognitz (ARC)
Sn001a	Solanum nigrum	1999	Wagenigen Netherlands	1.2.3.4.5.7.8.10.11	A1	1a	Henry van Raaij (Plant Research International

2.2.1 Preparation of rye agar medium

60g rye grains were soaked in distilled water for 24 hours at room temperature. The following day, the rye grain was boiled in a litre beaker covered with aluminium foil for 1 hour, with enough additional distilled water to cover the grain. The supernatant was filtered through four layers of cheesecloth and squeezed gently to remove residual liquid. The cheesecloth and sediment were discarded. The supernatant was mixed with 15 g agar and 20g sucrose, adjusted to 1 litre and autoclaved at 120°C for 20 minutes.

Figure 9: *P. infestans* growing on rye agar plate

2.2.2 Release of zoospores

7 to 14 days after inoculation of *P. infestans* on rye agar, sporangia were chilled with 20 ml of cold tap water and incubated in the fridge at 4°C for 2 hours to induce zoospore formation. After filtering through cheesecloth to remove mycelium, the zoospore concentration was determined microscopically using a hemacytometer and adjusted to $8x10^4$ spores/ml (Vleeshouwers et al., 2006).

To determine the spore concentration with haemacytometer, two sets of five grids (four corner and one center) were counted (10 grids x 1000 = spores/ ml).

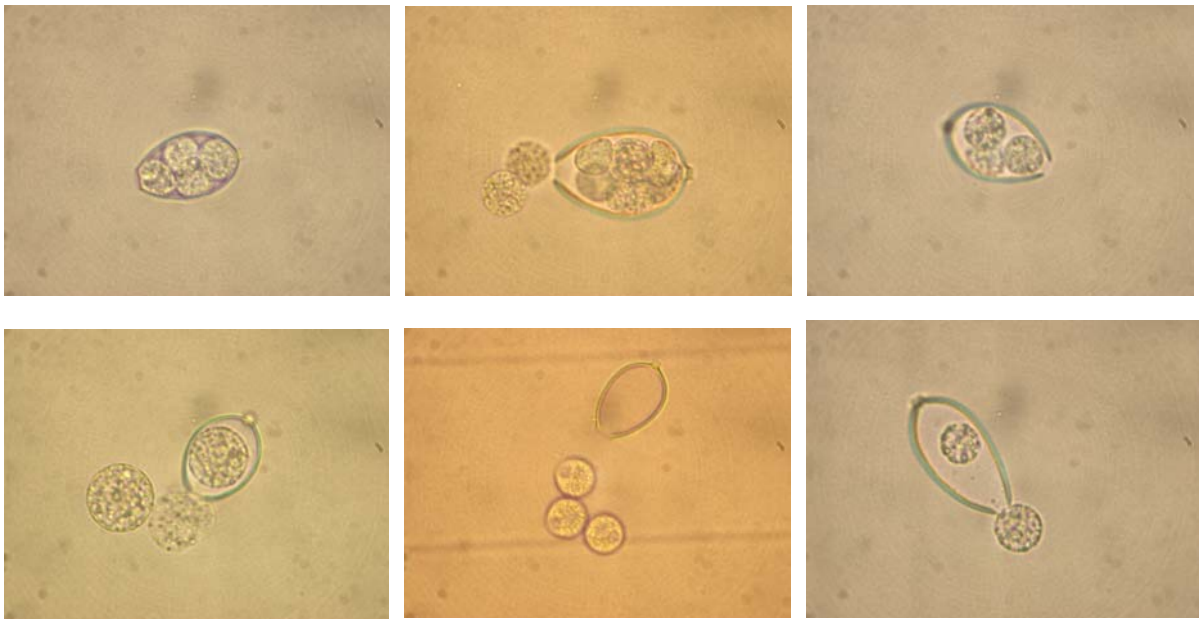


Figure 10: Sporangia releasing zoospores

2.3 Disease assay

2.3.1 Infection

Infection was achieved by spraying of zoospore suspension of 8 x 10^4 spores / ml on the lower side of leaves using a hand-held pump sprayer. For the first 18 hours, plants were kept at high humidity in the dark and subsequently in the light with a 16 h photoperiod at 18°C until evaluation. Relative humidity was maintained at 100% throughout the day and night.

Two days after inoculation, *S. nigrum* leaves were stained with trypan blue to visualize hyphae and hypersensitive reactions.

2.3.2 Staining

For cytological examination, the infected and uninfected leaf segments, about 4 cm in size, were fixed and stained in lacto phenol-trypan blue (10g phenol, 10ml glycerol, 10ml lactic acid, 10ml distilled water, and 20mg trypan blue). Leaf materials were heated at 95°C for 2 minutes in the staining solution (1 part lacto phenol-trypan blue and 2 parts 95% ethanol). After 48 hours of incubation in staining solution, the samples were cleared in chloral hydrate (50g chloral hydrate dissolved in 20ml distilled water) for 24 hours. Then they were mounted on cover slips in 50% glycerol, sealed, and microscopically examined. Leaf pieces were examined without sectioning. Trypan blue is the most common stain used to distinguish viable cells from nonviable cells; only nonviable cells absorb the dye and appear blue and may also appear asymmetrical. Conversely, live, healthy cells appear round and refractive without absorbing the blue-coloured dye (Keogh et al., 1980). The pathogen and affected host cells were located with bright field microscopy. Plant cells that were either dark blue or brown under bright field conditions were subsequently examined under UV light; cells exhibiting the HR fluoresced when exposed to UV light (Becktell et al., 2006).

Aniline blue is a specific stain used to visualize callose (Smith and McCully, 1978). Aniline blue stains callose and callose-like substances, such as is contained in pollen tubes, fungal hyphae, and corky zones of cell walls, thickened cell walls and cuticle as a response to stress. For 0.6 litter of Schreiter's solution; 1-2% of aniline blue were dissolved in 50 ml of water, 350 ml of a solution containing K3PO4*3H2O (7.08 g) (tri-potassium orthophosphate), 100 ml of a solution containing NaOH (4 g) and 100 ml of a detergent solution (diluted 1:10 of dish-washing detergent) were added. Infected and uninfected leaf segments were stained as follows: 1 sample / 10 Schreiter's solution (v/v) was heated on a boiling water bath for 10 to 30 min, depending on the strength of the leaves. 50% glycerol-water was added to the softened and cleared samples which were placed on a microscope slide, then covered with the cover slip and pressed gently to spread out the tissue to a thin layer. The observation was done under microscope using UV illumination and a fluorescence-exciting filter. Callose

stains bright yellow-greenish. Samples prepared in this way and covered with glycerol can be stored for 2-3 years. Samples were kept in the dark because aniline blue fades under light.

2.4 Isolation of genomic DNA

Genomic DNA was isolated from *S. nigrum* leaves according to a CTAB (Cetyltrimethylammoniumbromide) based protocol (Sambrook and Russel, 2001). Leaves were collected and frozen. Two grams from the harvested leaves were ground in liquid nitrogen using a mortar and pestle and incubated with 12.5 ml of 95°C preheated extraction buffer 2x CTAB containing 2% CTAB, 100 mM Tris-pH8, 20 mM EDTA and 1.4 M NaCl at 60°C for 10 min. 12.5 ml of a chloroform/isoamyl alcohol (24:1, v/v) solution was added, mixed and shaken for 15 min. Subsequently the sample was centrifuged for 10 min at 4300 rpm. The aqueous supernatant was transferred to a new tube. 0.1 volumes of 10% CTAB and 1 volume of chloroform: isoamyl alcohol (24:1) was added, mixed and shaken for 15 min and centrifuged for 10 min at 4300 rpm. The aqueous supernatant was transferred to a new tube once again. The DNA was precipitated by adding 2/3 volume of cold isopropanol. The tube was immediately inverted several times and then the DNA was collected by centrifuging at 4300 rpm for 20 min. The DNA pellet so obtained was washed first in 1 ml of 76% (v/v) ethanol with 0.2 M sodium acetate, 1µl of DNase free RNase (10 mg/ml) was then added to eliminate RNA. 2.5 ml original volume of pre-chilled 76% (v/v) ethanol and 3 mM ammonium acetate were then added to precipitate the DNA which was finally washed with 500µl of 70% ethanol. The DNA was dissolved in 100 µl 1x TE buffer.

2.5 RNA extraction

Total RNA was extracted from 100mg of leaves using Qiagen RNeasy Plant minprep kits (Qiagen, Hilden, Germany; http://www.qiagen.com) according to the producer's instructions. Leaves were ground with a mortar and pestle in the presence of liquid nitrogen. The powder was transferred into a liquid-nitrogen-cooled microcentrifuge tube and mixed vigorously with 450ml of buffer RTL. The lysate was transferred to a QIAshredder spin column, placed in a 2ml collection tube and centrifuged for 2 mn at full speed. To the supernatant was added 0.5 volume of 96% ethanol, mixed immediately by pipetting and transferred to an RNeasy spin column to bind RNA. To avoid genomic DNA contamination, RNase-free DNase was applied before the washing steps. 350 µl of RW1 buffer was added to the RNeasy spin column and centrifuged for 15 second at 10,000 rpm. Flow-through was discarded and 10 µl of DNase I mixed with 70 µl buffer RDD were added to the RNeasy spin column membrane and incubate at 25°C for 15 minutes. Then, another 350 µl buffer RW1 were added to the column and centrifuged again for 15 seconds at 10,000 rpm. The washing steps continued afterwards with 500 µl of buffer RPE for 15 second at 10,000 rpm and again with another 500

µl of buffer RPE for 2 minutes at 10,000 rpm. To elute RNA, RNase-free water was directly added to the spin column membrane and centrifuged for 1 mn at 10,000 rpm. The integrity and the quantity of RNA samples were analysed on an Agilent 2100 bioanalyzer.

2.6 Amplification of candidate genes

2.6.1 Polymerase chain reaction (PCR)

PCR was conducted in a reaction volume of 50µl. Each reaction consisted of 2µl of template DNA, 5µl of PCR buffer, 37.8µl of sterile distilled water, 1µl dNTPs (10mM), 0.2µl BioTherm Taq polymerase (5U/µl), 2µl forward primer (10µM) and 2µl reverse primer (10µM). These products were purchased from VBC Biotech.

The primers are listed in Table 3 as well as their product lengths and the annealing temperatures.

All reactions were carried in a PCR instrument (Multicycler PTC-200 and Peltier Thermal Cycler PTC-200 from MJ research). General PCR thermal cycling parameters were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of denaturation for 30 s at 94°C, annealing at 45 – 60°C (depending on each primer) for 30 s, and extension at 72°C for 1 min.

Amplified cation products were electrophoresed on a 0.8% agarose/1xTAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) gel containing 0.1 ng/ml ethidium bromide.

2.6.2 cDNA preparation

Total RNA was reverse transcribed into cDNA. First-strand cDNA was synthesized using 1µg of total RNA, 1µl oligo-d(T)primer (10µM), in a 14µl volume. The mixture was heated at 65°C for 5 minutes and incubated on ice for at least 1 minute. And then 4µl of 5x first-strand buffer, 1 µl of DTT (0.1M), 0.5 µl of RNase inhibitor (10U), and 0.5 µl of SuperScript III Reverse Transcriptase (200U/µl), from Invitrogen, were added to the contents of the microcentrifuge tube. The reaction was incubated at 25°C for 5 min. The incubation was ended at 50°C for 1h and the reaction was inactivated by heating 70°C for 15 min. Second strand cDNA was synthesized using gene specific primers (listed in Table 3) and performing PCR on 2µl of products of first strand cDNA in a 50µl volume with a PCR program of 94°C for 3 min, 94°C for 30s, 45 – 60°C (depending on each primer) for 30 s, and 72°C for 1 min for 35 cycles. The resulting PCR products were analyzed by agarose gel electrophoresis.

Amplified cation products were purified by the protocol described in the QIAquick PCR Purification Kit Protocol, and each then cloned in pENTR11.

Table 3: list of primers used to amplify candidate genes from *S. nigrum*

	5'-primer (5'-3')	3'-primer (5'-3')	An. temp. (°C)	Prod. length (bp)
CTR1	GCGAGAGAGGTGTTGGATGAAAGGC	GCCCAGCAAGCCTCAATAATAATCG	45	127
EDS1	CTTCGAGGGACGAAAGGA	GTCTGCAAGTTGGCTTGGAT	45	480
NDR1	CTCGACGAATTCATGGCCCTTCCATTC CGCCACCAG	CGCGCGCTCGAGCCCCTAAATTCTT CTCATTATTG	45	496
NPR1	GACCACGGCATCAAAACTCACC	GACTTCTTCGCTGATGCTAAGC	45	1014
RAR1	AGGAAAGCACACAACAGAAAAACC	GTGCCATCCTTTGGTGCATGGAGG	50	468
TGA1a	CGGGAATTCGGGTATATGCGATCCGATCCA TCA	CGGCTCGAGTTCCGCTTCACCAATTTGA GAATG	50	527
TGA2.1	AAATGCTAAGGTCCCACTGGCG	TAGTTCATTCGGTGAGTTGC	45	526
TGA2.2	CGGGAATTCGACATATTCAGGATAAAA GGGGA	CGGGGTACCTATTCCCGGGGGCGA GCAAGCCA	45	521
SGT1.1	GCCGTTGACCTCTACACTC	CCACCTCCTCTGGTTTCTG	50	2864
EDS1	AAGGTATGAGCTCCCAGATA	GGGGCATGGCAATATATGTG	45	441
JAR4	GCGGCGGTCGACGTAATATTTGGCCC TGATTTCC	GCGGCGGGATCCATTGCTTAACCGG CTG		292

(*An. Temp.*: annealing temperature; *Prod. length*: PCR product length)

2.6.3 DNA purification

Five times volume of buffer PB was added to the DNA template, mixed to the QIAquick column and centrifuged for 30 second with maximum speed to bind DNA. The pellet was washed with 0.75 ml of buffer PE by centrifuging one minute at 10.000 rpm. The centrifugation was repeated for a second time after discarding the flowthrough. For elution, 50 µl of TE buffer was added to the pellet and DNA was collected into a fresh 1.5 ml Eppendorf tube.

2.7 Cloning of PCR fragments into pENTR11 gateway vector

2.7.1 Construction of pENTR11Amp vector

pENTR11 (2744 bp) and pUC18 (2686 bp) were digested one by one with PstI and AatII for two hours. Each one of these restriction enzymes cuts only once in pENTR11 as well as in pUC18. The size of the products was verified by gel electrophoresis.

The fragment carrying the ampicillin resistance of pUC18 was ligated with the fragment containing the *att*L1-*ccd*B-*att*L2 cassette of pEntry11. The reaction was incubated for two hours at room temperature. The resulted new vector was transformed into *E. coli* DB 3.1 and selected on LB (Luria-Bertani medium, Sambrook and Russel, 2001) supplemented with ampicillin (100µg/ml).

The DB3.1 *E.coli* strain was used to propagate and maintain the pENTR11 vector as it is the only one strain resistant to *ccd*B effects and can support the propagation of plasmids containing the *ccd*B gene.

2.7.2 Construction of entry clones

To integrate the candidate genes, the entry vector was first digested with BsrGI (87 µl of plasmid DNA was mixed with 3 µl of BsrGI (10units/µl) and 10 µl of buffer and incubated at 37°C for 3 hours) which cut on each side from the outer part of the *ccd*B gene in order to replace it with the gene of interest. This will minimize the competition between the *ccdB* fragment and the gene of interest during the ligation process. The resulting fragments were checked by gel electrophoresis. They were purified and made blunt-end by filling in with Klenow (50µl of digested entry vector was mixed with 2µl Klenow (2U/µl), 2µl dNTPs (10mM) and 6µl orange buffer and incubated at 37°C for 30mn, then 20mn at 70°C to stop enzyme activity).

PCR products were also purified before cloning them into pENTR11Amp to remove the DNA polymerase (Taq DNA polymerase can fill in sticky ends and add bases of PCR products) and small DNA fragments such as primers, primer dimers, and excess dNTPs. PCR products generally do not have 5' phosphates and are not necessary blunt.

After purification, 1µl of the PCR product was mixed with 2µl of the digested entry vector, 1µl T4 DNA ligase (200U), 1µl ligation buffer, and 5µl ddH2O and left at room temperature for 2 hours. A mixture without insert and another mixture without insert and DNA ligase were used as controls.

In brief, this procedure is resumed in Figure 11.

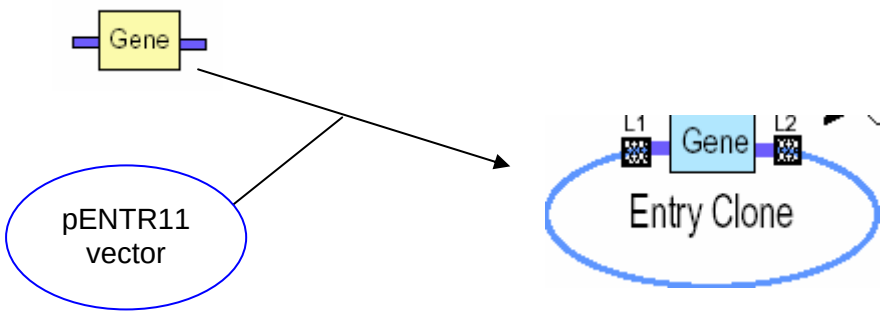


Figure 11: Cloning of PCR product into an entry vector

The resulted entry clone was transformed into *Escherichia coli* DBH10 to be multiplied. All transformations were conducted by electroporation (see subsection number 2.9.2). Selection was done on LB plates containing Ampicillin (100µg/ml). Colonies from each kind

of entry clones were picked and cultured overnight in LB liquid medium containing 100µg/ml ampicillin. Then, to verify the positive transformants, plasmid DNA were isolated using E.Z.N.A Plasmid Miniprep Kit and analyzed by PCR using the corresponding primers of each candidate gene listed in table 3. PCR was performed as described previously. The pENTR11Amp clones were analyzed by sequencing.

The primers used for sequencing the pENTR11Amp vector were:

- Forward primer (pENTR11-139F): 5'-GGCCTTTTTGCGTTTCTACA-3'
- Reverse primer (pENTR rev): 5'-GCAGCTGGATGGCAAATAAT-3'

2.8 Subcloning genes from entry clones into pTRV2 gateway

LR recombination reaction was performed to transfer the inserts from pENTR11Amp clones into pTRV-RNA2-Gateway.

pENTR11Amp clones carrying the candidate gene between attL1 and attL2 were mixed with the destination vector pTRV-RNA2-Gateway containing a cassette of the ccdB and kanamycin resistance (Kan^R) genes flanked by attR1 and attR2 and recombined in LR reaction. For each sample, 50 to 150 ng of Entry clone DNA were mixed with 150 ng/µl of the destination vector DNA (pTRV-RNA2-Gateway), 2µl of LR Clonase™ II enzyme mix, and filled up to 10µl with TE buffer (pH 8.0). The mixture was then left for 1 hour at room temperature. The enzymes were inactivated with 1µl of the Proteinase K solution (2 µg/ µl) for 10 min at 37°C. Gateway® LR Clonase™ II Enzyme Mix and Proteinase K solution were purchased from Invitrogen.

The reaction was used to transform DH10B chemically competent cells which were then spread on LB agar plates containing 50µg/ml kanamycin to select for an expression clone. Only recombinants can grow in DH10B because the ccdB gene, contained in the plasmid, is lethal.

2.9 Transformation

2.9.1 Transformation using chemically competent cells

DNA was mixed with 100 µl of chemically competent cells and incubated for 5 min on ice. Then, a heat shock was performed in a waterbath for 1 min at 42°C. Immediately after the heat shock, the cells were reincubated on ice for 5 min. After that, 400 µl of LB liquid medium without antibiotic were added and the culture was incubated at 37°C for 30 min. To select the transformed bacteria, they were plated on LB agar medium supplemented with the appropriate antibiotic(s) overnight at 37°C.

The chemically competent cells that I used were produced as follows: one colony of *Escherichia coli* was grown overnight in 5 ml LB liquid medium at 37°C. This preculture was

used to inoculate 500 ml LB liquid medium the following day and grown until the culture reached an optical density (OD_{600}) of 0.6. Bacteria were collected after centrifugation at 3000 rpm for 10 min at 4°C, resuspended in 20% volume (the original volume) of buffer TBF I (30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2$ $2H_2O$, 50 mM $MnCl_2$. $4H_2O$, and 15% glycerol, pH 5.8 with HAc) and centrifuged once again for 5 min. 4% volume of TBF II (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, and 15% glycerol, pH 6.5 with KOH) was added to the pellet and aliquots of competent cells were stored at minus 80°C. The cells were kept on ice all the time.

2.9.2 Transformation using electrocompetent cells

1µl (1-10µg/ml) of DNA and 20µl of TransforMax™ EC100 electrocompetent cells from Epicentre Biotechnologies, were mixed and put into a pre-chilled electroporation cuvette, then placed in the electroporator. The voltage of the electroporation device was set at 1800. Immediately after electroporation, 800µl of fresh SOC medium (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose, 10 mM MgCl2) was added to the cuvette and mixed gently by pipetting up and down 3 times. To recover the transformed cells, they were incubated at 37°C in a thermo shaker (300 rpm) for 1 hour before plating them on a LB agar plate containing the appropriate antibiotic. The plate containing the transformed bacteria was inverted and incubated at 37°C overnight.

2.9.3 Transformation of *Agrobacterium*

1µg plasmid DNA was added to 100µl of competent cells and frozen in liquid nitrogen. The mixture was incubated at 37°C for 5 min using water bath and then resuspended in 1 ml of YEB medium without any antibiotic. The culture was shaken for 90 min at 28°C, centrifuged for 30 s at 10,000 rpm, resuspended in 100 µl YEB and plated on YEB agar containing the appropriate antibiotics which is depending on the *Agrobacterium* strain, the helper plasmid,, and the binary vector that has been transformed.

The competent cells were prepared as follows: a single colony of *Agrobacterium* was grown in 5 ml YEB liquid medium containing antibiotics at 28°C overnight. Then, 50 ml of YEB with antibiotics was inoculated with 2 ml from this preculture and grown to an optical density (OD_{600}) of 0.6. The pellet was collected after centrifugation at 4300 rpm for 5 min at 4°C and resuspended in 1 ml of 25 mM $CaCl_2$. Aliquots of 100 µl were added to precooled 1.5 ml Eppendorf tubes and stored at minus 80°C.

2.10 Sequencing

Plasmids were isolated from *E.coli* by "Invisorb Spin Plasmid Mini Kit" and sent for sequencing (Some of them were sent to Austrian Research Center, some to International Atomic Energy Agency laboratory and some to the University of Chicago Cancer Research Center, DNA sequencing facility). Computer-aided sequence similarity searches were made with the BLAST suite of programs at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and sequences were aligned with published sequences from other *Solanaceae* species such as *Lycopersicum esculentum* (tomato), *Nicotiana tabacum* (tobacco), *S. tuberosum* (potato), and *Nicotiana bentamiana* downloaded from Genbank using Clustal W (http://www.ebi.ac.uk/clustalw).

2.11 Agroinfiltration

2.11.1 Leaf infiltration

Agrobacterium tumefaciens strains were plated on YEB agar and incubated overnight at 28°C. A single colony was cultured in 3 ml liquid YEB medium overnight at 28°C. Then 1 ml of this preculture was used to inoculate 100ml liquid YEB medium. The bacteria were collected after centrifugation at 4000 rpm for 5-10 minutes at room temperature. The harvested cells were resuspended in infiltration medium (10 mM MES, 10 mM MgCl2, 100µM acetocyringone) at an OD_{600} series of 0.03, 0.1, and 1 and incubated at room temperature for 2 hours before infiltration.

S. nigrum plants at the four true leaf stage were inoculated. Infiltration was done using a 1ml needleless syringe, as shown in Figure 12, by pressing the syringe on the underside of the leaf (to pass liquid through the stomata into the intercellular spaces) and exerting a counter-pressure with a finger on the other side.

Three leaves from each plant were infiltrated and three inoculation sites par leaflet were performed. Infiltrated plants were incubated at 16°C.

Source: www.nature.com

Figure 12: leaf infiltration

2.11.2 Vacuum infiltration:

All procedures for vacuum infiltration were the same as leaf infiltration; the only difference was that instead of using a syringe, the whole plant was infiltrated in infiltration medium using a desiccator which was evaporated with a vacuum pump.

All vacuum infiltration experiments were carried out using 2 week-old-plants grown from seeds.

2.11.3 Agrodrench

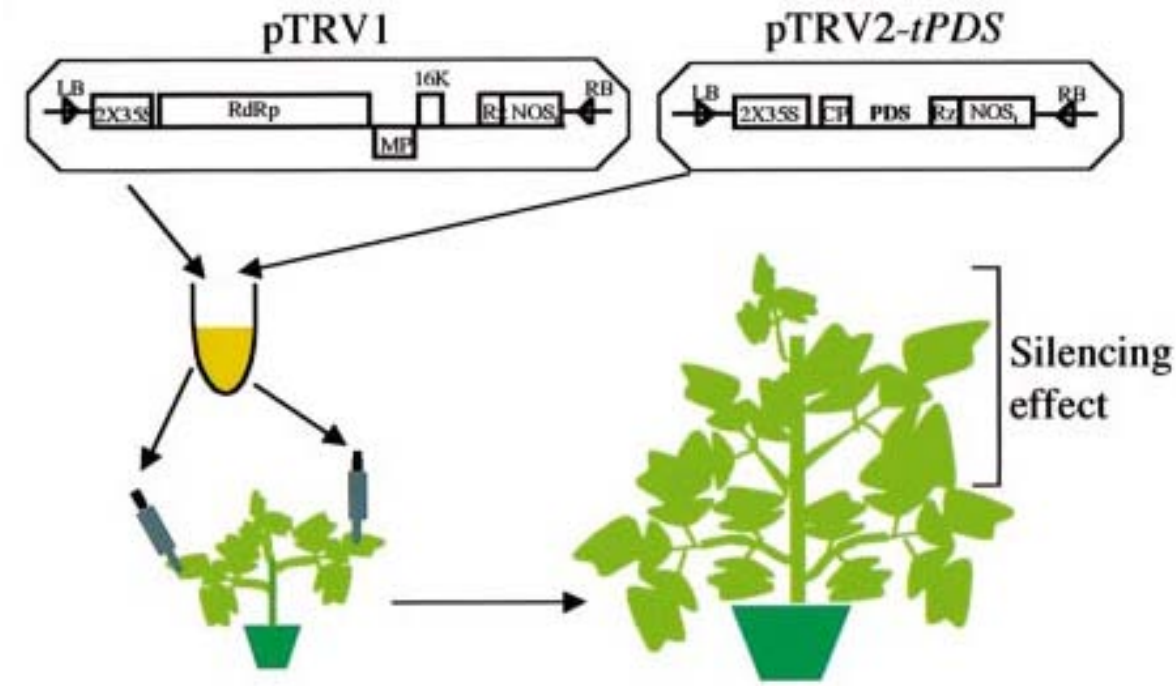
Agrodrench is a method of agroinoculation by drenching the plant rhizosphere with *A. tumefaciens* (Ryu et al., 2004). Instead of infiltration, the culture is applied directly onto the soil adjacent to the crown part of 2-3 week old *S. nigrum* plants.

2.12 Virus Induced Gene Silencing (VIGS)

I used TRV (bipartite RNA virus; pTRV-RNA1 and pTRV-RNA2) as a VIGS vector.

Agrobacterium strains containing pTRV1, pTRV2-empty, pTRV2-*PDS* and pTRV2 carrying the target sequences were propagated separately in different cultures; then collected by centrifugation and resuspended in infiltration medium (10 mM MES, 10 mM MgCl2, 100µM acetocyringone) at an $O.D_{600}$ of 0.6. An aliquot from each kind of pTRV2 was mixed one by one in a 1:1 ratio with pTRV1 and infiltrated into plants to initiate infection and VIGS. The infiltrated plants were placed at 16°C as the efficiency of VIGS is greater at lower temperature (between 16-20°C) (Ekengren et al., 2003).

A population of 15 plants was inoculated for each test. Uninoculated *S. nigrum* plants were used as a control.



Source: Liu et al. (2002)

Figure 13: Virus induced Gene Silencing system

2.13 Ploidy measurement of *Solanum nigrum*

The ploidy levels of *S. nigrum* accessions used in this study were measured using Flow cytometry (Partec PAS III particle analysing system). The reagent used was CyStain UV precise P. Leaves were frozen for storage and then chopped with a razor blade in extraction buffer and 2 volumes of DAPI staining solution were added. Each sample was immediately measured. Leaves from potato were used as standard and calibrator.

2.14 Chemicals and enzymes

Table 4: List of chemicals used in alphabetical order and their producers

Product	Supplier	Country
Acetic acid	Riedel-de Haen, Sigma Aldrich	Seelze, Germany
Acetosyringone	Fluka	Seelze, Germany
Agar	Fluka, Sigma Aldrich	Seelze, Germany
Agarose LE	Biozym	Oldendorf, Germany
Ammonium acetate	Fluka, Sigma Aldrich	Seelze, Germany
Asparagine	Fluka	Seelze, Germany
CaCl2 (Calcium chloride dehydrate)	Fluka, Sigma Aldrich	Seelze, Germany
Carbenicillin disodium	Duchefa	Haarlem, The Netherlands
Chloral hydrate	Riedel-de Haen	Seelze, Germany
Chloramphenicol	Fluka, Sigma Aldrich	Seelze, Germany
Chloroform	VWR (Norma pure)	Wien, Austria
CTAB (Cetyltrimethylammoniumbromide)	Fluka, Sigma Aldrich	Seelze, Germany
Daishin Agar	Duchefa	Haarlem, The Netherlands
DEPC (Diethylpyrocarbonat)	Roth	Karlsruhe, Germany
DNeasy tissue Kit	Qiagen	Helden, Germany
dNTPs	Fermentas	Harrington Court, Canada
D (+)-Saccharose	Prolabo	Fontenay sousbois, France
EDTA (Ethylendiamintetraacetat)	Fluka, Sigma Aldrich	Seelze, Germany
Ethanol	Merck	Darmstadt, Germany
Ethidium bromide	Sigma Aldrich	Seelze, Germany
Gentamycin sulphate	Duchefa	Haarlem, The Netherlands
Glucose	Fluka	Seelze, Germany
Glycerine (99.5%)	Roth	Karlsruhe, Germany
HAc	Apoka	
Invisorb Plasmid Mini Kit	Invitek	Berlin, Germany
Isoamylalkohol (3-Methyl-1-butanol)	Fluka, Sigma Aldrich	Seelze, Germany
Isopropanol	Fluka, Sigma Aldrich	Seelze, Germany
KAc	Fluka	Seelze, Germany
Kanamycin sulphate monohydrate	Sigma Aldrich	Seelze, Germany
KCl (Potassium chloride)	Sigma Aldrich	Seelze, Germany
KH2PO4	Merk	Darmstadt, Germany
KOH (potassium hydroxide)	Fluka, Sigma Aldrich	Seelze, Germany
Lysozym	Fluka	Seelze, Germany
Beta-Mercaptoethanol	Roth	Karlsruhe, Germany
MES monohydrate	Duchefa	Haarlem, The Netherlands
$MgCl_2$ 25mM (Magnesium chloride)	Promega	Mannheim, Germany
MgCl2 x7H2O	Fluka, Sigma Aldrich	Seelze, Germany
MgSO4 x7H2O	Fluka	Seelze, Germany
MnCl2 (Manganese chloride tetrahydrate)	Fluka, Sigma Aldrich	Seelze, Germany
Murashige & Skoog modification No. 1B (MSm medium)	Duchefa	Haarlem, The Netherlands
NaAc	Fluka	Seelze, Germany
NaCl	Roth	Karlsruhe, Germany

Product	Supplier	Country
NaOH (sodium hydroxide)	Fluka, Sigma Aldrich	Seelze, Germany
Peptone from vegetables	Fluka	Steinheim, Germany
Potassium acetate	Fluka, Sigma Aldrich	Seelze, Germany
QIAquick Gel Extraction Kit	Qiagen	Helden, Germany
QIAquick PCR Purification Kit	Qiagen	Helden, Germany
RbCl (Rubidium chloride)	Fluka, Sigma Aldrich	Seelze, Germany
Rifampicin	Duchefa	Haarlem, The Netherlands
RNeasy Plant Mini Kit	Qiagen	Helden, Germany
SDS	Fluka	Seelze, Germany
Sodium acetate	Duchefa	Haarlem, The Netherlands
Sodium hypochlorite solution	Fluka, Sigma Aldrich	Seelze, Germany
Spectinomycin sulphate	Duchefa	Haarlem, The Netherlands
Streptomycin sulphate	Sigma Aldrich	Seelze, Germany
SYBR Green I	Roche Diagnostics	Indianapolis, USA
Thiamine	Fluka	Seelze, Germany
Trypan blue	Fluka, Sigma Aldrich	Seelze, Germany
Tryptone	Duchefa	Haarlem, The Netherlands
Tris (Tris(hydroxymethyl)-aminomethan)	Duchefa	Haarlem, The Netherlands
Triton X-100	Fluka, Sigma Aldrich	Seelze, Germany
Yeast extract	Duchefa	Haarlem, The Netherlands

Table 5: List of restriction endonucleases

Enzyme	Recognition site	Supplier	Country
AatII	5'-GACGT↓C-3'	Fermentas	Harrington Court, Canada
Bsp1407 I (BsrGI)	5´-T↓GTACA-3´	Fermentas	Harrington Court, Canada
PstI	5'-CTGCA↓G-3'	Fermentas	Harrington Court, Canada
BamHI	5'-G↓GATCC-3'	Fermentas	Harrington Court, Canada

Table 6: List of additional enzymes

Enzyme	Supplier	Country
Biotherm *Taq* DNA polymerase	GeneCraft	Lüdinghausen, Germany
DNAse I	Fluka, Sigma Aldrich	Seelze, Germany
LR Clonase Enzyme Mix	Invitrogen	Carlsbad, USA
RNAse A	Qiagen	Helden, Germany
RNase Inhibitor	Promega	Mannheim, Germany
SuperScript III Reverse Transcriptase	Invitrogen	Carlsbad, USA
***Taq* DNA polymerase**	Promega	Mannheim, Germany
T4 DNA ligase	Fermentas	Harrington Court, Canada

2.15 Abbreviations

Amp	Ampicillin
Avr	Avirulence gene
bp	base pairs
CC-NB-LRR	Coiled-Coil Nucleotide Binding Leucin Rich Repeat
cDNA	complementary DNA
CTAB	Cetyltrimethylammoniumbromid
df	degree of freedom
PCD	programmed cell death
PCR	Polymerase Chain Reaction
DEPC	Diethylpyrocarbonat
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide 5`-triphosphate
dsRNA	double-stranded RNA
dpi	days post infection
EDS1	Enhanced Disease Susceptibility 1
EDTA	Ethylendiamintetraacetate
ESTs	Expressed sequence tag
ET	Ethylene
GUS	beta-glucuronidase
h	hour(s)
hai	hours after inoculation
HR	Hypersensitive response
JA	Jasmonic acid
Kan	Kanamycin
kb	Kilobase pairs
LB	Luria-Bertani medium, Sambrook and Russel
Le	*Lycopersicon esculentum*
MES	2-Morpholinoethanesulfonic acid
µg	Microgramm
µl	Microlitre
µm	Micrometer
min	Minute(s)
M	Molar
mM	Millimolar
ml	Milliliter
mRNA	Messenger ribonucleic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MSm	Murashige-Skoog-Medium modified
NB-LRR	Nucleotide Binding Leucin Rich Repeat
NDR1	Non-race-specific Disease Resistance
$O.D_{600}$	optical density at 600nm
PAD3	Phytoalexin Deficient 3
PAD4	Phytoalexin Deficient 4
PCR	Polymerase chain reaction

PDS	Phytoene Desaturase
PR	Pathogen related gene
Pto	Tomato *R*-gene resistance specifically to *Pseudomonas syringae*
PVX	Potato Virus X
RAR1	homolog of a barley gene required for *Mla* powdery mildew resistance
RNA	Ribonucleic acid
ROI	Reactive Oxygen Intermediates
Rpm	Rounds per minute
RT	Reverse transcription
s	second(s)
SA	Salisilic acid
SAG101	Senescence Associated gene 101
SAR	Systemic aquired resistance
siRNA	small interfering RNA
SDS	Sodium Dodecyl Sulphate
Sn	*Solanum nigrum*
SOC	Salt-optimized and carbon
T-DNA	Transferred DNA
TE buffer	Tris-EDTA buffer
TIR	Drosophila Toll and mammalian Interleukin-1 Receptor
TMV	Tomato Mosaic Virus
TRV	Tobacco Rattle Virus
V	Volts
Var.	variety
v/v	volume per volume
VIGS	Virus Induced Gene Silencing
wpi	weeks post infection
YEB	Yeast extract broth

3 Results

3.1 Screening of *Solanum nigrum* accessions for *Phytophthora infestans* tests

Durable resistance of a sufficiently high level against potato late blight is not yet available in agronomically acceptable cultivars of the cultivated potato (Colon *et al.,* 1993). *S. nigrum* is growing as a weed in potato fields. Colon and associates (1993) reported that *S. nigrum* may be regarded as a valuable source of resistance to potato late blight. In addition, Vleeshouwers and associates (2000) have seen that a HR was induced extremely fast (22 hai) in *S. nigrum*-SN18 after inoculation with *P. infestans*. They have found that *S. nigrum* was completely resistant. In most cases the HR response remained limited to one to three cells and *P. infestans* was not detected at 46 hai.

S. nigrum was regarded as a non-host for *P. infestans* (Vleeshouwers *et al.,* 2000). However, in Wales, blighted *S. nigrum* plants were found, and compatible *P. infestans* isolates have been collected (Flier *et al*., 2003). A nationwide late blight survey aimed at characterizing early outbreaks of late blight in the Netherland from 1999 to present has repeatedly confirmed the presence of sporulating lesions of *P. infestans* on *S. nigrum* (Flier *et al*., 2003).

To verify whether *S. nigrum* is resistant to *P. infestans,* I examined the interaction between twenty *S. nigrum* accessions which are listed in Table 1 and four aggressive *P. infestans* isolates of different origins (Table 2).

All *S. nigrum* accessions described in Table 1 were tested with *P. infestans* infection. Four different *P. infestans* isolates listed in Table 2 were used. Five plants of each accessions were inoculated within a trial; three trials were conducted. Non-inoculated *S. nigrum* served as a negative control and potato var. désirée as susceptible control.

Macroscopically, seven days postinoculation (dpi), all *S. nigrum* accessions tested with *P. infestans* isolates APiO2, LL8, and Sn001a gave no visible symptoms of disease (Figure 14A). There were no spreading lesions, nor sporulation on any of them, when examined under a binocular. In addition, no *P. infestans* hyphae were detected under microscop after Keogh staining. The désirée potato plants however were completely dead within a week (Figure 14D-F), sporulation was visible on the leaves just after a few days (from the fourth dpi) (Figure.15A). The isolate T04 was more virulent than the other three isolates. Infection with this isolate resulted in small (2-3mm^2) macroscopically visible necrotic spots (Figure.15B.) but did not cause plant *S. nigrum* death nor sporulation. This result indicated that resistance of *S. nigrum* against *P. infestans* was complete. This result also confirmed that *S. nigrum* is a non-host for *P. infestans* (resistance at species level).

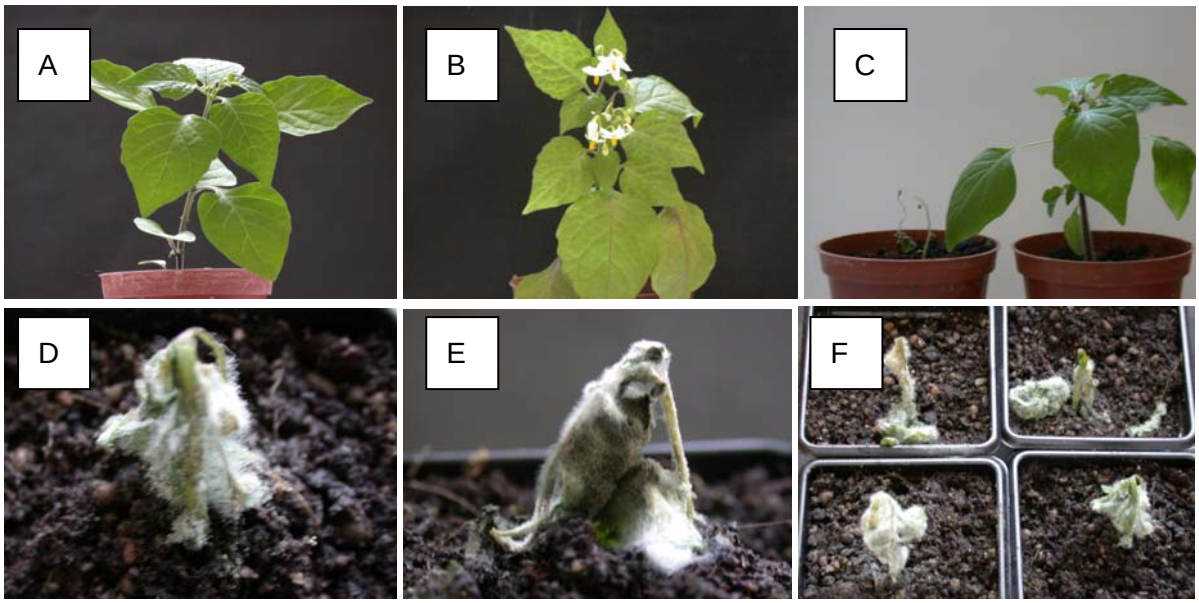


Figure 14: Potato and *S. nigrum* plants after infection with *P. infestans (*Api02*)*

(A)- *S. nigrum* 7dpi, (B)- *S. nigrum* 5 wpi C)- infected potato on the left and infected *S. nigrum* on the right, , (D through F)-.infected potato 7dpi

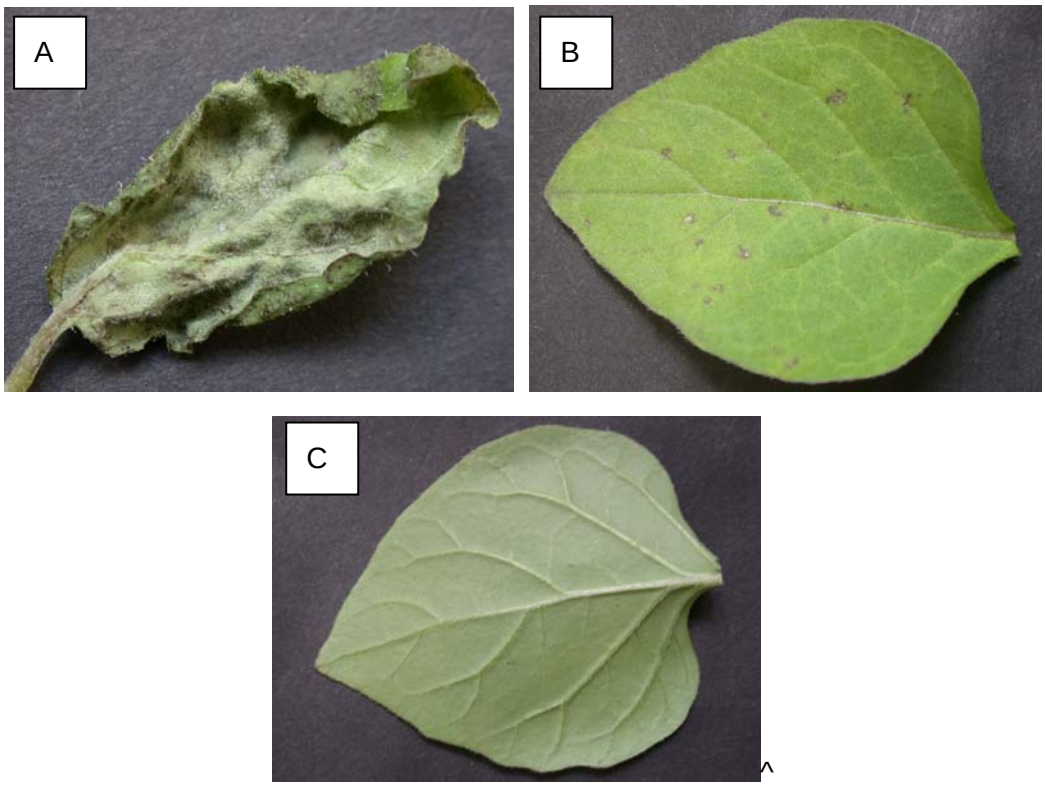


Figure 15: infected Potato and *S. nigrum* leaves 4 dpi (T04)

(A)- Potato leaf Leaf was overgrown by hyphae (hyphal growth was microscopically visible), (B)- *S. nigrum* leaf showing visible black spot, (C)- *S. nigrum* leaf control.

Microscopically, a very restricted hyphal growth (Figure 16) was visible on the leaves of some accessions infected with T04 isolate, whereas those infected with APiO2, LL8, and Sn001a, reacted with a HR which was confined to one to a few cells (Figure 18A,B).

In the case of the T04 isolate, the hyphae were able to penetrate further in the plant tissue (Figure.16) but were surrounded by necrotic cells. This trailing necrosis response successfully stopped further infection and became macroscopically visible as small necrotic regions on the leaves (Figure 15B). Staining of the tissue with aniline blue showed the presence of callose deposits to stop hyphal growth (Figure.16). Callose is an amorphous high-molecular weight ß-1, 3-glucan that is deposited in the form of papillae upon infection by fungal and oomycetous pathogens. It is thought to act as a physical barrier against fungal colonisation.

In the case of APiO2, LL8, and Sn001 isolates; secondary infection hyphae were not visible, indicating that the pathogen is restricted to the penetrated cell. The HR occuring in *S. nigrum* and *P. infestans* interaction have the typical characteristics of an HR induced in incompatible interaction: the granular structure of the cytoplasm (Figure 18A,B), thickened cell walls (Figure 18A,B), and autofluoresence under UV illumination (Figure 18C). This HR was generally limited to the penetrated epidermal cell and is consequently macroscopically invisible. Zhang and associates (2004) have reported that hypersensitive cell death is usually accompanied by the accumulation of fluorescent phenylpropanoid derivatives (phytoalexins) around the pathogen infection sites. The autofluorescence under UV might be due to these compounds.

In potato desirée cultivars, the pathogen colonized rapidly the plant tissue by spreading in all directions away from the penetration site and forming a dense network of intra- and extracellular hyphae. Then, the mycelia grew out of the stomata and produced sporangia (Figure 18E, F).

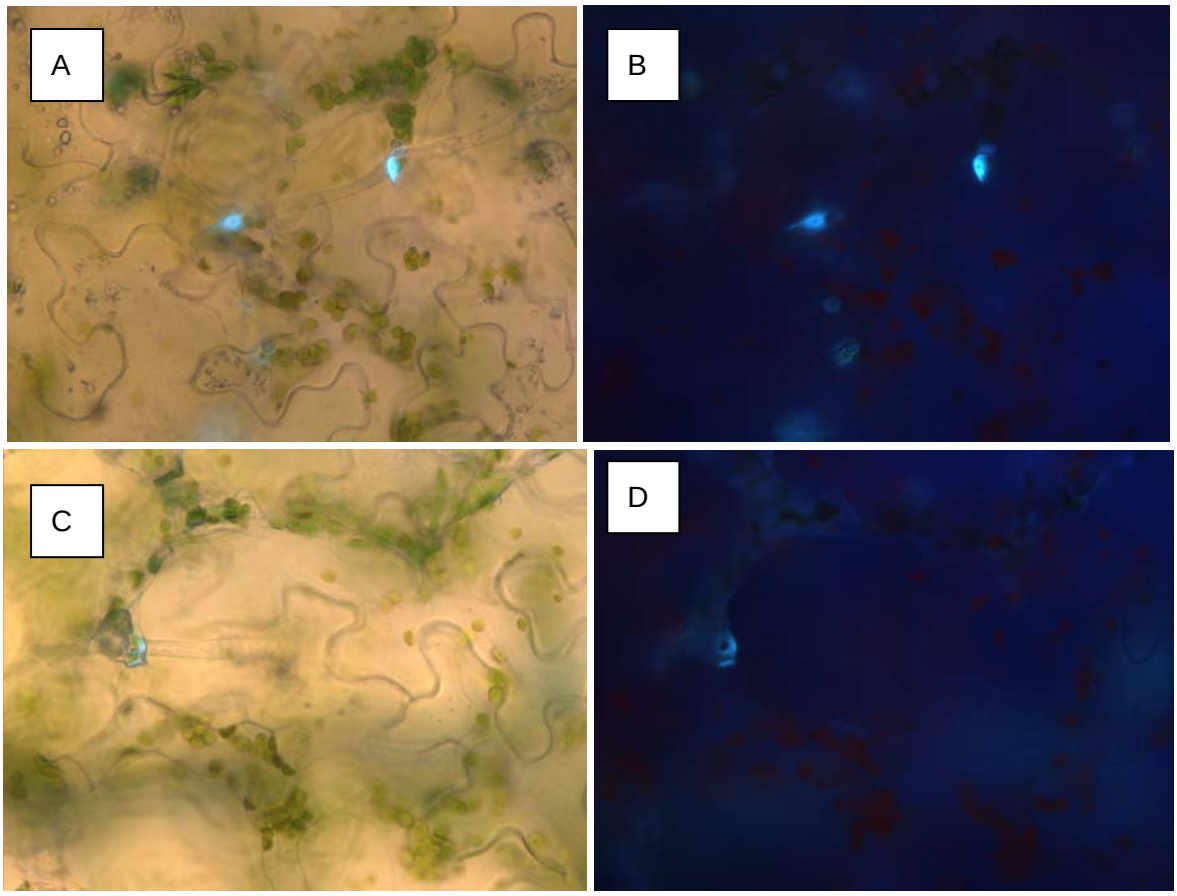


Figure 16: *S. nigrum* leaf with very limited hyphal growth of T04 isolate

(A through D)- Leaf disc stained with aniline blue, (B,D)- observation under UV light.

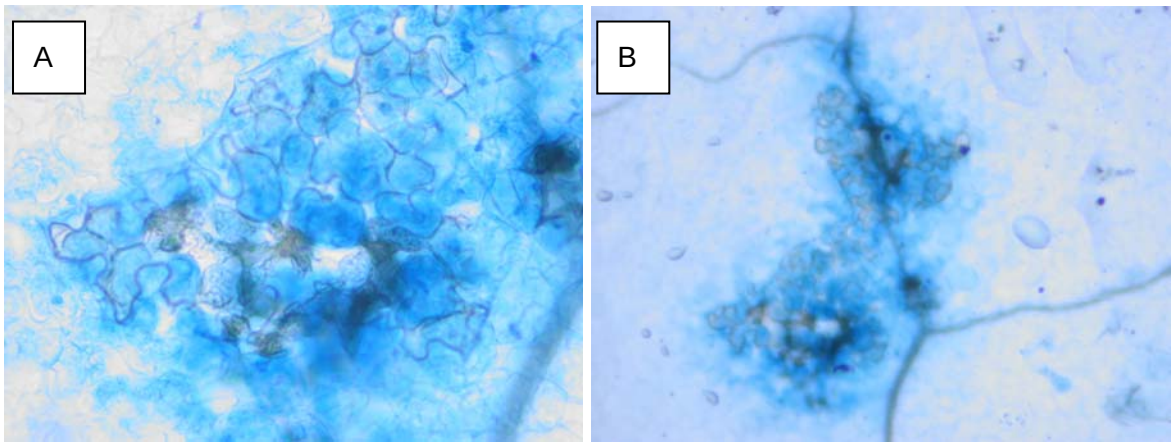


Figure 17: The macroscopically visible HR in T04-infected *S. nigrum* leaf after Keogh staining and observed under microscopy

(A)- 15x magnification, (B)- 10x magnification

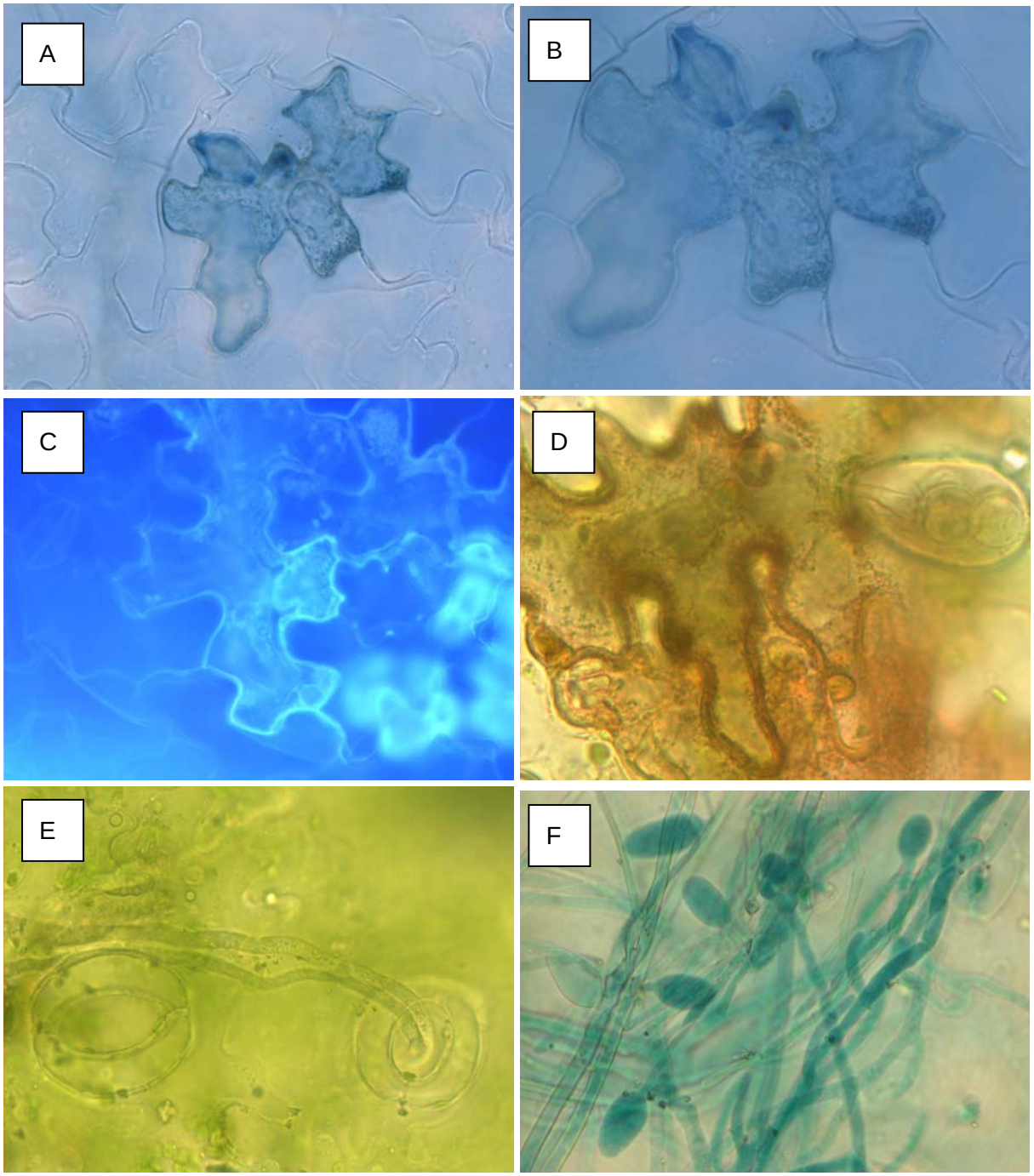


Figure 18: Microscopically examination of leaf discs after *P. infestans* (APi02) infection

(A,B)- HR response in a *S. nigrum* leaf showing the granular cytoplasm and thickened cell wall, without hyphal growth. Lacto phenol-trypan-blue stained leaf disc 7dpi. The penetration site is out of the focal plane and only the actual point of penetration can be seen as a dark blue area next to the stomata, (C)- Auto fluorescing cell wall (7dpi), this indicates the accumulation of phenolic compounds. (D through F)- Compatible interaction between potato and *P. infestans*, (D)- Extensive necrosis near the inoculation spot made further examinations impossible. The zoospore encysted inside the sporangial shell and the germ tube grew out of the opening of the shell to the surface of the leaflet (1dpi), (E)- Hyphae emerging through the stomatal opening, (F)- Potato leaf full of mycellium and lemon-shaped zoosporangia (4 dpi).

3.2 *Solanum nigrum* test with *Phytophthora infestans* elicitins

P. infestans and other *Phytophthora* species express a family of structurally related small 10-kDa extracellular proteins, known as elicitins, which induce the HR and other biochemical changes associated with defence responses in *Nicotiana spp*. but not in potato and tomato (Kamoun *et al*., 1993, 1997a; Ponchet *et al*., 1999; Ricci *et al*., 1989; Sasabe *et al*. 2000; Huitema *et al*. 2005). It was therefore tempting to speculate that the HR induced in *S. nigrum* during its interaction with *P. infestans* was due to the recognition of elicitins.

Recognition of elicitins is thought to be responsible for the resistance of *Nicotiana spp*. to a multitude of *Phytophthora* species, including *P. infestans*. Elicitins interact with a high-affinity binding site on the plasma membrane of tobacco, which appears to consist of a receptor complex of two glycoproteins of about 170 and 60 kDa in mass (Lebrun-Garcia *et al.*, 1999). However, the molecular genetic basis of elicitin perception remains unknown (Vleeshouwers *et al*., 2006).

To achieve the elicintin tests, *P. infestans* isolates listed in Table 2 were grown for three weeks in still liquid cultures in the synthetic medium containing per liter 0.5 g of KH2PO4, 0.25 g of MgSO47H2O, 1 g of asparagine, 1 mg of thiamine, 0.5 g of yest extract, and 25 g of glucose (modified from Bonnet *et al*., 1985) described by Kamoun and associates (1994) for elicitin production. The cultures, containing the elicitins, were harvested by filtration through cheesecloth and then through a 0.22 µl filter and infiltrated, using a needleless syringe, into the leaves of *N. benthamiana*, *N. tabacum* and all the twenty *S. nigrum* accessions listed in table 1.

Hypersensitive responses were induced in *Nicotiana* species but not in *S. nigrum* as shown in Figure 19. This result proved that elicitins are not responsible for the HR occurred on *S. nigrum* leaves after *P. infestans infection*. The resistance in *S. nigrum* against *P. infestans* is thus not due to elicitin recognition.

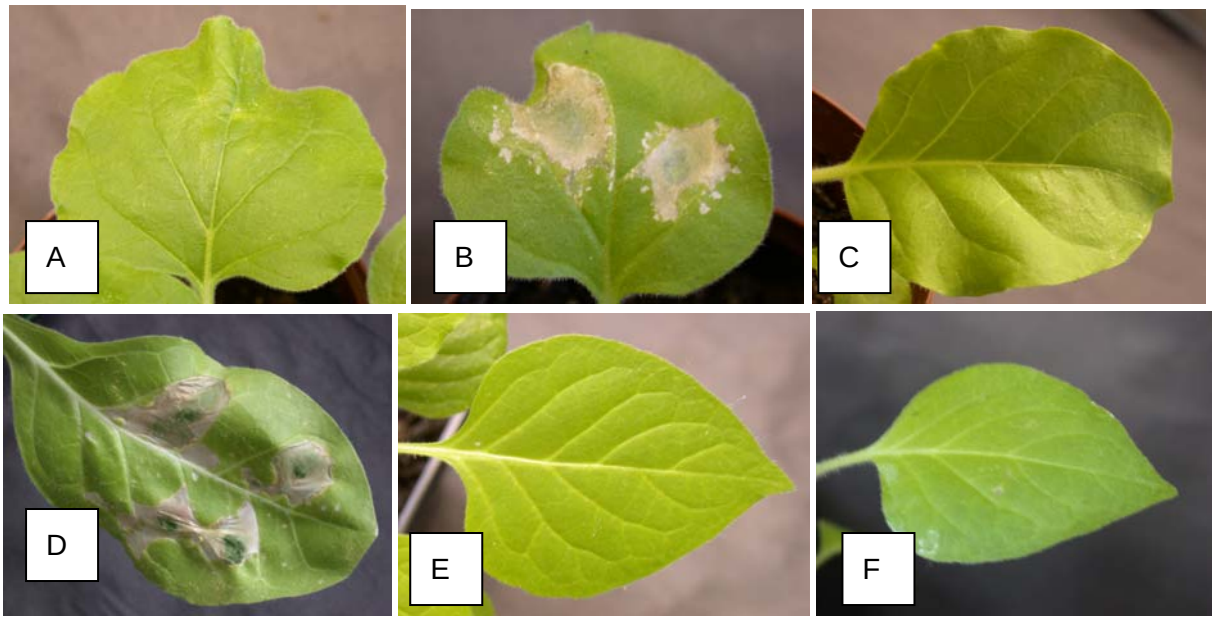


Figure 19: Infiltration of *P. infestans* elicitins into *N. benthamiana, N. benthamiana* and *S. nigrum* leaves

(A)- *N. benthamiana* control, (B) infiltrated *N. benthamiana* (C)- *N. tabacum* control, (D)- Infiltrated *N. tabacum* (E)- *S. nigrum* control, (F)- Infiltrated *S. nigrum.*

3.3 Virus Induced Gene Silencing (VIGS) to test the resistance of *Solanum nigrum*

VIGS offers an attractive and quick alternative for knocking down the expression of a gene without the need to use stable genetically transformed plants (Liu *et al*., 2002).

When a plant virus infects a host cell it activates an RNA-based defence that is targeted against the viral genome. VIGS is a virus vector technology that exploits this RNA defence mechanism (Lu *et al*., 2003). The approach involves the cloning of a short sequence of a targeted plant gene into a viral delivery vector (Baulcombe, 1999). Using this method, the virus vector carrying a partial sequence of a host gene is used to infect the plant (Liu *et al*., 2002) and natural defence mechanisms of the plant directed at suppressing virus replication result also in specific degradation of mRNAs from the endogeneous plant gene that is targeted for silencing (Baulcombe, 1999). Thus, the symptoms in the infected plant would reflect the loss of the function in the encoded protein (Lu *et al*., 2003). Therefore, VIGS can be used for reliable gene silencing (Robertson, 2004).

TRV is one of a handful of plant viruses that have been modified to serve as a vector to facilitate the silencing of endogenous target genes in host plants (Liu *et al*., 2002; Ratcliff *et al*., 2001). TRV is able to spread vigorously throughout the entire plant, including meristems tissue, yet the overall symptoms of infection are mild compared with other viruses. TRV is a bipartite single-stranded RNA tobravirus; the genome has two RNA components (RNA1 and RNA2). Thus, there are two versions of the TRV vector(TRV-RNA1 and TRV-

RNA2 also named TRV1 and TRV2 respectively) (Robertson, 2004). To expedite cloning and transfer to plant hosts, cDNA versions of TRV sequences have been introduced into an *Agrobacterium* T-DNA binary vector. Multicloning sites or recombination are used to insert a fragment of an endogenous gene of interest, such that it is converted to dsRNA as part of viral replication. Endogenous gene silencing by TRV-based VIGS is facilitated by *Agrobacterium*-mediated delivery of TRV vectors into host plant cells (Lu *et al*., 2003). The presence of dsRNA complementary to endogenous transcripts results in the degradation of these dsRNAs along with the silencing of their endogenous mRNA counterparts (Hileman, 2005). Silencing is coincident with viral spread and is usually greatest 2-3 weeks after inoculation (Liu *et al*., 2002).

Using VIGS in *Solanaceae* (*Nicotiana, Lycopersicon*, and *Petunia*), a number of genes have been functionally characterized. VIGS has been especially powerful for dissecting signalling components involved in disease resistance (Liu *et al*., 2002a; Peart *et al*., 2002; Robertson, 2004). In this study, I also used VIGS in an attempt to knock down the gene responsible for non-host resistance against *P. infestans* in *S. nigrum,* which belongs to *Solanaceae* as well, in silencing genes for *R*-gene signal transduction. I amplified the fragments of candidate genes from *S. nigrum*, cloned them into *pTRV-RNA*2 gateway vector and then introduced them into *S. nigrum* plants by *Agrobacterium*-mediated infiltration method in order to silence these genes. The candidate genes have been chosen from public databases. In many cases, LRR-containing resistance genes have been found to be dependent on either *Eds1* in the case of TIR-NB-LRR genes or on *Ndr1* in the case of CC-NB-LRR genes. If these genes are required for disease resistance against *P. infestans*, I anticipated that the virus vector-infected *S. nigrum* plants would become partially or completely susceptible to the pathogen.

3.3.1 Establishment of agroinfilttration techniques

Agroinoculation is the most potent way of introducing cDNA-derived viral RNA into plants (Lu *et al*., 2003). Agroinoculation involves the use of Ti plasmid vectors of *A. tumefaciens* in which a region (the T-DNA) is transferred to the genome of infected plant cells. This process can be exploited for inoculation of plant viruses if the full-length viral cDNA is present in the T-DNA and between a promoter and a transcriptional terminator that is active in plant cells. Agroinoculation has advantages over *in vitro* transcription because the virus vector cDNA does not have to be isolated, digested, or transcribed (Lu *et al*., 2003). The *Agrobacterium* cells carrying the insert can be inoculated directly into the plant. Presumably there are cells at the site of inoculation that are transformed to be infected with the virus vector that is

represented in the T-DNA. These cells would then serve as a reservoir of infection that spreads systemically throughout the plant (Lu *et al*., 2003).

The problem in using agroinfiltration is that some combinations of plant species/varieties and different *Agrobacterium* strains are incompatible and lead to an HR. I have therefore tested different *Agrobacterium* strains with *S. nigrum* using the leaf infiltration method. Eight strains of *A. tumefaciens* (*GV3101pMP90, LBA4404 pAL4404 Z1, LBA4404 pAL4404 Z71, LBA4404 W, MOG101 pMOG101, UIA pMP90, AGL1 and LBA1101*) at different concentration (Optical Density $O.D_{600nm}$: 0.03, 0.1 and 1) have been used.

Among the eight tested *A. tumefaciens* strains, only GV3101 and MOG 101 gave no signs of a plant reaction (Figure 20C, D). All other strains caused necrosis (Figure 20B) around the inoculation sites and could not be used for agroinfiltration assay. 4-week-old plants showed the most consistent results.

To assess the effectiveness of agroinfiltration, *S. nigrum* leaves were also infiltrated with *A. tumefaciens* strains (GV3101 and MOG101) containing a CaMV-GUS construct. GUS staining was performed 4 days after infiltration and they gave a good GUS expression. GUS activity was visible within the infiltrated area of the leaves (Figure 20E). Therefore, I decided to use GV3101 and MOG101 for the subsequent experiments.

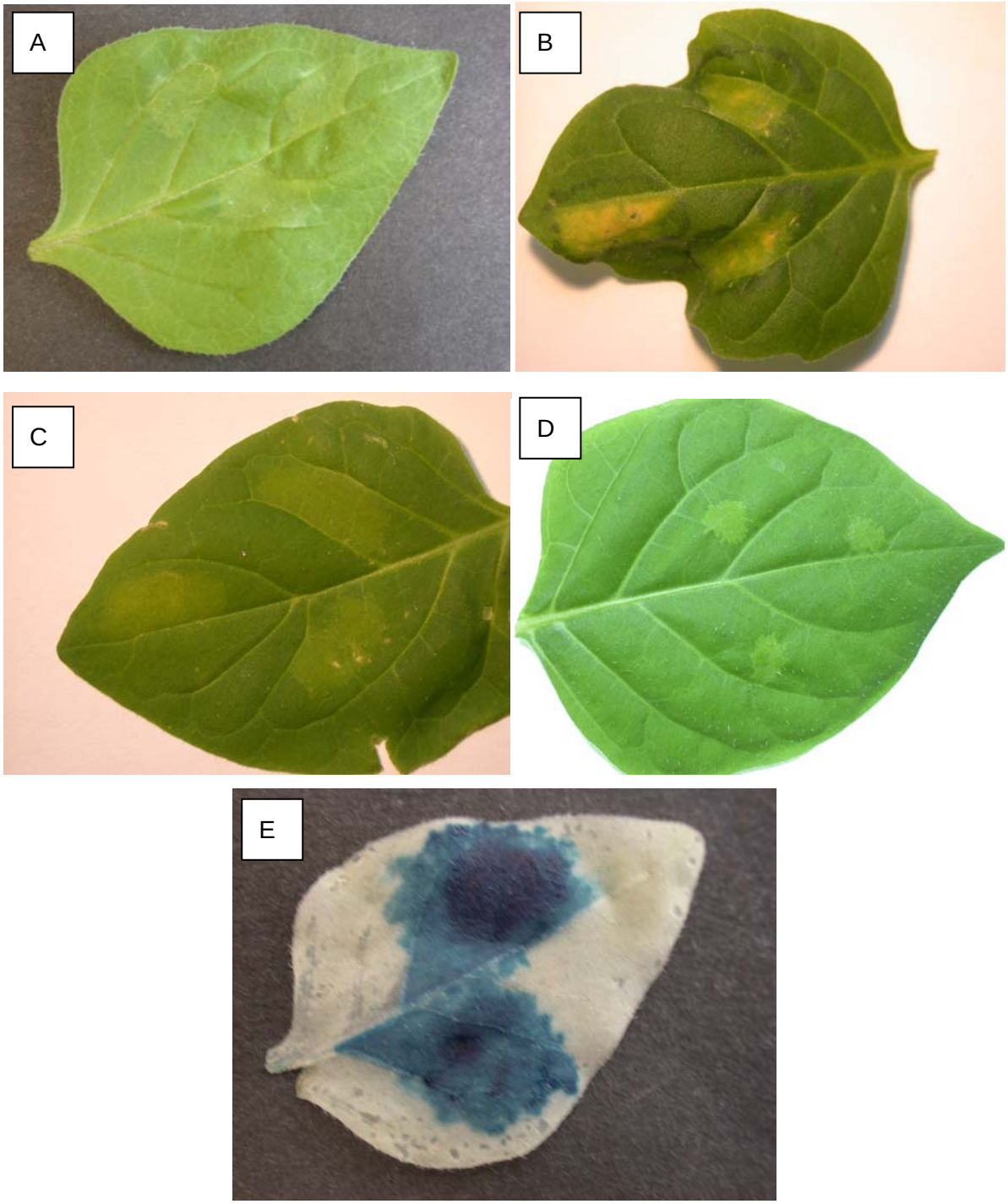


Figure 20: Infiltrated *S. nigrum* leaves;

(A)- Control: *S. nigrum* leaf infiltrated with water, (B)- *S. nigrum* leaf infiltrated with AGL1 showing disease symptoms, (C): infiltrated with MOG101, (D) infiltrated with GV3101, (E)- Control: *S. nigrum* leaf infiltrated with GV3101 containing a CaMV-GUS (after GUS staining).

3.3.2 Silencing of the PDS gene in *Solanum nigrum* using a TRV-VIGS vector

Most viruses used for VIGS have a limited number of hosts, and the virus-host combination seems to be a crucial factor in determining the efficacy of silencing. Some of the viruses used in VIGS can cause symptoms that might mask the phenotype caused by the silencing of the target gene. Tobacco Rattle Virus (TRV) -VIGS in *Solanaceae* overcomes many of these problems. PVX (Potato Virus X), TMV (Tobacco Mosaic Virus) and many other viruses do not infect plant meristems, but TRV seems to infect almost all tissues of the plant, including meristems and floral organs, and produces mild symptoms. The widespread distribution of TRV throughout the plant probably makes TRV-VIGS the better system. TRV vectors have been used for gene silencing in several species of the *Solanaceae*, for instance *N. bentamiana*, tomato, tobacco, potato pepper, and Petunia (Gianinna Brigneti et al., 2004), they can systemically move in *Solanaceae* species (Ryu et al., 2004). One vector that seems to be especially effective is the TRV-based vector described by Liu and Kumar (Liu et al., 2002) and this is the vector I used in this experiment. The TRV genome comprises two RNAs: RNA1, which encodes several genes, including the RNA-dependent RNA polymerase; and RNA2, which encodes the coat protein. The target sequence is inserted into RNA2, downstream of the gene that encodes the coat protein, and this modified RNA is co-inoculated with unmodified RNA1 into a plant to generate an infection and induce VIGS. The two versions TRV-RNA1 and TRV-RNA2 are carried separately on Ti plasmid vectors in different strains of *Agrobacterium* (Lu et al., 2003). TRV has a wide host range and so in principle could serve as an efficient vector for VIGS in a diverse array of plant species (Hileman, 2005). I would expect also that the TRV-based vector and VIGS system worked well in *Solanum nigrum*.

The phytoene desaturase (*PDS*) gene encodes an enzyme involved in carotenoid biosynthesis and silencing of this gene results in photobleaching of plant leaves (Liu et al., 2002a). The leaves turn white due to lack of carotenoids and destruction of chlorophyll by photo-oxidization (Kumigai et al., 1995). Therefore I have chosen *PDS* as a visual indicator of gene silencing and also as a positive control for TRV- induced gene silencing of R-gene signal transduction.

In the beginning, leaf infiltration was used to deliver the TRV vectors, carrying *PDS,* using two kinds of *Agrobacterium* strains (GV3101 and MOG101). I optimised the VIGS procedure by using different optical densities of *Agrobacteria* in infiltration medium ($O.D_{600}$ of 0.6, 1 and 1.5). Photobleaching was observed on the newly developed leaves of *Solanum nigrum* plants four weeks after infiltration. Photobleaching must be caused by the absence of the photoprotective carotenoid pigments that require phytoene desaturase for their synthesis. *PDS* was silenced in about 80% of infiltrated plants. Silencing was monitored in the upper leaves of plants. This demonstrates that agroinfiltration of pTRV-*PDS* achieves gene

silencing in *S. nigrum*. The best result (strong photobleaching) was obtained with the plant leaves infiltrated with the lowest concentration of *Agrobacterium* GV3101 (OD of 0.6). However, all plants infiltrated with MOG101 did not show any photobleached leaf. Therefore, I used only GV3101 strain for the subsequent experiments and infiltration medium was always adjusted to an O.D of 0.6.

In order to obtain more percentage of photobleached plants, I also compared the effectiveness of VIGS by agrodrench, vacuum infiltration and the commonly used leaf infiltration method. The efficacy of VIGS was determined by counting all leaves (small and big), including leaves of secondary shoots, that showed photobleaching. It was found that vacuum infiltration of 2 week-old seedlings gives the best results (Figure 21C-E). 100% of the inoculated plants showed the silencing phenotype and almost all newly developed leaves were bleached. Also the appearance of photobleached leaves started 3 weeks after inoculation through vacuum infiltration; it was delayed by more than one week in agrodrench and one week with leaf infiltration. Perhaps, this result is caused by plant stages; at the time of infection they were 2 weeks old for vacuum infiltration and 4 weeks old for leaf infiltration and agrodrench. Plant stage is an important factor being advantageous for efficient VIGS (Chung *et al*. 2004). The delay in silencing through agrodrench may be due to the length of time required for the virus or the silencing signal to move from the site of infection (roots) to the upper parts of the plants (leaves) as well (Ryu *et al*., 2004). Based on this result, I decided to use vacuum infiltration in subsequent experiments.

These results showed that recombinant TRV can efficiently replicate and spread systemically in *S. nigrum* plants. Thus, VIGS system can be used to silence gene(s) of interest in *S. nigrum*.

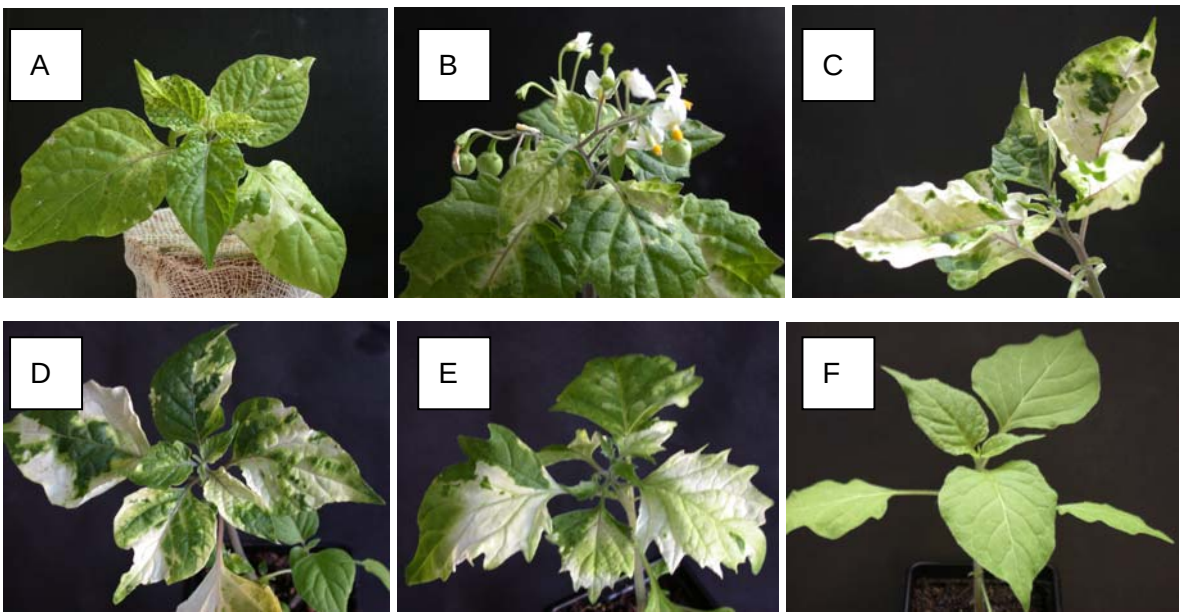


Figure 21: Silencing of *PDS* gene in *S. nigrum*

(A)- Infiltrated plant by agrodrench, (B)- leaf infiltration, (C through E)- Whole plant vacuum infiltration, (F)- uninfiltrated plant.

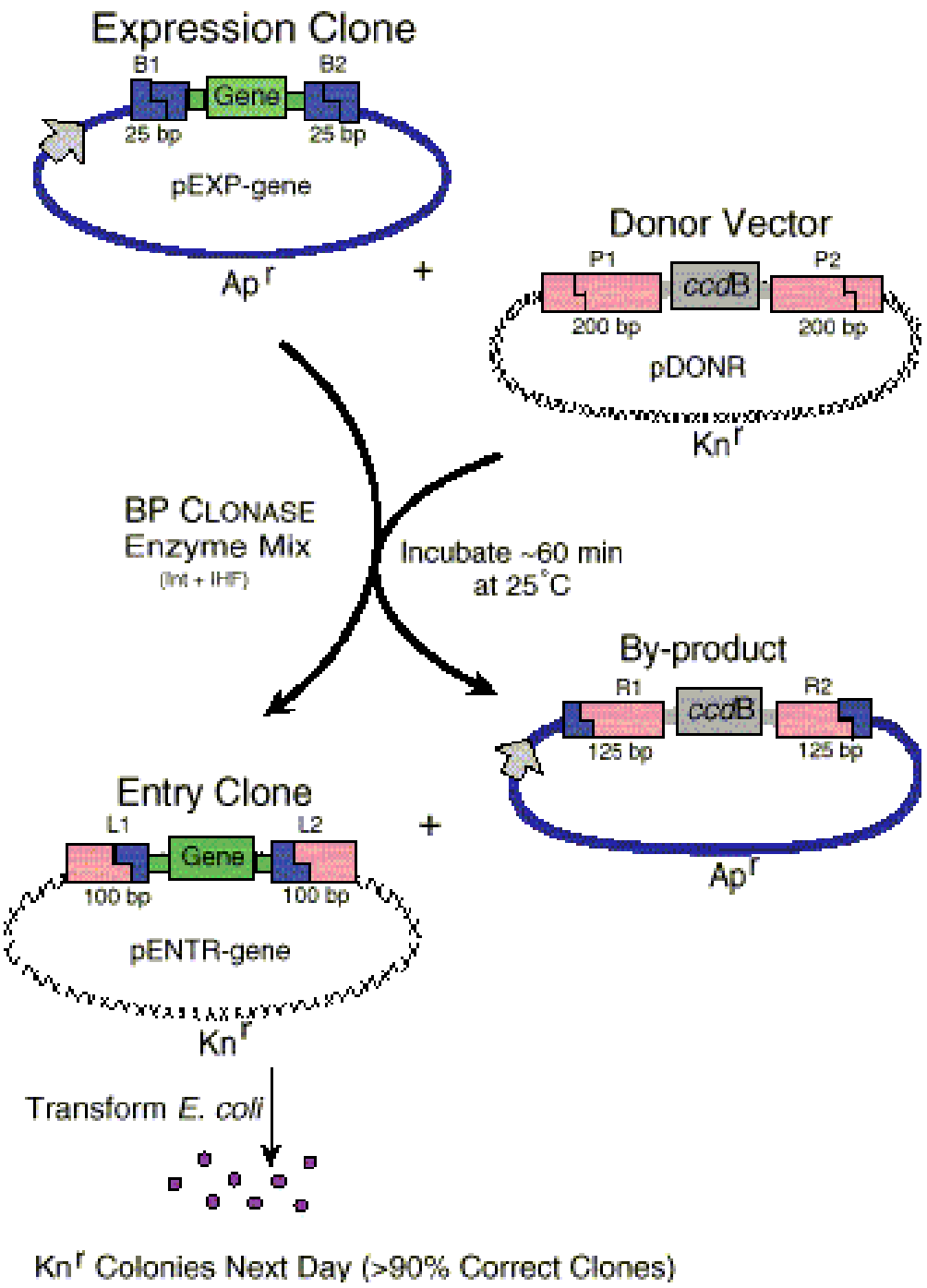
3.3.3 Gateway procedure:

The Gateway Cloning Technology is a universal cloning system that provides a rapid and highly efficient route to move and express a gene of interest (Landy 19989), instead of restriction enzymes and ligase. The technology is based on the enzymatic machinery of the *Escherichia coli* bacteriophage lambda site-specific recombination system, which facilitates the transfer of heterologous DNA sequences between vectors. Lambda-based recombination involves two major components: the DNA recombination sequences (*att* sites) and the proteins that mediated the recombination reaction (i.e. Clonase™ enzyme mix). The *att* sites serve as the binding site for recombination proteins and have been well-characterized (Weiberg and Landy, 1983).

There are two recombination reactions: BP reaction (lysogenic pathway) and LR reaction (lytic pathway) (Ptashne, 1992).

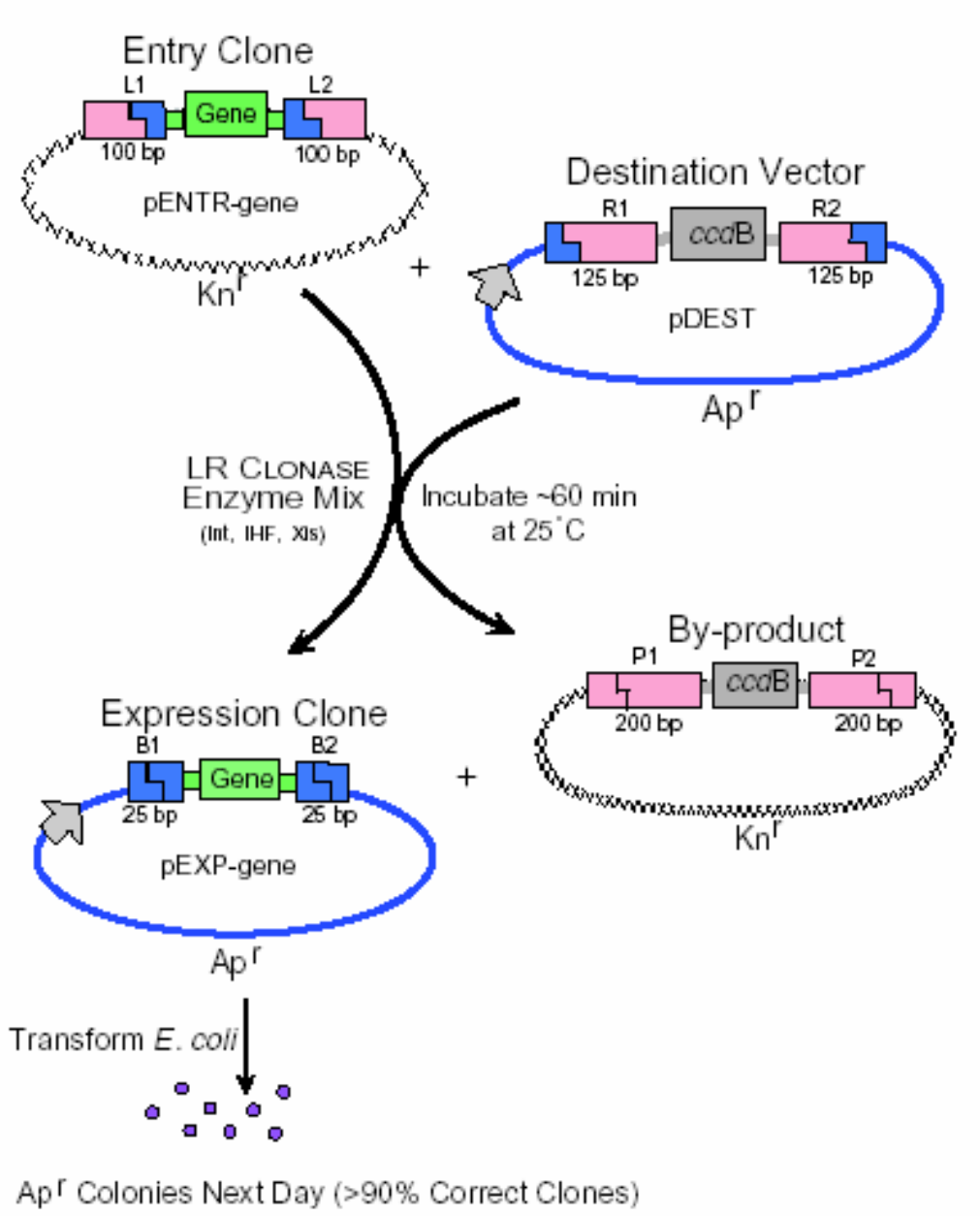
- **The BP reaction** consists of recombination between *att*B (25 bp) and *att*P (200 bp) sites of the expression vector and the donor vector respectively to generate *att*L (100bp) sites of the entry clone and *att*R (125 bp) sites of the by-product. This Reaction is catalysed by the BP Clonase™ enzyme (Figure 22).
- **The LR reaction** (Figure 23) is the process in the opposite direction which is the recombination between *att*L and *att*R sites of the entry clone and the destination vector respectively to create an *att*B-containing expression clone.

This reaction is catalysed by the LR Clonase™ enzyme mix. In this way, the gene can be transferred from the Entry clone into an Expression clone while maintaining its orientation and frame throughout this subcloning (Sasaki et al., 2004).



Source: http://bxaf.biosino.cn/bxaf/bm/bm_cg6.php

Figure 22: BP reaction



Source: http://bxaf.biosino.cn/bxaf/bm/bm_cg6.php

Figure 23: LR reaction

Recombination is conservative (i.e. there is no net gain or loss of nucleotides) and requires no DNA synthesis. The DNA segments flanking the recombination sites are switched, such that after recombination, the *att* sites are hybrid sequences comprised of sequences donated by each parental vector. Strand exchange occurs within a core region that is common to all sites.

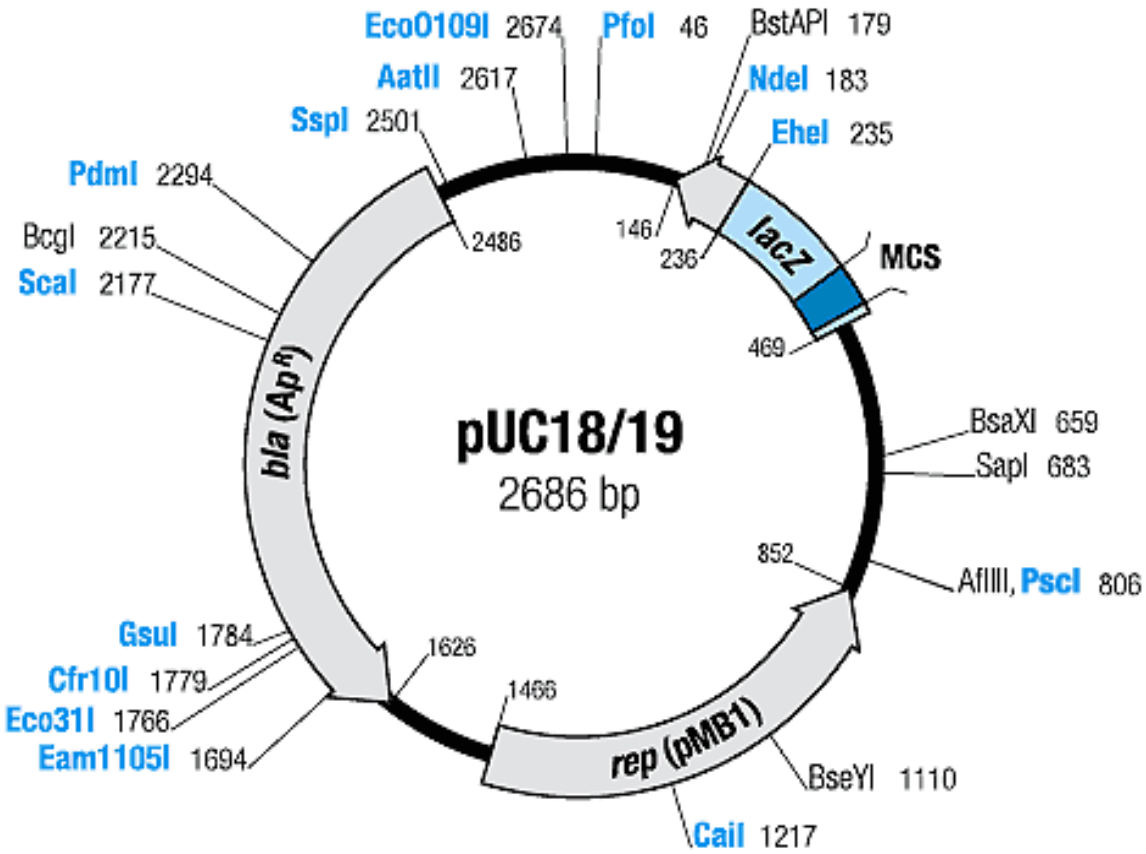
The power of this system is that a gene can be subcloned while maintaining the orientation and reading frame of the gene throughout the procedure.

Furthermore, a gene or multiple genes, can be subcloned in parallel into one or more vectors in a simple, rapid, in vitro recombination reaction. In these reactions, a high percentage (>95%) of the *E. coli* colonies produced contain the desired clone. The high percentage of success is facilitated by the killing effect for *E.coli* of the toxic gene, *ccd*B, remaining in unreacted vectors.

3.3.4 Construction of a new pENTR11 vector

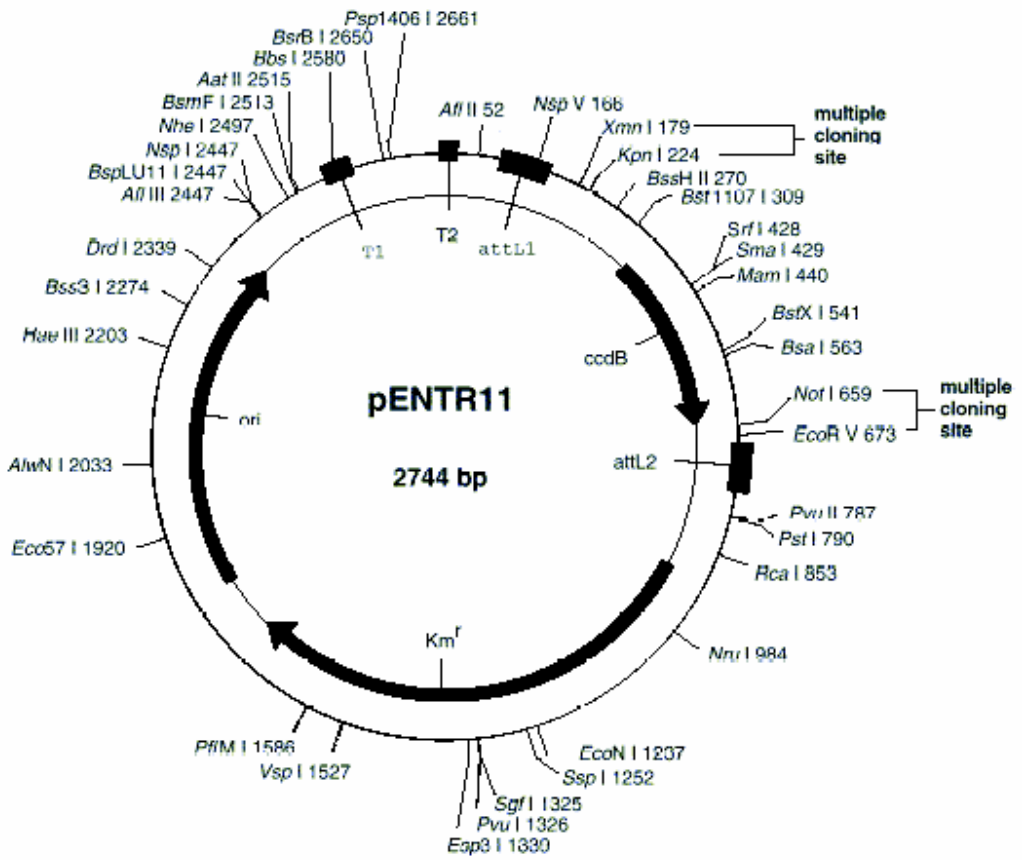
As described previously, pENTR11 vector allows restriction cloning of the gene of interest from entry into the Gateway™ system. It contains *att*L1 and *att*L2 sites (for site-specific recombination of the entry with a Gateway destination vector) and two *E. coli* ribosome binding sites for efficient initiation of translation in prokaryotic cells. The presence of a pUC origin inside this plasmid allows high-copy number replication and maintenance of plasmid in *E. coli*. The *ccd*B gene, which is located between the two *att*L sites of this vector, is very useful for negative selection of clones. The *ccd*B gene encodes a protein that interferes with *E. coli* DNA gyrase, thereby inhibiting the growth of standard *E. coli* hosts. After the recombination between an entry clone and a destination vector, the *ccdB* gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the *ccdB* gene, or by-product molecules that retain the *ccdB* gene, will fail to grow. This allows high-efficiency recovery of only the desired clones.

The pENTR11 has kanamycin resistance (Kan^R) gene for selection in *E. coli,* however, since I used a destination vector (pTRV-RNA2-Gateway) which is also kanamycin resistant, I constructed a new vector which has another antibiotic resistance than kanamycin for further selection. I transferred the ampicillin resistance of pUC18 vector (Figure 24) into pENTR11 (Figure 25).



Source: http://www.fermentas.com/techinfo/nucleicacids/mappuc1819.htm

Figure 24: pUC18 map



Source:
http://www.embl.de/ExternalInfo/protein_unit/draft_frames_save/flowchart/cloning_strategy/gateway/ENTR_vectors/Pentr11_map.pdf

Figure 25: Map of the pENTR11 vector.

After digestion with PstI and AatII restriction enzymes, the fragment carrying the ampicillin resistance of pUC18 (2206 bp) was successfully ligated with the fragment containing the *att*L1-*ccd*B-*att*L2 cassette of pEntry11 (1019 bp).

The colonies of transformed *E. coli* DB 3.1 were well grown on LB medium supplemented with ampicillin 100µg/µl. This result indicated that the new vector, named pEntry11Amp, has ampicillin resistance. A pENTR11Amp analysis was done by restriction enzymes and also by sequencing (The DNA sequence of pEntry11Amp is given in supplementary material).

The new vector was digested once again with PstI and AatII for verification and with BamHI which cut once in pENTR11 fragment and once in pUC18 fragment. The result confirmed that I have successfully ligated the correct fragments from pENTR11 and pUC18. All fragments from each restriction enzyme gave the expected size (Figure 26); 1019 bp and 2206 bp from PstI/AatII and 595 bp and 2630.bp from BamHI.

The map of this new pENTR11 vector is presented in Figure 27.

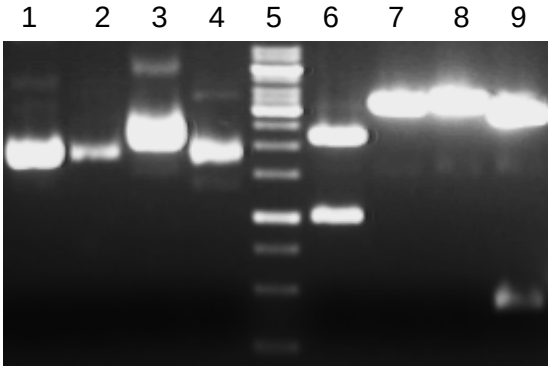


Figure 26: Analysis of pENTR11Amp

(1,2)- pENTR11, (3)- pENTR11Amp, (4)- pUC18, (5)- DNA ladder 1kb, (6)- pENTR11Amp digested with AatII/PstI, (7)- pENTR11Amp digested with AatII, (8)- pENTR11Amp digested with PstI, (9)- pENTR11Amp digested with BamHI.

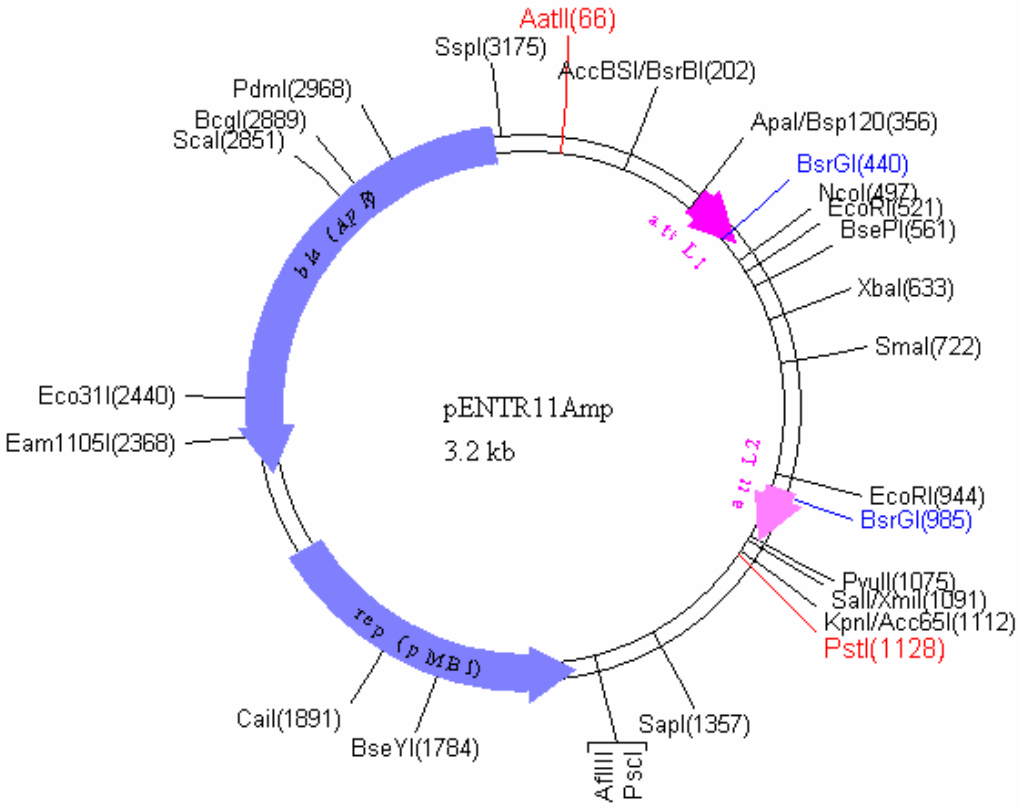


Figure 27: Map of pENTR11Amp

3.3.5 Cloning of genes for R-gene signal transduction from *Solanum nigrum*

All primers listed in table 3 were used to amplify candidate genes from genomic DNA of *S. nigrum*. After checking by electrophoresis, only *Eds1* and *Ndr1* gave the correct size, 530 and 496 bp respectively. The rest were with introns. Thus, I had to amplify them form cDNA. However, only *Ctr1, Npr1, Rar1, Sgt1.1, Tga1a, Tga2.2* gave positive results.

3.3.5.1 Construction of entry clones

Among the PCR products obtained from *Solanum nigrum*, *Eds1*, *Ndr1,* and *Rar1* were successfully integrated into pENTR11Amp after ligation as described in material and methods. Each one of them replaced the *ccd*B gene flanked between attL1 and attl2 sites of pENTR11Amp. These entry clones were transformed into E. coli using electrocompetent cells EC100 for multiplication and analysed by PCR using *Eds1, Ndr1*, and *Rar1* gene-specific primers. The PCR product size for each gene (Figure 28) indicated that *Eds1, Ndr1,* and *Rar1* were successfully cloned into pEntr11Amp.The results from positive transformants analyses are shown in Table 7.

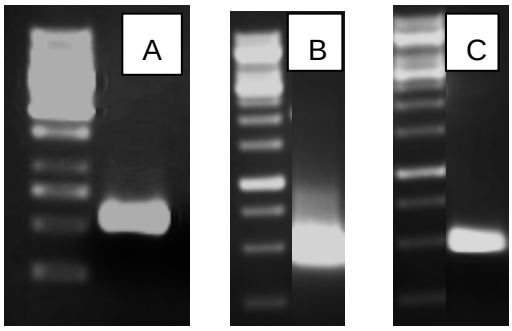


Figure 28: PCR products from entry clones

(A)- pENTR11Amp-*Eds1*, (B)- pENTR11Amp-*Ndr1*, (C)- pENTR11Amp-*Rar1*

Table 7: Positive entry clones

Primers	Number of colonies checked	Positive clones from genomic DNA	Positive clones from cDNA
EDS1	139	6	6
NDR1	167	6	2
RAR1	72		11

The pENTR11Amp clones were analysed using sequencing as well to confirm the presence and orientation of the insert. The primer used was pENTR11-139F (5'-GGCCTTTTTGCGTTTCTACA-3'). The results from the sequencing are as follows:

EDS1

5′-
AGGTATGAGCTCCCAGATAGCTTCGAGGGACGAAAGGACTGGATACAACTAGGGACACAGTTTCGCAG
GCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACATGAAGAATGAAGATACTGGACCTTACA
TGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGATGGTTAGAGCATGCTGAGAAAGTGCAA
GCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAGAAGTGGAGGAACTTAGAAACAAGCAATTTGCAGA
AGGGCAAAACAGGGTTTTGAGTTTAGAAACAACTGCAAGTGGCTGGATCCAGAGTGGCCTTCTTGGCA
ATGATGTTTTCTTCCCTGAGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGACTG
GCATCTTGGGTATCAGGGAAAATATCTTCTTAGCGCCTTGCGCCCTAATATTTTGAGAAGGATGATAT
CCAAGCCAACTTGCAGACACCTAAAACATTCCACCACATATATTCCCATGCCCC -3′

***Solanum nigrum (Sn)* - Eds1 sequences alignment with Tomato (*Le*), tobacco (*Nt*) and *Nicotiana benthamiana (Nb)*:**

```
NtEDS1          AAGGTATGAGCTCCCAGATAGGTTCGAGGGACGAAAGGAATGGATAAAACTAGGGACACA 60
NbEDS1          -AGGTATGAGCTCCCAGATAGTTTTGAGGGAAGAAAGGAATGGATAAAACTAGGGACGCA 59
SnEDS1          AAGGTATGAGCTCCCAGATAGCTTCGAGGGACGAAAGGACTGGATACAACTAGGGACACA 60
LeEDS1          AAGGTATGAGCTCCCAGATAGCTTCGAGGGACGAAGAGACTGGATAGAACTAGGGACACA 60
                ******************** ** ****** ***  ** ****** ********** **

NtEDS1          GTTCCGCCGGCAAGTTGAGCCCTTGGATATTGCAAACTATTACAGGCACTCGAAGAATGA 120
NbEDS1          GTTCCGCAGGCAAGTTGAGCCCTTGGATATTGCAAACTATTACAGGCATTTGAAGAATGA 119
SnEDS1          GTTTCGCAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACATGAAGAATGA 120
LeEDS1          GTTTCGCAGGCAAGTTGAGCCCTTGGATATTGCAAACTATTACAGGCACTTGAAGAATGA 120
                *** *** ********************** *****************   *********

NtEDS1          AGATACAGGACCTTACATGATCAGGGCTAGGCCGAAGCGTTATAGGTTCACACAACGATG 180
NbEDS1          AGATACAGGACCTTACATGATCAGGGCTAGGCCGAAGCGTTATAGGTTCACACAACGATG 179
SnEDS1          AGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGATG 180
LeEDS1          AGATACTGGACCTTACTTGATCAGGGCTAGGCCTAAGCGTTATCGGTTCACACAGAGATG 180
                ****** ********* *************** *********  *********  ****

NtEDS1          GTTAGAGCATGAAGAAGAGGGTGCAAACAGGTGAACGCTCTGAGTCTTGTTTTTGGGCAG 240
NbEDS1          GTTAGAGCATGAAGA-GAGGGTGCAAACAGGTGAACGCTCTGAGTCTTGTTTTTGGGCAG 238
SnEDS1          GTTAGAGCATGCTGA-GAAAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 239
LeEDS1          GTTAGAGCATTTTGA-TAGAGTGCAAGCTGGTGCACGTTCCGAGTCTTGTTTCTGGGCAG 239
                **********   **  *  ****** * ***   ** ** *********** *******

NtEDS1          AAGTGGAGGAACTAAGAAACAAGCCAATTATGGAAGTGCAAAACAGGATTTTGAGTTTAG 300
NbEDS1          AAGTGGAGGAACTAAGAAACAAGCCAATTATGGAAGTGCAAAACAGGATTTTGAGTTTAG 298
SnEDS1          AAGTGGAGGAACTTAGAAACAAGCAATTTGCAGAAGGGCAAAACAGGGTTTTGAGTTTAG 299
LeEDS1          AAGTTGAGGAACTAAGAAACAAGCCATTTGCACAAGTGCAAGACAGGGTTTTGAATTTAG 299
                **** ******** ********** * **    *** **** ***** ****** *****

NtEDS1          AAACAAACCGCACGGGATTGGTCCCAGAGTGGCCTTCTGGGCGATGATGTTTTCTTCCCT 360
NbEDS1          AAACAAAG-GCATGGGATTGGTCCCAGAGTGGCCTTCTGGGCGATGATGTTTTCTTCCCT 357
SnEDS1          AAACAACT-GCAAGTGGCTGGATCCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCT 358
LeEDS1          AAACAGCT-GCAAATGGCTGGATCCAGAGTAGCCTTCTTGGCGATGATATTTTCTTCCCT 358
                *****    ***   *  ***  ******* ******* *** ***** ***********

NtEDS1          GAGTCTACCTTTACCAAATGGTGGAAACAACTCCCTACTCAGCACAAACTGACATCTTGG 420
NbEDS1          GAGTCTACCTTTACCAAATGGTGGAAACAACTCCCTACTCAGCACAGAATGACATCTTGG 417
SnEDS1          GAGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGACTGGCATCTTGG 418
LeEDS1          GAATCTACCTATACCAAGTGGTGGAAAACACTCCCACCTCAGCACAAACAGGCATCTTGG 418
                ** ******* ****** *********  ******  ********* *  * ********

NtEDS1          ATATCAAGGAAAATAAATTCTTAGCGTCTTTTACAGCTCCATATTATGCCATTGCCAACG 480
NbEDS1          ATATCAGGGAAAGTAAATTCTTAGCGTCTTTTACAGCTCCATATTACGCCAGCGCCAGCG 477
SnEDS1          GTATCAGGGAAAATATCTTCTTAGCGCCTT--GCGCCCTAATATTTTGAGAAGGATGATA 476
LeEDS1          GTGTCAAGGAAAATAACTCCTTAGCGCCTT--GCGCCCTAATATCTTGATAAAGATGGTA 476
                 * *** ***** **  * ******* ***   *  *   ****   *  *  *

NtEDS1          CCCTAATGTTTTGATAATGATGGTGTCCTAGCCAGCTTGCTGGTACTCCTAGTCCAATTC 540
NbEDS1          CCCTAATGTTTTGATAATGATT-TGTCCTAGCCAGCTTGCGGGTACTCCTAGTCCAATGC 536
SnEDS1          TCCAAGCCAACTTGCA--GACA----CCTA--AAACATTCCACCACATATATTCCCATGC 528
LeEDS1          TCCCAGCCAACTCACA--GACA----CCTA--TAACATTCCACCACATATGTTGCAATGC 528
                 ** *      *   *  **      ****   * * * *    **   *  * * ** *

NtEDS1          CC- 542
NbEDS1          ---
SnEDS1          CCC 531
LeEDS1          CCC 531
```

NDR1

5′-
CTCGACGAATTCATGGCCCTTCCATTCCGCCACCAGCCAAAACCTACCACCGTAGCCGCGGNGGTGGT
AGTTGTTGCAACCCATGTAGTTGTCTCTTCAATTGTCTTTGTACTTGTATTTGCCAAATTATCTTCAC
CCTTGTCATCATCCTCGGAGTCATTGCATTAGTCCTCTGGCTTGTTCTACGTCCTAACAAAGTCAAAT
TTTACGTGACAGATGCCACGTTGACACAATTCGATTATTCCTCAACAAACAACACCCTCTACTACGAT
CTAGCCCTCAACATGACCATTAGGAACCCCAATAAACGCGTNGGGATCTACTACGANNCAATTGAAGT
GAGAGCTATGTATGGTGGCCAGAGATTTGCTAGTCAAAATTTGGAACCGTTTTATCNGGGNCNTAAAA
ATACTANNANTTTGCATCCGGCGTTNTAAAGGACAGAGTTTTGGTTCTATTGGGAGATAGANAGAAAT
CAAATTTNTAATAATGAGAAGAATTTAGGGGCTCGAGAGCGCG-3′

***Solanum nigrum* - Ndr1 sequences alignment with Tomato (*Le*) and potato (*St*)**

```
LeNDR1          ATTTCTTTCTACAATGGCAGAGCAACAAAGAATTCATCCAGTACCTGAACCACAACATGA 60
StNDR1          ATTTTTTCTCCCTATGGCAGAGCAACAAAGAATGCATCCAGTACCTGAACCAGAACATGA 60
SnNdr1          ------------------------------------------------------------

LeNDR1          ACTTCCAGTACAAAAACCCTCTGTCCCTTTAGTGCCAAAGGGCTCTTTCAGTTCAGAAAA 120
StNDR1          ACTTCCAGTACAAAAACCCTCTGTCCCTTTAGTGCCAAAGGGTTCTTTCAGTTCAGAAAA 120
SnNdr1          --------------------------------------------------CTCGACGAAT 10
                                                                   *  *  **

LeNDR1          AGGTGACCCTGAGAAACAACAGCAGCAGCAGCAACCACCTTTTAGAAGAACAATCCCATA 180
StNDR1          AGGTGATCCTGAGAAGCAGCAGCAGCAGCAAGAACCACCTCTTAGAAGAACAATCCCATA 180
SnNdr1          TCATGGCCCTTCCATTCCGC--CACCAGCCAAAACCTACCACCGTAGCCGCGGN-----G 63
                   **  ***   *  *  *  ** ****   ****  *      *    *

LeNDR1          TTATCCACCATTAAAACCACCAAAGAGAAGATGTTGTTCTTGCAAAAGATGTCTATGTTG 240
StNDR1          TTATCCACCATTAAAACCACCAAAGAGAAGATGTTGTTCTTGCAAAAGATGTCTATGTTG 240
SnNdr1          GTGGTAGTTGTTGCAACCCATGTA--------GTTGTCTCTTCAATTGTCTTTGTACTTG 115
                 *        **  ****     *        *****   * ***  *   *     ***

LeNDR1          CACATGTCGTCTGTGGCTCATCATCATCATAATCATTGCTGCTTTATCTGCTGCTTTTTA 300
StNDR1          CACATGTTGTCTGTTGTTGCTCATCATCATAATCATTGCTGCTTTATCTGCTGCTTTTTA 300
SnNdr1          TATTTGCCAAATTATCTTCACCCTTGTCATCATCCTCGGAGTCATTGCATTAGTCCTCTG 175
                 *  **     *     *   * *  **** *** * *  *   *  *    *   * *

LeNDR1          CTTTGTCTTCCAGCCAAAAATACCAAAGTACTCCGTTGACAGTATGAGAATCACACAGTT 360
StNDR1          CTTTGTCTTCCAGCCAAAAATACCAAAGTACTCTGTTGACAGTATGAGAATCACACAGTT 360
SnNdr1          GCTTGTTCTACGTCCTAACAAAGTCAAATTTTACGTGACAGATGCCACGTTGACACAATT 235
                  ****  * *  ** ** * *   ** *  *  **      *   *   * ***** **

LeNDR1          CAATGTCAATAATGACATGAGCTTATTGTTTGCTAC--TTTTAATGTTAACATCACTGCA 418
StNDR1          CAATTTCAATAATGACATGAGCTTATTATTTGCTAC--TTTTAATGTTAACATCACTGCA 418
SnNdr1          CGATT--ATTCCTCAACAAACAACACCCTCTACTACGATCTAGCCCTCAACATGACCATT 293
                * **   * *  * *    *    *   * * ****  * *     * ***** **

LeNDR1          AGAAACCCCAACAAGAAGATTGGGATTTACTATGAAAATGGTAGCCAATTGAGTGTTTGG 478
StNDR1          AGAAACCCCAACAAGAAAATTGGGATTTACTATGAAAGTGGTAGCCAATTGAGTGTTTGG 478
SnNdr1          AGGAACCCCAATAAACGCGTNGGGATCTACTACGANNCAATTG---AAGTGAGAGCTATG 350
                ** ******** **     * ***** ***** **      *    ** **** * *  *

LeNDR1          TATACTGGTGTTCAGCTGTGTGAAGGGTCATTGCCAAGATTTTATCAG-GGTCATCAGAA 537
StNDR1          TATACTGGTGTTCAGCTTTGTGAAGGGTCATTGCCAAGATTTTATCAGCGGTCATCAGAA 538
SnNdr1          TATGGTGGCCAGAGATTTGCTAGTCAAAATTTGGAACCGTTTTATCNG-GGNCNTAAAAA 409
                ***  ***        *   *         ***  *   ******* * ** * * * **

LeNDR1          TACTACTGTACTCAGTTTGGATTTATCA-GGACAAACACAGAATGTTACTGGATTGTTG- 595
StNDR1          TACTACTGTACTTAGTTAGGATTTATCA-GGACAAACACAGAATGTTACTGGATTGTTG- 596
SnNdr1          TACTANNANTTTGCATCCGGCGTTNTAAAGGACAGAGTTTTGGTTCTATTGGGAGATAGA 469
                *****      *   *  **  ** * * ***** *       *  ** ***    * *

LeNDR1          CA--------------------------------------------------------- 597
StNDR1          CAGGCACTGCAGGTGGATCACAGAGAGGAAGTATTCCATTGAATCTCAGAGCAAAAAGTT 656
SnNdr1          NAGAAATCAAATTTNTAATAATGAGAAGAATTTAGGGGCTCGAGAGCGCG---------- 519
                 *

LeNDR1          ---------------------------
StNDR1          CCAGTGAAGCTCAAGATTGGGAACTTG 683
SnNdr1          ---------------------------
```

RAR1

5′-
AGGAAAGCACACAACAGAAAAACCAGTGATTGCAAAGCCACCTATTACCATAAATAAAGCAATTCATG
CGCCTGCGCCTGTGGCAGCTCCTATGACCAATGCATCAGCGAAAGAAGCCTGTCCTAGATGCCGCCAG
GGATTTTTTTGTTCTGATCATGGTTCACAACCCAGGGAAGCAATTCCGAAAGCATCAAATACAGTAAC
ATCTGTACCTTCTGAGAGCAATACAGATGTAAAGCAAGACCATCCGGCCCCAGTGAAGAAGAAAATTG
ATATTAACGAGCCTCAAATTTGTAAAAATAAGGGCTGTGGTAAGACCTTTAAAGAAAAGGAAAATCAT
GACACTGCTTGCAGTTACCATCCTGGCCCCGCTATCTTCCATGACCGGATGAGAGGATGGAAATGCTG
TGATATTCACGTCAAAGAATTTGACGAGTTCATGAGCATACCTCCCTGCACCAAAGGATGGCAC-3′

***Solanum nigrum* - Rar1 sequences alignment with potato (*St*), tomato (*Le*), and tobacco (*Nt*)**

```
StRAR1          AGGAAAACACACAACAGAAAAACCAGTGATCGCAACACCATCTGCTACCAAAAATAAAGC 60
LeRAR1          AGGAAAGCACACAACAGAAAAACCAGTGATTGCAACACCATCTGCTACCAAAAATAAAGC 60
SnRAR1          AGGAAAGCACACAACAGAAAAACCAGTGATTGCAAAGCCACCTATTACCATAAATAAAGC 60
NtRAR1          AGGAAAGCACACAACAGAAAAACCAGTGATAGCAAATCCAGCTGCTAACCGGAATAGAGC 60
                ****** ********************** ****  *** **  ** *   **** ***

StRAR1          AATTCCTGTGCCGGTGAGTGTGCCAGCTCCTATGACCAATGCATCACCGAAAGAAGCCTG 120
LeRAR1          AGTTCCTGTGCCAGTGCGTGTGCCAGCTCCTATGACCAATGCATCACCGAAAGAAGCCTG 120
SnRAR1          AATTCATGCGCCTGCGCCTGTGGCAGCTCCTATGACCAATGCATCAGCGAAAGAAGCCTG 120
NtRAR1          AATTCCTGCACC------------AACTTCTACAACCAATGTATCACCGAAAGAAGCTTG 108
                * *** **  **            * ** ***  ******* **** ********** **

StRAR1          TCCTAGATGCCACCAGGGATTCTTTTGTTCTGATCATGGTTCACAACCCAGAGAAGCAAT 180
LeRAR1          TTCTAGATGCCACCAGGGATTCTTTTGTTCTGATCATGGTTCACGACCCAGAGAAGCAAT 180
SnRAR1          TCCTAGATGCCGCCAGGGATTTTTTTGTTCTGATCATGGTTCACAACCCAGGGAAGCAAT 180
NtRAR1          TCCTAGATGCCGCCAGGGATTCTTTTGTTCTGATCACGGTTCACAACCCAGAGAAGTAAT 168
                * ********* ********* ************** ******* ****** **** ***

StRAR1          TCCAAAAGCATCAAATACAGTAGCATCTGTACCTTCTGGGAGCAATACAGTTGTACAGCA 240
LeRAR1          TCCAAAAGCATCAAATACAGTAACATCTCTACCTTCTGAGAGCAATACAGTTGTACAGCA 240
SnRAR1          TCCGAAAGCATCAAATACAGTAACATCTGTACCTTCTGAGAGCAATACAGATGTAAAGCA 240
NtRAR1          TCCAAAAGCATCAAATACAGTAACATCTGTACCTTCTGAGAGCAATACAGATGTACAGCA 228
                *** ****************** ***** ********* *********** **** ****

StRAR1          AGACCATCCAGCTCCAGTGAAGAAGAAAATTGATATAAATGAGCCCCAAATTTGTAAAAA 300
LeRAR1          AGACCATCCAGCTCCAGTGAAAAAGAAAATTGATATAAATGAGCCCCAAATTTGTAAAAA 300
SnRAR1          AGACCATCCGGCCCCAGTGAAGAAGAAAATTGATATTAACGAGCCTCAAATTTGTAAAAA 300
NtRAR1          ATGCCATCCTGCTCCGGTGAAGAAGAAAGTTGATATAAACGAACCCCAAATTTGTAAAAA 288
                *  ****** ** ** ***** ****** ******* ** ** ** **************

StRAR1          CAAGAGCTGCAGTAAGACCTTTACAGAAAAGGAAAATCACGACACTGCTTGTAGTTACCA 360
LeRAR1          CAAGAGCTGCGGTAAGACCTTTACAGAAAAGGAAAATCACGACACTGCTTGTAATTACCA 360
SnRAR1          TAAGGGCTGTGGTAAGACCTTTAAAGAAAAGGAAAATCATGACACTGCTTGCAGTTACCA 360
NtRAR1          TAAGGGCTGTGGTAAGACCTTTACAGAAAAGGAAAATCATGACGCTGCTTGCAGTTACCA 348
                 *** ****  ************ *************** *** ******* * ******

StRAR1          TCCTGGCCCTGCTATCTTCCATGACCGGATGAAAGGATGGAAATGCTGTGATGTTCATGT 420
LeRAR1          TCCCGGCCCCGCTATCTTCCATGACCGGATGAAAGGATGGAAATGCTGTGATGTTCATGT 420
SnRAR1          TCCTGGCCCCGCTATCTTCCATGACCGGATGAGAGGATGGAAATGCTGTGATATTCACGT 420
NtRAR1          TCCTGGCCCCGCTATCTTCCATGATCGGATGAGAGGATGGAAATGCTGTGATATTCATGT 408
                *** ***** ************** ******* ******************* **** **

StRAR1          CAACGAATTTGATGAGTTCATGACCATACCGCCATGCACCAAAGGATGGCAC 472
LeRAR1          CAAAGAATTTGACGAGTTCATGACCATACCTCCATGCACCAAAGGATGGCAC 472
SnRAR1          CAAAGAATTTGACGAGTTCATGAGCATACCTCCCTGCACCAAAGGATGGCAC 472
NtRAR1          CAAAGAATTTGATGAGTTCATGAGCATATCGCCATGCACCACAGGATGGCAC 460
                *** ******** ********** **** * ** ******* **********
```

Blue colours indicate primer sequences

Sequences of *EDS1* and *RAR1* from *Solanum nigrum* showed more than 90% identity to *EDS1* and *RAR1* from other Solanaceae spieces such as tomato, tobacco, potato and *Nicotiana benthamiana*, but only 65% for *NDR1*.

3.3.5.2 Subcloning genes from entry clones into pTRV2 gateway

The fragment of the candidate genes which are flanked between *att*L1 and *att*L2 in pENTR11Amp (entry vector) were transferred into pTRV2 (destination vector) that contain compatible recombination sites *att*R1 and *att*R2 in a reaction mediated by the Gateway™ LR Clonase™ Enzyme Mix (Invitrogen).

The transfers of the attL-flanked PCR products to the destination vector pTRV-RNA2 Gateway by LR clonase were essentially carried out according to the methods described previously and after transformation into DH10B, some colonies were grown on Kanamycin plates. The resulted expression clones had the same size as pTRV2 control (Figure 29).

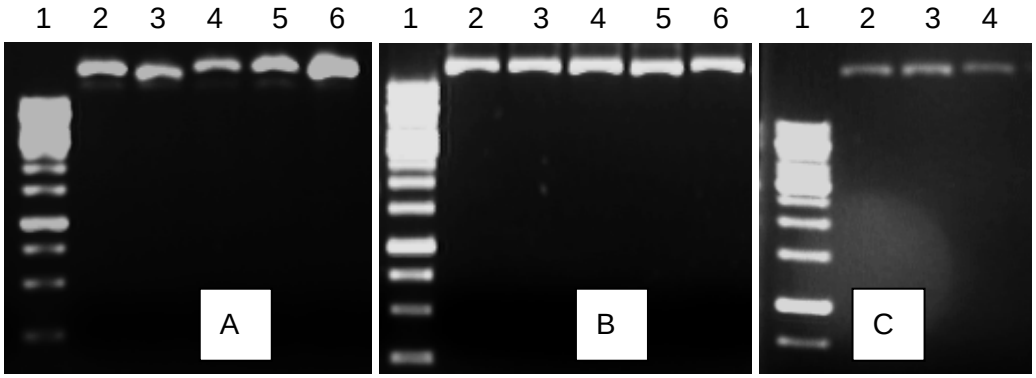


Figure 29: Expression clones isolated from *E. coli* DH10B

(A)- TRV-RNA2-Gateway-*Eds1* plasmids (number 2 – 5: 4 expression clones) from E.coli (the last one is TRV control), (B)- TRV-RNA2-Gateway-*Ndr1* plasmids (number 2 – 5: 4 expression clones) from E.coli (the last one is TRV control) E.coli, (C)- TRV-RNA2-Gateway-Rar1 plasmids (number 2 and 3: 2 expression clones) from E.coli (the last one is TRV control and number 1 is DNA ladder 1 kb).

- **Analysis of positive transformants using PCR and sequencing**

Inserts-containing clones were verified by PCR using *EDS1, NDR1* and *RAR1* specific primers. The resulting fragments were electrophoresed and the results confirmed the presence of the inserts (Figure.30).

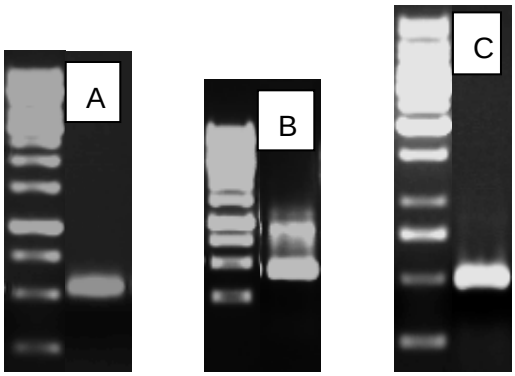


Figure 30: PCR products from expression clones using *Eds1, Ndr1* and *Rar1* primers

(A)- *EDS1;* (DNA ladder 1kb), (B)- *NDR1*, (C)- *RAR1*

These expression clones were introduced into the *A. tumefaciens* strain GV3101 for subsequent use in silencing the target genes.

3.3.5.3 Transformation into *Agrobacterium*

All three constructs (pTRV-*Eds1*, pTRV-*Ndr1* and pTRV-*Rar1*) were introduced into the GV3101 strain of *A. tumefaciens* following the protocol described in material and methods (subsection number 2.9.3). To verify whether *A. tumefaciens* were transformed, plasmids from some colonies were isolated using QIAprep Spin Miniprep Kit and checked by PCR using the specific primers for *Eds*1, *Ndr*1 and *Rar*1. The fragment size of each DNA on gel electrophoresis (Figure 31) proved that pTRV-*Eds*1, pTRV-*Ndr*1 and pTRV-*Rar*1 were successfully introduced into GV3101.

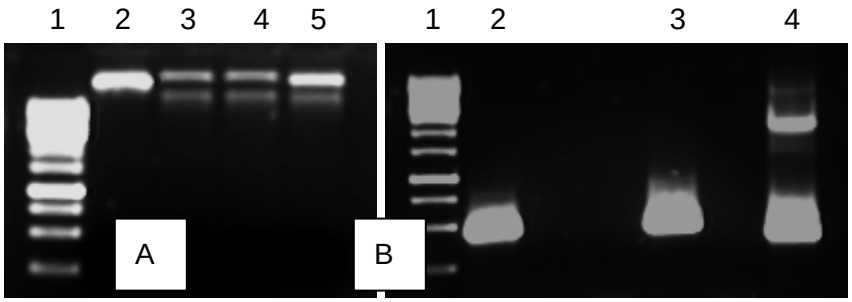


Figure 31: Results from *Agrobacterium* transformation

(A)- Plasmids isolated from GV3101; (1)- DNA ladder 1 kb, (2)- pTRV2 control, (3) pTRV2-*Ndr1*, (4)- pTRV2-*Rar1* (5)- pTRV2-*Eds1*, (B)- PCR products of expression clones from GV3101; (2)- *Eds1*, (3)- *Ndr1*, (4)- *Rar1*

3.3.6 Silencing of genes for R-gene signal transduction in *Solanum nigrum*

A. tumefaciens GV3101 containing pTRV2-*Eds1*, pTRV2-*Ndr1* and pTRV2-*Rar1* were infiltrated into *S. nigrum* plants through vacuum infiltration. As a negative control, similar plants were inoculated with pTRV-empty vector and with pTRV-*PDS* as a visual indicator of gene silencing. Infiltrated plants were incubated at 16°C with 24h light per day. Each experiment included 15 independent plants.

3.4 Solanum nigrum susceptibility tests with *Phytophthora infestans*

3.4.1 Effect of VIGS at mRNA level:

VIGS is associated with a reduction in the level of the targeted mRNA (Peart *et al*., 2002). I therefore carried out RNA extraction to check whether pTRV-*Eds1*, pTRV-*Ndr1*, and pTRV-*Rar1* caused a reduction of *SnEds1*, *SnNdr1*, and *SnRar1* mRNA, respectively. Total RNA was harvested from the youngest leaves of silenced and non-silenced plants when leaves treated with pTRV-*PDS* turned white. Reverse Transcriptase RT-PCR was performed to prepare cDNA. Gene-specific primers for *Eds1, Ndr1*, and *Rar1* (Table 3) were used to amplify the respective genes and PCR was performed for 21,24,27,30, 33 and 35 cycles. Control PCR using template derived RNA without reverse transcription was performed for 30 cycles. The results from the electrophoresis gel of this control (Figure 32) showed that the DNA from the leaves of each one of them was completely digested. The analyse of the resulting PCR products by agarose gel electrophoresis showed that the amount of *EDS1, NDR1*, and *RAR1* PCR products obtained from inoculated leaves were not lower than those from uninoculated control (Figure 33).

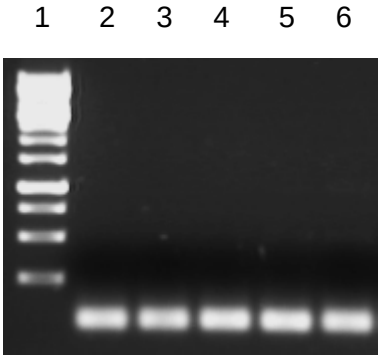


Figure 32: PCR products from RNA of inoculated *S. nigrum* plants

(1)- DNA ladder 1kb, (2)- uninfiltrated plant, (3)- pTRV2-*Eds1*, (4)- pTRV2-*Ndr1,* (5) - pTRV2-*Rar1*, and (6)- p-TRV2-empty.

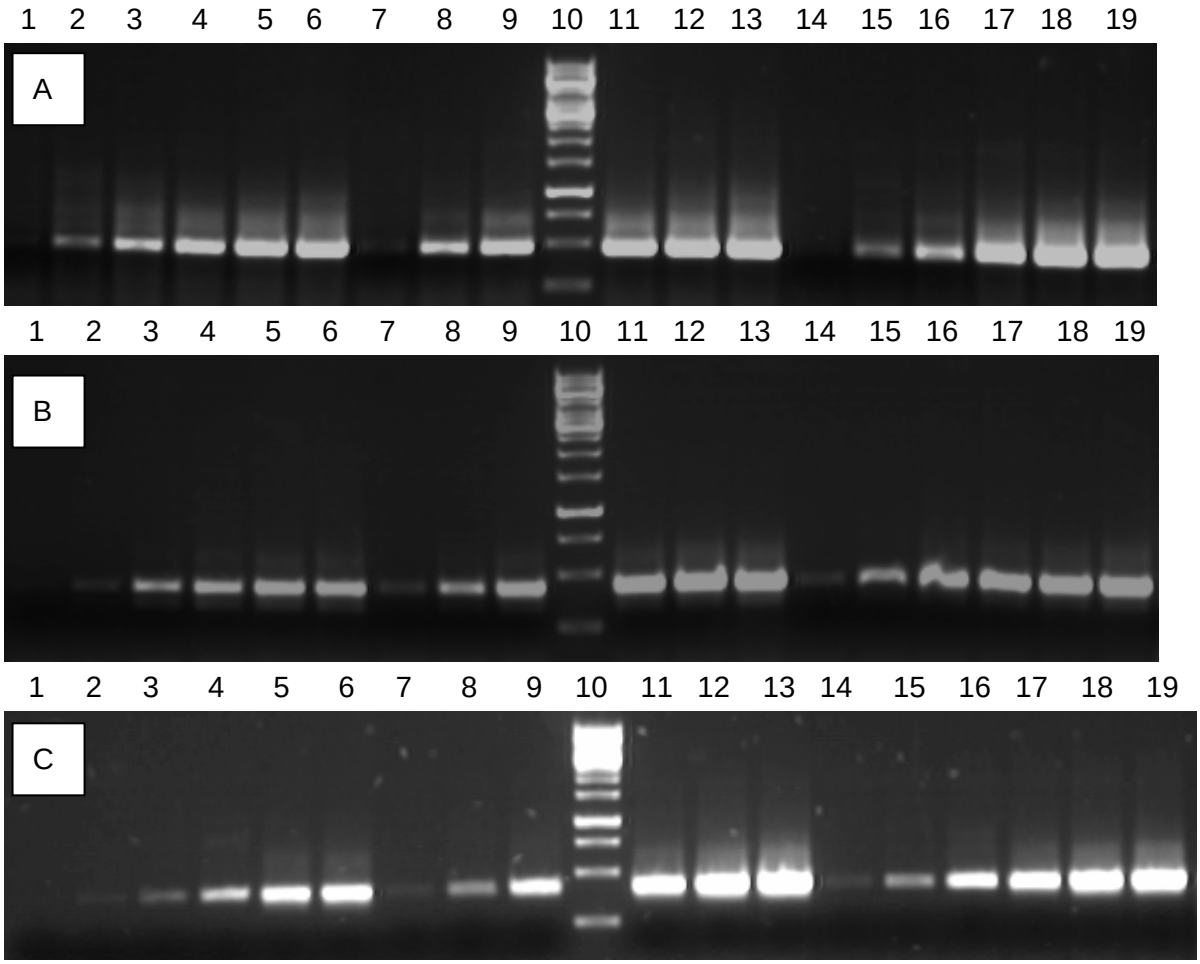


Figure 33: PCR products from cDNA of inoculated *S. nigrum* plants

(A7-9 and A11-13)- pTRV2-*Eds1,* (B7-9 and B11-13)- pTRV2-*Ndr1* and (C7-9 and C11-13)- pTRV2-*Rar1*. The numbers 1 to 6 for A-B-C are uninfected plants and the number 14 to 19 are p-TRV2-empty-infected plants. All number 10 are the DNA ladder 1 kb.

3.4.2 Phenotype effect of VIGS:

The plants were challenged with *P. infestans* zoospores as well when the leaves of pTRV-*PDS* infected plants turned white. I would not expected any visible phenotype changes, but surprisingly, seven days after *Phytophthora* infection (APi0_2, T04), I found lesions on the leaves (Figure 34 A, B) of some plants inoculated with pTRV-*Eds1* and pTRV-*Rar1*, whereas the plants infiltrated with pTRV-*Ndr1* as well as pTRV-empty exhibited normal development and morphology (Figure 34C, D) but the potato plants were dead. This phenotype was consistently seen either in the APi02- or T04-infected plants but the number of plants that exhibited lesions was higher with T04. About 34% (Figure 35) of the pTRV-*Eds1*-infiltrated plants were showing lesions on their leaves after infection with T04, but only about 14% after APi02 infection (Figure 36). In addition, there were much more pTRV-*Eds1* infiltrated-plants showing lesions on the leaves than pTRV-*Rar1* plants (Figure 35, 36) in

both cases (infection with T04 and APi02). The statistical analysis (see subsections number 3.4.3.1 and 3.4.3.2) proved that the result obtained from pTRV- *Eds1* infiltrated-plants after T04 infection was significant compared to the controls (Table 11), whereas those from pTRV- *Rar1* infiltrated-plants - T04, pTRV- *Eds1* infiltrated-plants – Api02 and pTRV- *Rar1* infiltrated-plants – Api02 were not significant (Table 12).

Figure 34: *S. nigrum* plants inoculated with pTRV2 carrying the candidate genes

(A)- pTRV2-*Eds1,* (B)-pTRV2-*Rar1*, (C)- pTRV2-*Ndr1*, and (D)- pTRV2-Empty vector.

Table 8: Number of leaves and plants showing lesions after T04 infection:

Sets	Control	pTRV:*Eds1*	pTRV:*Ndr1*	pTRV:*Rar1*	pTRV:Empty
Total number of infiltrated plants	15	15	15	15	15
Number (Nb) plants with lesions	0	5	0	2	0
Nb plants without lesions	15	10	15	13	15
Nb leaves with lesions Plant1	0	3	0	2	0
Nb leaves with lesions Plant2	0	2	0	1	0
Nb leaves with lesions Plant3	0	1	0	0	0
Nb leaves with lesions Plant4	0	1	0	0	0
Nb leaves with lesions Plant5	0	1	0	0	0
Nb leaves with lesions Plant6	0	0	0	0	0
Nb leaves with lesions Plant7	0	0	0	0	0
Nb leaves with lesions Plant8	0	0	0	0	0
Nb leaves with lesions Plant9	0	0	0	0	0
Nb leaves with lesions Plant10	0	0	0	0	0
Nb leaves with lesions Plant11	0	0	0	0	0
Nb leaves with lesions Plant12	0	0	0	0	0
Nb leaves with lesions Plant13	0	0	0	0	0
Nb leaves with lesions Plant14	0	0	0	0	0
Nb leaves with lesions Plant15	0	0	0	0	0

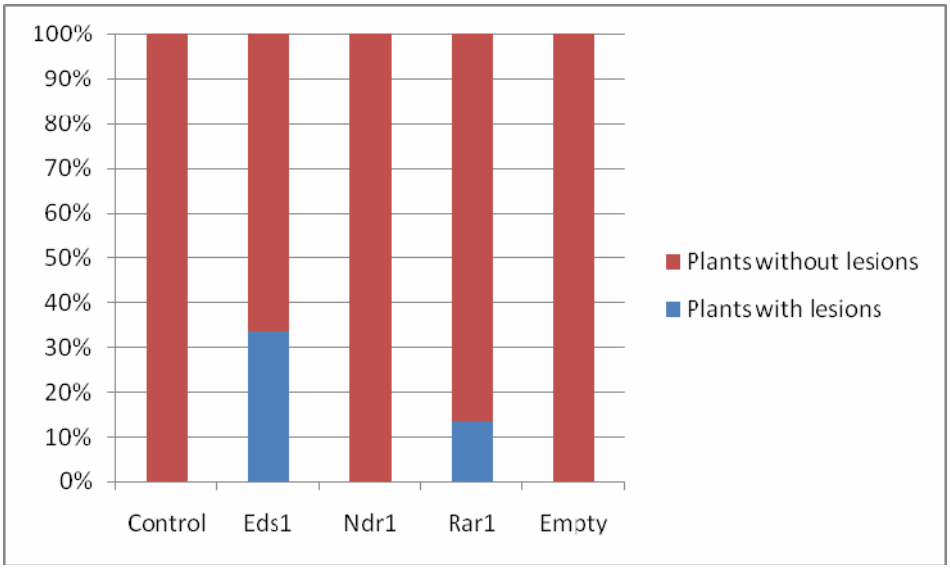


Figure 35: Percentage of plants having lesions on their leaves after infection with T04.

Table 9: Number of leaves and plants showing lesions after APi02 infections:

Sets	Control	pTRV:*Eds1*	pTRV:*Ndr1*	pTRV:*Rar1*	pTRV:Empty
Total number of infiltrated plants	15	15	15	15	15
Nb plants with lesions	0	2	0	1	0
Nb plants without lesions	15	13	15	14	15
Nb leaves with lesions Plant1	0	2	0	1	0
Nb leaves with lesions Plant2	0	1	0	0	0
Nb leaves with lesions Plant3	0	0	0	0	0
Nb leaves with lesions Plant4	0	0	0	0	0
Nb leaves with lesions Plant5	0	0	0	0	0
Nb leaves with lesions Plant6	0	0	0	0	0
Nb leaves with lesions Plant7	0	0	0	0	0
Nb leaves with lesions Plant8	0	0	0	0	0
Nb leaves with lesions Plant9	0	0	0	0	0
Nb leaves with lesions Plant10	0	0	0	0	0
Nb leaves with lesions Plant11	0	0	0	0	0
Nb leaves with lesions Plant12	0	0	0	0	0
Nb leaves with lesions Plant13	0	0	0	0	0
Nb leaves with lesions Plant14	0	0	0	0	0
Nb leaves with lesions Plant15	0	0	0	0	0

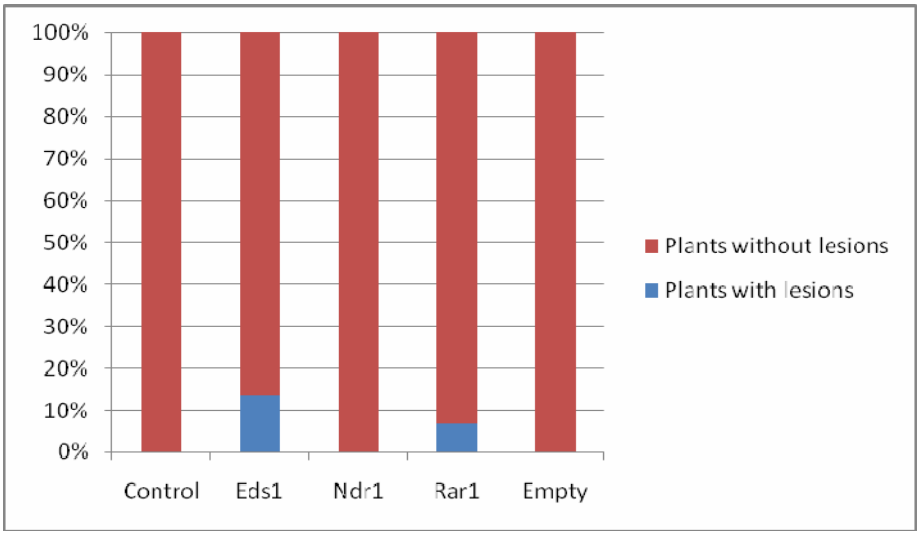


Figure 36: Percentage of plants having lesions on their leaves after infection with APi02.

3.4.3 Statistical analysis:

Analysis of lesions per plant in general (without considering any kind of infiltration, silenced with pTRV-*Eds1* or *Ndr1* or *Rar1*) using Kolmogorov-Smirnov test:

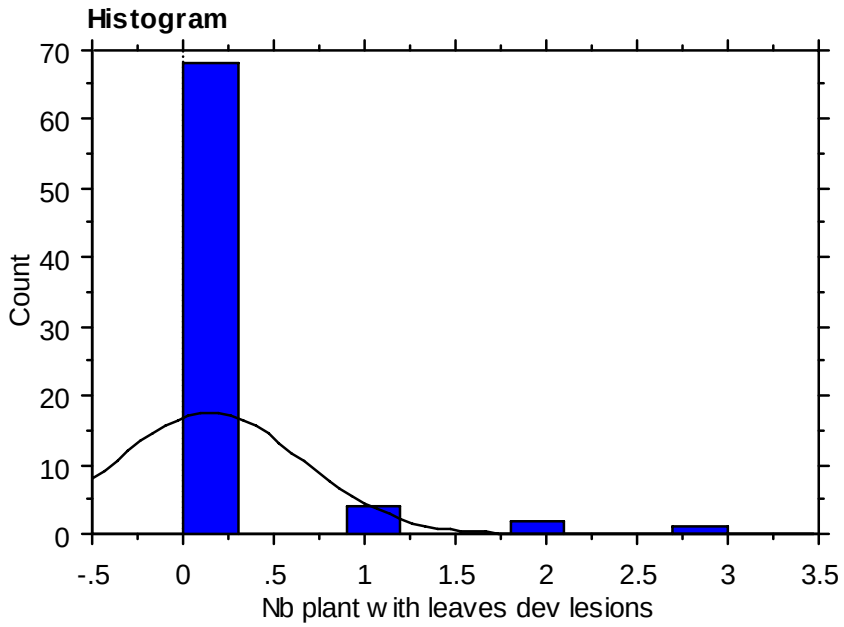


Figure 37: Distribution of the samples.

Descriptive Statistics

	Nb plant w ith leaves dev lesions
Mean	.147
Std. Dev.	.512
Std. Error	.059
Count	75
Minimum	0.000
Maximum	3.000
# Missing	0
Variance	.262
Coef. Var.	3.490
Range	3.000
Sum	11.000
Sum Squares	21.000
Geom. Mean	•
Harm. Mean	•
Skew ness	3.879
Kurtosis	15.365
Median	0.000
IQR	0.000
Mode	0.000
10% Tr. Mean	0.000
MAD	0.000

The mean is 0.147 and the median is 1.5. If the distribution of the samples is symetric, the mean (the point on the horizontal axis where the distribution would balance) and the

median (that value on the horizontal axis which has exactly 50% of the data to the left and also to the right) should be nearly equal. Here the mean is skewed to the left, the mean and median are not the same. This result indicates that the distribution of the samples is not symetric. Therefore, the parametric test such as ANOVA or T-test cannot be used in this case. Statistical analysis should be made with non-parametric tests and Kruskal-Wallis and Mann-Whitney tests were used to analyse these data. The Kruskal-Wallis test is a nonparametric test used to compare three or more samples. It is the analogue to the F-test used in analyses of variance. While analysis of variance tests depend on the assumption that all populations under comparison are normally distributed, the Kruskal-Wallis test places no such restriction on the comparison. It is a logical extension of the Mann-Whitney test. Mann-Whitney Test is one of the most powerful of the nonparametric tests for comparing only two populations.

3.4.3.1 Kruskal-Wallis test

Table 10: Number of leaves with lesions from 15 plants infected with T04

Plant number	Control	pTRV-*Eds1*	pTRV-*Ndr1*	pTRV-*Rar1*	pTRV-Empty
Plant1	0	3	0	2	0
Plant2	0	2	0	1	0
Plant3	0	1	0	0	0
Plant4	0	1	0	0	0
Plant5	0	1	0	0	0
Plant6	0	0	0	0	0
Plant7	0	0	0	0	0
Plant8	0	0	0	0	0
Plant9	0	0	0	0	0
Plant10	0	0	0	0	0
Plant11	0	0	0	0	0
Plant12	0	0	0	0	0
Plant13	0	0	0	0	0
Plant14	0	0	0	0	0
Plant15	0	0	0	0	0

Test Statistics

	N_LESION
Chi-Square	14,887
df	4
Asymp. Sig.	**,005**

a Kruskal Wallis Test

b Grouping Variable: GENE

This result indicated that there is a very high significant differences between the five variables which are the control, pTRV-*Eds1*, pTRV-*Rar1*, pTRV-*Ndr1* and pTRV-empty (the level of significance is 0.5%). The results from Mann-Whitney test below proved that this very high significant differences is due to pTRV-*Eds1* as there is significant difference between pTRV-*Eds1* and controls (the level of significance is 1.6%, see Table 11) but no significant difference between pTRV-*Rar1* and controls (the level of significance is 15%, see Table 12).

3.4.3.2 Mann-Whitney test

Table 11: Comparison between pTRV-*Eds1* and controls :

Test Statistics

	N_LESION
Mann-Whitney U	75,000
Wilcoxon W	195,000
Z	-2,398
Asymp. Sig. (2-tailed)	**,016**
Exact Sig. [2*(1-tailed Sig.)]	,126

a Not corrected for ties.
b Grouping Variable: GENE

Table 12: Comparison between pTRV-*Rar1* and controls:

Test Statistics

	N_LESION
Mann-Whitney U	97,500
Wilcoxon W	217,500
Z	-1,438
Asymp. Sig. (2-tailed)	**,150**
Exact Sig. [2*(1-tailed Sig.)]	,539

a Not corrected for ties.
b Grouping Variable: GENE

Similarly, there is no significant difference between pTRV-*Eds1* and controls as well as pTRV-*Rar1* and controls for the plants infected with Api02.

4 Discussion

4.1 Screening of *Solanum nigrum* accessions for *Phytophthora infestans* tests

S. nigrum was regarded as a non-host for *P. infestans* at the beginning of this project (Pieterse *et al.,* 1994; Platt, 1999; Vleeshouwers *et al*., 2000). A nationwide late blight survey aimed at characterizing early outbreaks of late blight in the Netherlands from 1999 to the present has repeatedly confirmed the presence of sporulating lesions of *P. infestans* on *S. nigrum* (Flier *et al.,* 2003). The first European report on the incidence of *P. infestans* on *S. nigrum* dates back to 1950s (Hirst and Stedman, 1960) but confirmed records of field infections are scarce (Flier *et al.,* 2003). The present population of *P. infestans* in the Netherlands is pathogenic on *S. nigrum,* oospores were produced on the leaves following infection with A1 and A2 isolates (Flier *et al.*, 2003). However, sporulation density is higher on potato, tomato and *S. dulcamara* compared to *S. nigrum* and observations of blighted *S. nigrum* and *S. dulcamara* plants in the field remain very rare (Flier *et al*., 2003).

Based on the presence of field infections and results obtained in detached leaf inoculation studies, the present status of *S. nigrum* as a non-host plant for *P. infestans* needs to be reconsidered.

For this reason, I have screened twenty *S. nigrum* accessions for responses against four *P. infestans* isolates. These isolates sporulated well on the susceptible host potato désirée but hardly at all on the *S. nigrum* accessions. The hyphae in the susceptible cultivar have penetrated deeper into the tissue and the aerial hyphae form a thin mat on the surface of the host. All twenty *S. nigrum* accessions responded to *P. infestans* infection by inducing a rapid cell death, characteristic of HR, in epidermal cells. This phenomenon occurred with all of the four *P. infestans* isolates used in this study and most of them are macroscopically invisible.

In the case of the *T04* isolate, penetration and mycelial development occurred in some *S. nigrum* accessions and necrosis of invaded and adjacent cells can be seen macroscopically as black spots, but there was no sporulation. Cells were activated after penetration by the pathogen. The HR is induced and the pathogen is contained within a group of dead plant cells. This might be due to the delay of its response to the pathogen; the hyphae penetrate the tissue beyond the cells of initial infection before the HR occurs. Colon and associates (1993) have already reported that penetration and limited intercellular growth of *P. infestans* may occur in *S. nigrum* leaves. These results support the designation of *S. nigrum* as non-host of *P. infestans*.

This is in accordance with Niks (1987), who defines a non-host as a plant species of which most genotypes cannot be infected by most genotypes of the pathogen. It is also

confirmed here that non-host resistance of *S. nigrum* to *P. infestans* is associated with the HR. HR was restricted to the epidermis. In many non-host plants, the HR is induced extremely fast, and only one or two plant cells are sacrificed (Colon et al., 1993). Previously, responses in plants exhibiting race-specific and race-nonspecific resistance to *P. infestans* were reported to be similar (Wilson and Coffey, 1980). The HR is not unique to specific resistance but is a general expression of highly incompatible interactions (Gees and Hohl, 1987). During the HR a conserved programmed cell death (PCD) mechanism is activated (Heath, 1998). Vleeshouwers and associates (2000) have hypothesized that PCD is activated during the HR in the *P. infestans* - *Solanum* interaction. This hypothesis is confirmed with the *P. infestans* isolates and *S. nigrum* accessions that I have used in this study.

Hypersensitive cell death is usually accompanied by the accumulation of fluorescent phenylpropanoid derivatives around the pathogen infection sites (Dorey et al., 1997; Zhang *et al*., 2004). The infected *S. nigrum* accessions in this experiment also showed autofluorescing under UV. Vleeshouwers *et al.,* (2000) reported that autofluorescing compounds were found to accumulate during *P. infestans* - *S. nigrum* and *P. infestans* - *Arabidopsis* interactions. *Phytophthora* produces glucanases which are essential for maintaining hyphal tip growth. The resistant host produces glucanase inhibitors and it has been suggested that the phytoalexins produced in response to *P. infestans* have such an activity (Kuc 1972) and would inhibit growth of the *P. infestans* within the plant tissue (Hohl and Stössel, 1975).

4.2 *Solanum nigrum* tests with *Phytophthora infestans* elicitins

The HR that was induced in the interaction of *S. nigrum* with *P. infestans* might be due to elicitin recognition as in the case of *Nicotiana spp – P. infestans* interactions. To determine whether this is the case, all twenty accessions were tested with elicitin produced from *P. infestans* isolates (Api02, LL8, SN001a and T04).

Recognition of elicitin is thought to be one component of resistance of *Nicotiana spp*. to *P. infestans*. In 1993, Kamoun and associates have already reported that elicitins, secreted abundantly by *P. infestans* species, induce the HR in non-host plant species of the genus *Nicotiana*. Also Kamoun and associates (1998) have found that *P. infestans* strains deficient in the elicitin *INF1* induced disease lesions on the wild tobacco species *N. benthamiana* and they suggested that *INF1* conditions resistance in this plant species. In contrast, *INF1*-deficient strains remained unable to infect other *Nicotiana spp*. such as tobacco. In this case, tobacco was hypothesized to react to additional elicitors, perhaps other elicitin-like proteins (Kamoun 2001; Kamoun et al., 1998b, 1999c).

In this study, I tested the response of *N. tabacum*, *N. benthamiana* and twenty *S. nigrum* accessions to *P. infestans* elicitins. *N. tabacum* and *N. benthamiana* leaves showed necrosis but *S. nigrum* leaves did not have any reaction. The presence of necrosis on *Nicotiana* plants confirmed Kamoun's finding that elicitins produced by *P. infestans* induce HR in non-host plants species of the genus *Nicotiana* (Kamoun, 1993). The absence of necrosis in all *S. nigrum* plants indicated that this species does not respond to elicitins. Therefore, elicitin was not responsible for the HR induced in *S. nigrum* during the *S. nigrum* - *P. infestans* interaction.

Since the hypersensitive response of *S. nigrum* was not due to elicitin recognition, an attempt to silence genes for *R*-gene signal transduction of *S. nigrum* was done. Candidate genes were amplified from *S. nigrum* genomic DNA and cDNA, cloned into the *pTRV-RNA*2 gateway vector and introduced into *S. nigrum* plants by *Agrobacterium*-mediated infiltration in order to silence the endogenous genes. These candidate genes have been chosen from public databases. *Eds1* (enhanced disease susceptibility) (Parker *et al*., 1996)*, Ndr1* (non-race-specific disease resistance) (Century *et al.*, 1997)*,* and *Rar1* (homolog of a barley gene required for *Mla* powdery mildew resistance) (Muskett et al., 2002; Tornero *et al*., 2002) have been chosen.I In many cases, LRR-containing resistance genes have been found to be dependent on either *Eds1* in the case of TIR-NB-LRR genes or on *Ndr1* in the case of CC-NB-LRR genes. *Rar1* is required for the function of both TIR-NB-LRR and CC-NB-LRR subclasses. If these genes are required for disease resistance against *P. infestans*, the virus vector-infected *S. nigrum* plants would become partially or completely susceptible to the pathogen.

4.3 Virus Induced Gene Silencing (VIGS) to test the resistance of *Solanum nigrum*

The most established technologies used for loss-of-function studies in plants are chemical mutagenesis and the use of transposons or *Agrobacterium* T-DNA insertions to create disruptions of coding sequences. These technologies have been widely and successfully used and are still the method of choice for the model plant *Arabidopsis*. There are, however, a few important points to consider that limit the adoption of these approaches in other plant species. First, these techniques require the generation of large populations to screen for mutations in a gene of interest. Even with new technologies such as Targeting Induced Local Lesions In Genomes (TILLING) (reviewed in Henikoff and Comai, 2003), many mutations are missed and the function of the gene under study remains unknown. To isolate the desired mutation away from other unlinked mutations it is often necessary to perform several backcrosses, a process that can be labourious and time-consuming. VIGS, which avoids most of these difficulties, can therefore be seen as a complementary approach

to traditional approaches. A significant advantage of VIGS as a functional genomics tool is that it can identify a loss-of-function phenotype for a specific gene within a short time. Because the gene of interest is directly targeted in VIGS; there is no need for screening large populations to identify a mutation in a specific gene. In a forward genetics VIGS screen, only a single plant is needed to identify a phenotype and an initial result can be rapidly scaled up and repeated. Also, the gene responsible for an interesting phenotype can be quickly sequenced from the VIGS vector and identified. VIGS avoid plant transformation. VIGS is a transient method that does not rely on the generation of transgenic plants, a procedure that is difficult in many plant species (Liu et al., 2004b).

With a virus vector carrying a fragment of a host gene, the VIGS is targeted against the corresponding host mRNA (Baulcombe, 1999). In this study, *Eds1, Ndr1*, and *Rar1* were successfully cloned from *Solanum nigrum* genomic DNA (*Eds1*) and cDNA (*Ndr1* and *Rar1*) into pTRV-RNA2 gateway and inoculated into *S. nigrum* plants through Agroinfiltration.

pTRV2-*PDS* was also infiltrated into *S. nigrum* plants for two reasons; the first reason was to test whether the TRV vector can efficiently replicate and spread systemically in *Solanum nigrum* plants. The obtained results validated the use of VIGS for identification of genes required for disease resistance in this species because the inoculation of pTRV2-*PDS* has successfully silenced the *SnPDS*, i.e. the *S. nigrum* leaves were photobleached as the *PDS* gene encodes an enzyme involved in carotenoid biosynthesis and silencing of this gene results in photobleaching of plant leaves (Liu et al., 2002a). The leaves turn white due to lack of carotenoids and destruction of chlorophyll by photo-oxidization (Kumigai et al., 1995). The second reason was to use it in parallel with the candidate genes to be referred as a visual indicator of gene silencing and as a positive control for TRV- induced gene silencing.

4.3.1 *Eds1*

Eds1 encodes an essential component of disease resistance conferred by a subset of *R* -genes that condition resistance to bacterial and oomycete pathogens (Falk *et al.,* 1999). The *Eds1* protein is likely to operate within a convergent pathway that is modulated through specific *R-Avr* protein recognition (Falk *et al*., 1999). *Eds1* encodes a lipase-like protein (Falk *et al.* 1999; Jirage *et al.* 1999) and functions within the defence pathways that regulates salicylic acid (SA) accumulation (Feys *et al.* 2001; Zhou *et al.* 1998). Rusterucci and associates (2001) reinforce the observations reported by Feys and associates (2001) that *Eds1* has a role in initiation of localized HR, and in potentiation of early defence signals (Borhan *et al*., 2004).

In *Arabodopsis, Eds1* is critical for disease resistance conferred by TIR-NB-LRR, as mutations in *Eds1* result in enhanced disease susceptibility to virulent pathogens (*Pseudomonas syringae* and *Peronospora parasitica*) (Peart *et al*., 2002). Liu and associates

(2002) reported that *Eds1* functions in resistance pathways in the Solanaceous species as well. For instance, resistance against tobacco mosaic virus mediated by the tobacco N gene, encoding a TIR-NB-LRR protein, was *Eds1*-dependent (Peart *et al*., 2002). Also, *LeEds1* is important for basal defenses in tomato. However, no enhanced disease susceptibility was detected in the *Eds1*-silenced *Nicotiana benthamiana* (Peart *et al*., 2002; Gongshe *et al*., 2005). It appears from the VIGS results in *N. benthamiana* that *NbEds1* is not required to limit colonisation by the virulent pathogens PVX, TMV and *P. syringae tabaci*. This also seems to be the case in *S. nigrum*; silencing of *Eds1 in S. nigrum* plants did not enhance disease susceptibility. There was no reduction of the level of *SnEds1*mRNA but surprisingly some plants developed large necrosis on their leaves. However, there was no sporulation of *P. infestans* observed on these lesions. It may be, that, although in the RT-PCR assay no reduction of *SnEds1* transcripts was detected, there was still a partial reduction in *SnEds1* transcript levels in some parts of the plants which led to increased susceptibility to *P. infestans*. Alternatively, *S. nigrum* being a hexaploid, might contain genes similar to *SnEds1* which were partly silenced but not detected in the RT-PCR assay. Further experiments are needed to adress this phenomenon.

SnEds1 possibly interacts with *PAD4* (Phytoalexin Deficient) (Borhan *et al*., 2004). Both *Eds1* and *Pad4* encode lipase-like proteins (Falk et al., 1999; Jirage et al., 1999) and function within the same defence pathway (Feys *et al*., 2001; Zhou *et al*., 1998). Feys and associates (2001) showed that the *Eds1* and *Pad4* proteins interact directly with each other and both affect the resistance response mediated by the same spectrum of *R*-gene (Borhan *et al*., 2004). *Eds1* and *Pad4*, together, are required for salicylic acid (SA) accumulation and for defence potentiation involving the processing of ROI-derived signals around infection foci. SA itself contributes to the expression of both *Eds1* and *Pad4* as part of a positive feedback loop that appears to be important in defence amplification. In TIR-NB-LRR-protein-triggered responses, *EDS1* and its partners are needed for expression of hypersensitive plant cell death (Wiermer *et al*., 2005). Certain non-TIR-NB-LRR type R proteins such as *RPW8* and *HRT* have been shown to require *Eds1* and *Pad4* for full resistance, possibly reflecting their requirement for SA-pathway amplification (Wiemer et al., 2005). Besides, *Arabidopsis Eds1*, *Pad4* and a newly identified *in vivo Eds1*-interactor, *SAG101* (Senescence Associated Gene 101)*,* constitute a regulatory mode that couples *R*-protein-mediated pathogen recognition to activation of basal defences (Wiemer et al., 2005). Lipka *et al.,* (2005) have also shown that the combined *Pad4* and *SAG101* contribute to postinvasion nonhost resistance that greatly exceeds those of the single components *eds1*, *pad4,* or *sag101* in nonhost resistance to the grass powdery mildew fungus (*Blumeria graminis* hordei). It is possible that silencing all three genes (*Eds1*, *Pad4,* and *SAG101*) might lead to pronounced susceptibility.

4.3.2 Ndr1

In contrast to *Eds1*, *Ndr1* is required by non-TIR-containing *R* proteins (Aarts et al., 1998). Peart *et al.* (2002) reported that *Ndr1* is required for disease resistance specified by CC-NBS-LRR proteins encoded by *Rpm1*, *Rps2* and *Rps5* but *Eds1* mediates resistance conferred by the TIR-NB-LRR proteins encoded by *Rpp1*, *Rpp2*, *Rpp4*, *Rpp5*, and *Rps4* and it was suggested that *R* proteins of the CC-NBS-LRR class signal through *Ndr1* while the TIR-NB-LRR class of *R* proteins utilize *Eds1* (Aarts *et al*., 1998; McDowell *et al*., 2000; Peart *et al*., 2002). Yun *et al*. (2003); Zimmerli *et al*. (2002); and Gongshe et al. (2005) confirmed that *Eds1* is generally necessary for resistance genes conferred by TIR-NBS_LRR proteins, whereas *Ndr1* functions in concert with members of the CC-NBS-LRR subclass. In Arabidopsis, three genes, *Eds1, Pad4*, and *Ndr1,* have been identified as important components of the NBS-LRR R gene-mediated response to specific pathogens but *Eds1* and *Pad4* are typically required for resistance mediated by different set of R genes than is *Ndr1*. *Eds1* additionally functions in non-host resistance (Yun *et al*., 2003; Zimmerli *et al*., 2002; Gongshe *et al*., 2005). In this study, silencing of *SnNdr1* did not cause any reduction in the level of *SnNdr1* mRNA or visible phenotype changes of *S. nigrum* plants. One possible interpretation of this result is that *Ndr1* is not involved in the signal transduction pathway of the resistance of *S. nigrum* against *P. infestans*.

4.3.3 *Rar1*

Rar1, originally isolated from barley (Shirasu *et al*., 1999), encodes an intermolecular Zn^{2+}- binding protein and is required for several *R*-gene-mediated responses against different pathogen classes in monocotyledonous and dicotyledonous plant species. *Rar1* is required for the function of two subtypes of NB-LRR proteins, TIR-NB-LRR and CC-NB-LRR subclasses. This is in contrast to *Eds1* and *Ndr1,* which appear to be preferentially engaged by either the TIR-NB-LRR or the CC-NB-LRR subclasses. The biochemical roles of *Rar1* in NB-LRR-mediated resistance are poorly understood (Freialdenhoven *et al*., 2004).

Silencing of *SnRar1* did not reduce *SnRar1* mRNA level as well but extended lesions also appeared on some leaves of very few numbers of plants (only one among the 15 plants tested with P. infestans Api02 isolate and two from 15 plants tested with T04). No sporulations were found on these lesions. Peart *et al*. (2002) have also reported that *Rar1* mutation in barley on its own was not sufficient to support enhanced growth of the inappropriate fungi. However, they suggested that *Rar1* is required for non-host interactions of barley. Perhaps, like in the case of *Eds1*, the hexaploid *S. nigrum* might contain genes similar to *SnRar1* which were partly silenced but not detected in the RT-PCR assay. Further experiments are needed to adress this phenomenon as well.

Likewise, a possible interactor of *Rar1* is *Sgt1*. A function for the *Rar1* interactor *Sgt1* in nonhost resistance has been shown in *N. benthamiana* interactions with inappropriate *Xanthomonas spp*. (Peart *et al*., 2002). Azevedo *et al.,* (1999) have already reported that *Rar1* interact directly with *Sgt1. Sgt1* and *Rar1* may function also as co-chaperones of heat shock protein *HSP90* to assemble or to regulate multi-protein signalling complexes in plant disease resistance (Hubert et al., 2003; Shirasu and Schulze-Lefert, 2003; Takahashi *et al*., 2003; Zhang *et al*., 2004).

Sgt1 was one of the candidate genes I have amplified from *S. nigrum* cDNA but it could not be integrated into the pEntr11Amp vector, this might be due to its very large size because it is 2864 bp.

5 Summary

Potato is the fourth most important crop grown globally in terms of acreage, yield, and value, with annual production exceeding 320 million tons (online FAOSTAT data for 2004). Late blight caused by *P. infestans* is historically the most costly and damaging disease affecting potatoes in the world (Abad *et al.*, 1995). This disease is best known as being responsible for the irish potato famine in the 1840's. Between 1846 and 1860, more than a million Irish people starved to death as a consequence of the blight; another 1.5 million were forced to emigrate overseas to Europe and America. This aggressive, fungus-like, *P. infestans*, has been also ravaging more than 70% of the Russian crop in the late 1990s (Garelik, 2002). This disease is still a major constraint nowadays; it is estimated to cost growers US $ 5 billion per year (Judelson and Blanco, 2005). In combating late blight, in many area of the world, the number of fungicide application has increased because potato late blight has become more severe. The resistance to fungicides evolved rapidly in the population of *P. infestans*. However, fungicides are harmful for the environment and human health. The most appropriate alternative to increasing the intensity of fungicide usage is the use of potato cultivars with high levels of resistance to late blight. Along with this demand for resistant cultivars comes the concern for how durable this resistance is. Resistance to *P.infestans* in currently available cultivars of potato is race-specific resistance, which is mainly derived from *S. demissum* and is considered non-durable.

Improving the genetic resistance to late blight is therefore a major issue in breeding new varieties of potato. Since major gene resistance from *S. demissum* is considered non-durable, it can be argued that either the mechanism or the induction of durable resistance should be different from that which is derived from *S. demissum*. The search for new sources of resistance is still ongoing. Rate-reducing resistance seems to be durable, but its level is too low to be of practical value.

Several wild *Solanum* species have been found resistant to *P. infestans*, including *S. demissum, S. bulbocastanum, S. berthaultii (Ballvora et al., 2002), S. microdontum, S. stoloniferum* (Vleeshouwers et al., 2000), *S. edinense* (Trognitz 1998), *Solanum phureja* (Trognitz et al., 2001), *S. caripense* (Trognitz , 2004), and *Solanum nigrum* (Kamoun et al., 1998).

S. nigrum was regarded as a valuable source of resistance to potato late blight (Colon et al., 1993; Vleeshouwers et al., 2000). It was considered as a non-host for *P. infestans*. Although Flier and associates (2003) reported the presence of sporulating lesions of *P. infestans* on *S. nigrum*, all twenty *S. nigrum* accessions tested in this study were resistant to the four *P. infestans* isolates used. Traits employed to distinguish resistant and susceptible plants were; survival or death and a hypersensitive resistance reaction. The most important

phenotypic characteristic for susceptible is the production of sporangia on infected leaf tissue (Black 1952).

The interactions between four *P. infestans* isolates (APiO2, LL8, T04 and Sn001) of different origins and twenty *S. nigrum* accessions in this study resulted in a rapid hypersensitive response limited to one to three epidermal cells. No sporulation of *P. infestans* was detected at all although the hyphae were able to penetrate further in the plant tissue of some accessions in the case of the T04 isolate. These hyphae were subsequently surrounded and stopped by necrotic cells which are macroscopically visible as black spots on the leaves. T04 was more virulent but did not cause *S. nigrum* death nor sporulation. However, plants of the potato variety Désirée, which were used as a control in all infection experiments, were completely dead within a week with sporulation visible on the leaves just after a few days (from the fourth dpi). Thus, the twenty *S. nigrum* accessions tested with *P. infestans* infections in this study were resistant against all the four isolates used. This result confirmed the consideration of *S. nigrum* as a non-host for *P. infestans* (resistance at species level). Additionally, the infected *S. nigrum* plants, that Flier et al (2003) have found, appear to be a relatively rare event. However, taking into account the presence of these compatible *P. infestans* isolates in the Netherlands, I would suggest that the response of *S. nigrum* towards *P. infestans* infection depends on gene-for-gene interaction.

The hypersensitive responses found on the leaves of these *S. nigrum* accessions were not caused by the recognition of *P.infestans* elicitins because none of them responded with an HR when infiltrated with elicitins from *P. infestans*. whereas *N. benthamiana* and *N. tabacum* responded with a strong necrosis. The HR is an important characteristic associated with *R*-gene resistance responses (Alvarez, 2000; Durrant and Dong, 2004; Feys et al., 2001). Therefore, the resistance of *S. nigrum* against *P. infestans* might be due to *R*-gene(s). An attempt to silence genes for *R*-gene signal transduction, which might be involved in this resistance, using VIGS was done in this study.

The fragments of the candidate genes were amplified from *S. nigrum* genomic DNA and cDNA and successfully cloned into pENTR11Amp vector (a pENTR11 that contains ampicillin resistance). The candidate genes have been chosen from public databases containing a range of ESTs for genes from the *Solanaceae* gene family that have been shown to be necessary for the function of resistance genes. The pENTR11Amp vector was constructed and used in this study because the original pENTR11 was Kanamycin resistant and cannot be used as the pTRV-RNA2 gateway vector contains also Kanamycin resistance. This ampicillin resistance was transferred from pUC18 into the pENTR11 which was originally kanamycin resistant. Through gateway procedures, using LR clonase enzyme mix, *Eds1, Ndr1* and *Rar1* were successfully subcloned into pTRV-RNA2 from the entry clone

pENTR11Amp. The introduction of pTRV-*Eds1*, pTRV-*Ndr1*, pTRV-*Rar1*, pTRV-*PDS* (as positive control) and pTRV-empty (as negative control) vectors via *Agrobacterium*-mediated transfer into *S. nigrum* plant cells was successfully accomplished through vacuum infiltration of *Agrobacterium* GV3101.

The result showed that a vector based on TRV is effective for silencing the endogenous phytoene desaturase (*SnPDS*) gene in *S. nigrum*. The infiltrated leaves were photobleached about three weeks after infiltration. This indicated that recombinant TRV can efficiently replicate and spread systemically in *S. nigrum* plants. This vector did not compromise the growth of *S. nigrum* plants and did not display viral symptoms. Therefore, I conclude that VIGS is an effective method which can be used for knocking down resistance genes in *S. nigrum* species.

It was also shown during this study that agroinoculation is an efficient technique that can be applied in *S. nigrum* to deliver TRV-based vectors carrying the gene of interest into *S. nigrum* plants and vaccuum infiltration was the best and quickest method. Through vaccuum infiltration, 100% of the inoculated plants showed the silencing phenotype and almost all newly developed leaves were at least partly photobleached starting from the third week after infiltration. However, the plants that were infected through agrodrench and leaf infiltration started to be bleached only after 4 weeks and the percentage of plants exhibiting photobleached leaves was less than 80% for both techniques. Additionally, only a few leaves per plant were bleached and the bleaching was not as strong as was the case with the plants from vacuum infiltration. Thus, agroinfiltration is the method of choice for *S. nigrum* and was used throughout this work.

Concerning the *Agrobacterium* strains, among the eight *A. tumefaciens* strains that I have tested; only GV3101 and MOG 101 gave no signs of a plant reaction but they gave a good GUS expression which is visible within the infiltrated area. All other strains caused necrosis around the inoculation sites and could not be used for agroinfiltration assay. Therefore, GV3101 and MOG101 would be the *Agrobacterium* strain of choice when working with *S. nigrum* species. However, I did not get any photobleached leaves when using MOG101 to deliver pTRV-*PDS* into *S. nigrum* plants. Based on this result, GV3101 is the only one efficient *A. tumefaciens* strain which could be used to transfer the TRV vector carrying the gene of interest into *S. nigrum*.

The infiltrated plants (either with pTRV-*Eds1,* pTRV-*Ndr1* or pTRV-*Rar1*) did not show any mRNA reduced levels of *SnEds1, SnNdr1* and *SnRar1* although the leaves of pTRV-*PDS*-infiltrated plants were bleached. However, some pTRV-*Eds1* and pTRV-*Rar1* infiltrated plants developed lesions on their leaves. No sporulation was found on these lesions. *S. nigrum*, being a hexaploid, might contain genes similar to *SnEds1* and *SnRar1* which were partly silenced but not detected in the RT-PCR. Further experiments are needed to adress

this phenomenon. In addition, as it was reported by other researchers that *Eds1*, *Pad4* and *SAG101* contribute to nonhost resistance (Lipka *et al.,* 2005; Wiemer *et al*., 2005), future studies should reveal whether co-infiltration of *Eds1* and/or *Rar1* with other gene such as *Pad4* and/or *Sag101*, *eds1-pad4-sag101* for instance, might lead to enhanced disease susceptibility in *S. nigrum* plants.

6 References

Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J., & Parker, J. E. (1998). Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 95(17): 10306-10311.

Abad, Z.G., Abad, J.A., and Ochoa, C. (1995) Historical and Scientific Evidence that Support the Modern Theory of Peruvian Andes as the center of Origin of *Phytophthora infestans*. *Phytophthora infestans* 150: 239-245.

Alvarez, M. E. (2000). Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant Molecular Biology* 44(3): 429-442.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I., & Doolittle, **W. F.** (2000). A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* **290(5493)**: 972-977.

Ballvora, A., Ercolano, M. R., Weiß, J, Meksem, K, Bormann, C.A., Oberhagemann, P., Salamini, F. and Gebhardt (2002) The R1 gene for potato resistance to late blight (Phytophthora infestans) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *Plant Journal* **30** (3): 361-371.

Baulcombe, D. (1999). Viruses and gene silencing in plants. *Archives of Virology*, 189-201.

Becktell, M.C., Smart, C.D., Haney, C.H., and Fry, W.E. (2006) Host-Pathogen interactions between *Phytophthora infestans* and *Solanaceous* Hosts *Calibrachoa* x *hybridus*, *Petunia* x *hybrida*, and *Nicotiana benthamiana*. *The American Phytopathological Society* **90** (1): 24-32

Bendahmane, A., Kanyuka, K., & Baulcombe, D. C. (1999). The *Rx* gene from potato controls separate virus resistance and cell death responses. *Plant Cell*, 11(5): 781-791.

Birch, P.R.J., Avrova, A.O., Duncan, J.M., Lyon, G.D., and Toth, R.L. (1999) Isolation of potato genes that are induced during an early stage of Hypersensitive response to Phytophthora infestans. *MPMI* **12**(4): 356-361.

Bonnet, P., Poupet, A., & Bruneteau, M. (1985). Toxicity to Tobacco of Different Fractions from A Culture Filtrate of *Phytophthora cryptogea*. *Agronomie* 5(3): 275-282.

Borhan, M.H., Holub, E.B., Beynon, J.L., Rozwadowski, K., and Rimmer, R. (2004) The *Arabidopsis* TIR-NB-LRR Gene *RAC1* confers resistance to *Albugo candida* (white Rust) and is dependent on *EDS1* but not *PAD4*. *MPMI* **17**(7): 711-719.

Brigneti, G., Martin-Hernandez, A.M., Jin, H., Chen, J., Baulcombe, D.C., Baker, B., Jones, J.D.G. (2004) Virus-induced gene silencing in *Solanum* species. *The Plant Journal* **39:** 264-272.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B. and Dinesh-Kumar, S.P. (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The plant journal* **39**: 734-746.

Cai-Cheng Huang, Ton Groot, Fien Meijer-Dekens, Rients, E.N., and Pim Lindhout (1998) The resistance to powdery mildew (*Oidium lycopersicu*,) in *Lycopersicon* species is mainly associated with hypersensitive response. *European Journal of Plant Pathology* **104**: 399-409.

Caten, C. E. & Jinks, J. L. (1968). Spontaneous Variability of Single Isolates of *Phytophthora Infestans* .I. Cultural Variation. *Canadian Journal of Botany*, 46(4): 329-&.

Century, K. S., Shapiro, A. D., Repetti, P. P., Dahlbeck, D., Holub, E., & Staskawicz, B. J. (1997). *NDR1*, a pathogen-induced component required for *Arabidopsis* disease resistance. *Science*, 278(5345): 1963-1965.

Chung, S.M., Frankman, E.L. and Tzfira, T. (2005) A versatile vector system for multiple gene expression in plants. *Trends in Plant Science* **10(8):** 357-361.

Chung, S. M., Frankman, E. L., & Tzfira, T. (2005). A versatile vector system for multiple gene expression in plants. *Trends in Plant Science*, 10(8): 357-361.

Chung, E., Seong, E., Kim, Y.C., Chung, E.J., Oh, S.K., Lee, S., Park, J.M, Joung, Y.H, and Doil Choi (2004) A method of High frequency Virus-Induced Gene silencing in Chili Pepper (*Capsicum annuum* L. cv. Bukang) *Mol. Cells* **17(2)**: 377-380.

Coffey, M.D. and Wilson, U.E. (1983) An ultrastructure study of late blight fungus *Phytophthora infestans* and its interaction with the foliage of two potato cultivars possessing different levels of general (field) resistance. Can. J. Bot. **61**: 2669-2685.

Colon, L.T., Eijlander, R., Budding, D.J., van Ijzendoorn, M.T., Pieters, M.M.J., and Hoogendoorn, J. (1993) Resistance to potato late blight (*Phytophthora infestans* (Mont.) de Bary) in *Solanum nigrum*, *S. villosum* and their hybrids with *S. tuberosum* and *S demissum*. *Euphytica* **66**: 55-64.

Colon, L. T., Jansen, R. C., & Budding, D. J. (1995a). Partial Resistance to Late Blight (Phytophthora-Infestans) in Hybrid Progenies of 4 South-American Solanum Species Crossed with Diploid *Solanum tuberosum*. *Theoretical and Applied Genetics*, **90(5)**: 691-698.

Colon, L. T., Turkensteen, L. J., Prummel, W., Budding, D. J., & Hoogendoorn, J. (1995). Durable Resistance to Late Blight (*Phytophthora Infestans*) in Old Potato Cultivars. *European Journal of Plant Pathology* , **101(4)**: 387-397.

Dangl, J. L. & Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature*, **411(6839)**: 826-833.

Da-Qi Fu, Zhu, B.Z., Zhu, H.L., Zhang, H.X., Xie, Y.H., Jiang, Wei-Bo, Zhao, X.D., and Luo, Y.B. (2006) Enhancement of Virus Induced Gene silencing in Tomato by low temperature and low humidity. *Mol. Cells* **21(1)**: 153-160.

De, Maine, M.J., Carroll, C.P. Stewart, H.E. Solomon, R.M. & Wastie, R.L. (1993). Disease resistance in *Solanum phureja* and diploid and tetraploid *S. tuberosum* × *S. phureja* hybrids. *Potato Res* **36**: 21–28.

De Merlier, D., Chandelier, A., Debruxelles, N., Noldus, M., Laurent, F., Dufay, E., Claessens, H. and Cavelier, M. (2005) Characterization of Alder *Phytophthora* isolates from Wallonia and development of SCAR primers for their specific detention. *J. Phytopathology* **153**: 99-107.

Dorey, S., Baillieul, F., Pierrel, M. A., Saindrenan, P., Fritig, B., & Kauffmann, S. (1997). Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitively to a fungal glycoprotein elicitor. *Molecular Plant-Microbe Interactions* **10(5)**: 646-655

Durrant, W. E. & Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology* **42**: 185-209.

Ebstrup, T., Saalbach, G. and Egsgaard, H. (2005) A proteomics study of in vitro cyst germination and appressoria formation in *Phytophthora infestans*. *Proteomics* **5**: 2839-2848.

Edgar Huitema, Vleeshouers, V.G.A.A., Cahit Cakir, Sophien Kamoun, and Francine Govers (2005) Differences in intensity and specificity of hypersensitive response induction in *Nicotiana spp*. by *INF1*, *INF2A*, and *INF2B* of *Phytophthora infestans*. MPMI **18(3)**: 183-193.

Edmonds, J.M. and Chweya, J.A. (1997) Black nightshades, *Solanum nigrum* L. and related species. *International Plant Genetic Resources Institute* 1-112.

Ehrlich, M.A and Ehrlich, HG. (1966) Ultrastructure of the hyphae and haustoria of *Phytophthora infestans* and hyphae of *P. parasitica*. *Canadian Journal of Botany* **44**: 1495-1503.

Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P. and Martin, G.B. (2003) Two *MAPK* cascades, *NPR1* and *TGA* transcription factors play a role in Pto-mediated disease resistance in tomato.. *The Plant Journal* **36**: 905-917.

Erwin, D.C. and Ribeiro, O.K (1996) *Phytophthora* diseases Worldwide. *APS Press*: 346-353.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J., and Parker, J.E. (1999) *EDS1*, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Plant Biology* **96**: 3292-3297.

Feys, B. J., Moisan, L. J., Newman, M. A., & Parker, J. E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins, *EDS1* and *PAD4*. *Embo Journal*, **20(19)**: 5400-5411.

Flier, W.G. , Van den Bosch, G.B.M and Turkensteen (2003) Epidemiological importance of *Solanum sisymbriifolium*, *Solanum nigrum* and *Solanum dulcamara* as alternative hosts for *Phytophthora infestans*. *Plant Pathology* **52**: 595-603.

Freialdenhoven, A., Orme, J., Lahaye, T., and Schulze-Lefert, P. (2005) Barley Rom1 reveals a potential link between race-specific and non-host resistance responses to powdery mildew fungi. *Molecular Plant-Microbe Interactions* **18(4)**: 291-299.

Fu, D.Q, Zhu, B.Z, Zhu, H.L., Zhang, H.X., Xie, Y.H., Jiang, W.B., Zhao, X.D., and Luo, Y.B. (2006) Enhancement of Virus-induced gene silencing in tomato by low temperature and low humidity. *Molecules and Cells* **21(1)**: 153-160.

Gabriels, S.H.E.J., Vossen, J.H., Ekengren, S.K., van Ooijen, G., Abd-El-Haliem, A.M., van den Berg, C.M., Rainey, D.Y., Martin, G.B., Takken, F.L.W., de Wit, P.J.G.M and Joosten, M.H.A.J (2007) An NB-LRR protein required for HR signaling mediated by both extra- and intracellular resistance proteins. *Plant Journal* **50(1)**: 14-28.

Garelik, G. (2002) Agriculture - Taking the bite out of potato blight. *Science*, **298(5599)**: 1702-1704.

Gees, R. and Hohl, H.R. (1987) Cytological comparison of specific (*R3*) and general resistance to late blight in potato leaf tissue. *Phytopathology* **78**: 350-357.

Gisi, U. & Cohen, Y. (1996) Resistance to phenylamide fungicides: A case study with Phytophthora infestans involving mating type and race structure. *Annual Review of Phytopathology* **34**: 549-572.

Glazebrook, J. (2001) Genes controlling expression of defense responses in Arabidopsis - 2001 status. *Current Opinion in Plant Biology*, **4(4)**: 301-308.

Gongshe Hu, deHart, A.K.A., Yansu Li, Ustach, C., Handley, V., Navarre, R., Hwang, C., Aegerter, B. J., Williamson, V.M., and Baker, B., (2005) EDS1 in tomato is required for resistance mediated by TIR-class R genes and the receptor-like *R* gene *Ve*. *The Plant Journal* **42**: 376-391.

Grünwald, N. and Flier, W.G. (2005) The biology of Phytophthora infestans at its center of origin. *Annual Review Phytopathology*. **43**: 10.1-10.20.

Grünwald, N.J., Hinojosa, M.A.C., Covarrubias, O.R., Pena, A.R., Niederhauser, J.S., and Fry, W.E. (2002) Potato cultivars from the Mexican National Program: sources and durability of resistance against late blight. *Phytopathology* **92**(7): 688-692.

Hammond-Kozack, K.E. and Jones, J.D.G. (1996) Inducible plant defence mechanisms and resistance gene function. *The Plant Cell* **8**: 1773-1791.

Hardham, A.R. (2001) The cell biology behind Phytophthora pathogenicity. *Australasian Plant Pathology* **30**: 91-98.

Heath, M. C. (1998) Involvement of reactive oxygen species in the response of resistant (hypersensitive) or susceptible cowpeas to the cowpea rust fungus. *New Phytologist*, **138(2)**: 251-263.

Heath, M. C. (2000). Nonhost resistance and nonspecific plant defenses. *Current Opinion in Plant Biology* **3(4)**: 315-319.

Henikoff, S. & Comai, L. (2003). Single-nucleotide mutations for plant functional genomics. *Annual Review of Plant Biology*, 54: 375-401.

Henri Nicholls (2004) Stopping th Rot. *PLoS Biology* **2**: 0891-0895.

Hileman, L.C., Drea, S., de Martino, G., Litt, A and Irish, V.F. (2005) Virus-induced gene silencing is an effective tool for assaying gene function in the besal eudicot species *Papaver somniferum* (opium poppy). *The Plant Journal* **44(2)**: 334-341.

Hirst, J. M. and Stedman, O. J. (1960) The epidemiology of *phytophthora infestans*. *Annals of Applied Biology* **48(3)**: 471-488.

Huang, S., Vleeshouwers, V.G.A.A., Visser, R.G.F., and Jacobsen, E. (2005) An accurate in vitro assay for high-throughput disease testing of *Phytophthora infestans* in potato. *Plant Disease* **89(12)**: 1263-1267.

Huang, S., Vleeshouwers, V.G.A.A., Werij, J.S., Hutten, R.C.B., van Eck, H.J., Visser, G.F., and Jacobsen, E. (2004) The *R3* resistance to Phytophthora infestans in potato is conferred two closely linked *R* genes with distinct specificities. *MPMI* **17(4)**: 428-435.

Hubert, D. A., Tornero, P., Belkhadir, Y., Krishna, P., Takahashi, A., Shirasu, K., & Dangl, J. L. (2003). Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein. *Embo Journal* **22(21)**: 5679-5689.

Huitema, E., Vleeshouwers, V.G.A.A., Cakir, C., Kamoun, S., and Govers, F. (2005) Differences in Intensity and Specificity of Hypersensitive response Induction in *Nicotiana* spp. by INF1, INF2A, and INF2B of *Phytophthora infestans*. *MPMI* **18(3)**: 183-193.

Huitema, E., Vleeshouwers, V. G. A. A., Francis, D. M., & Kamoun, S. (2003) Active defence responses associated with non-host resistance of Arabidopsis thaliana to the oomycete pathogen Phytophthora infestans. *Molecular Plant Pathology* **4(6)**: 487-500.

Hohl, H.R. (1991) Surface-related host-pathogen interactions in Phytophthora. *British Mycological Society* **6**: 70-89.

Hohl, H.R. and Balsiger, S. (1986) A model system for the study of fungus-host surface interactions: adhesion of Phytophthora megasperma to protoplasts and mesophyll cells of soybean. *NATO ASI series* **4**: 259-271.

Hohl, H.R. and Stössel, P. (1975) Host-parasite interfaces in a resistant and susceptible cultivar of *Solanum tuberosum* inoculated with *Phytophthora infestans*: tuber tissue. *Can. J. Bot* **54**: 900-912.

Hohl, H.R. and Suter, E. (1976) Host-parasite interfaces in a resistant and a susceptible cultivsr of Solanum tuberosum inoculated with *Phytophthora infestans*: leaf tissue. *Can. J. Bot*. **54**: 1956-1970.

Jadwiga Sliwka (2004) Genetic factors encoding resistance to late blight caused by *Phytophthora infestans* (Mont.) de Bary on the potato genetic map. *Cellular & Molecular Biology Letters* **9**: 855-867.

J**irage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., Ausubel, F. M., & Glazebrook, J.** (1999). *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proceedings of the National Academy of Sciences of the United States of America* **96(23)**: 13583-13588.

Jones, D. A., Thomas, C. M., Hammondkosack, K. E., Balintkurti, P. J., & Jones, J. D. G. (1994) Isolation of the Tomato *Cf-9* Gene for Resistance to Cladosporium-Fulvum by Transposon Tagging. *Science* **266(5186)**: 789-793.

Judelson, H. S. (1997) The genetics and biology of Phytophthora infestans: Modern approaches to a historical challenge. *Fungal Genetics and Biology* **22(2)**: 65-76.

Judelson, H.S. and Blanco, F.A. (2005) The spores of *Phytophthora*: weapons of the plant destroyer. *Nature reviews microbiology* **3**: 47-58.

Kamoun, S. (2001). Nonhost resistance to Phytophthora: novel prospects for a classical problem. *Current Opinion in Plant Biology* , **4(4)**: 295-300.

Kamoun, S., Huitema, E. and Vleeshouwers, V. G.A.A. (1999) Resistance to Oomycetes: a general role for the hypersensitive response? *Trends in Plant Science* **4(5)**: 196-200.

Kamoun, S., vanWest, P., deJong, A. J., deGroot, K. E., Vleeshouwers, V. G. A. A., & Govers, F. (1997). A gene encoding a protein elicitor of Phytophthora infestans is down-regulated during infection of potato. *Molecular Plant-Microbe Interactions* **10(1)**: 13-20.

Kamoun, S., van west, P., Vleeshouwers, V.G.A.A., de Groot, K.E., and Govers, F. (1998) Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by recognition of the elicitor protein *INF1*. *The Plant Cell* **10**: 1413-1425.

Kamoun, S., Young, M., Glascock, C. B., & Tyler, B. M. (1993) Extracellular Protein Elicitors from *Phytophthora* - Host-Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens. *Molecular Plant-Microbe Interactions* **6(1)**: 15-25.

Kamoun, S., Young, M., Forster, H., Coffey, M. D., & Tyler, B. M. (1994) Potential Role of Elicitins in the Interaction Between *Phytophthora* Species and Tobacco. *Applied and Environmental Microbiology* **60(5)**: 1593-1598.

Karimi, M., Inzé, D., and Depicker, A. (2002) GatewayTM vectors for *Agrobacterium*-mediated plant transformation. *Trends in Plant Science* **7(5)**:193-195.

Keller, H., Pamboukdjian, N., Ponchet, M., Poupet, A., Delon, R., Verrier, J. L., Roby, D., & Ricci, P. (1999) Pathogen-induced elicitin production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. *Plant Cell* **11(2)**: 223-235.

Keogh, R.C., Deverall, B.J. and MacLeod, S. (1980) Comparison histological and physiological responses to *Phakopsora pachyrhizi* resistant and susceptible soybean. *Trans. Brit. Mycol. Soc*. **74**: 329-333

Kmecl, A., Mauch, F., Winzeler, M., Winzeler, H. and Dudler, R. (1995) Quantitative field resistance of wheat to powdery mildew and defence reactions at the seedling stage: identification of a potential marker. Physiological and Molecular Plant Pathology **47**: 185-199.

Knoester, M., van Loon, L. C., van den Heuvel, J., Hennig, J., Bol, J. F., & Linthorst, H. J. M. (1998) Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. *Proceedings of the National Academy of Sciences of the United States of America* **95(4)**: 1933-1937.

Kuc, J. (1972) Phytoalexins. *Annual Review of Phytopathology* **10**: 207-&

Kumar, H.Y.P.P. (2003) Post-transcriptional gene silencing in plants by RNA. *Plant Cell Rep* **22**: 167-174.

Lebrun-Garcia, A., Bourque, S., Binet, M. N., Ouaked, F., Wendehenne, D., Chiltz, A., Schaffner, A., & Pugin, A. (1999). Involvement of plasma membrane proteins in plant defense responses. Analysis of the cryptogein signal transduction in tobacco. *Biochimie* **81(6)**: 663-668.

Leister, D., Ballvora, A., Salamini, F., & Gebhardt, C. 1996. A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. *Nature Genetics* **14(4)**: 421-429.

Leonards-Schippers, C., Gieffers, W., Schafer-Pregl, R., Ritter, E., Knapp, S.J., Salamini F. and Gebhardt C. (1994) Quantitative Resistance to Phytophthora infestans in Potato: A Case Study for QTL Mapping in an Allogamous Plant Species. *Genetics* **137**: 67-77.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiemer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S., Schulze-Lefert, P. (2005) Pre- and Postinvasion Defenses Both Contribute to Nonhost Resistance in Arabidopsis*. Sciences* **310:** 1180-1183.

Liu, Y., Schiff, M., and Dinesh-Kumar, S.P. (2004) Involvement of *MEK1 MAPKK, NTF6* **MAPK, WRKY/MYB** transcription factors, *COI1* and *CTR1* in N-mediated resistance to tobacco mosaic virus. *The Plant Journal* **38**: 800-809.

Liu, Y., Schiff, M., and Dinesh-Kumar, S.P. (2002) Virus-induced gene silencing in tomato. *The Plant Journal* **31(6)**: 777-786.

Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., and Baulcombe, D.C. (2003) Virus-innduced gene silencing in plants*. Methods* **30**: 296-303.

Marcel Wiermer, Feys, B.J., and Parker, J.E. (2005) Plant immunity: *EDS1* regulatory node. *Current Opinion in Plant Biology* **8**: 383-389.

Martin, G. B., Devicente, M. C., & Tanksley, S. D. (1993) High-Resolution Linkage Analysis and Physical Characterization of the *Pto* Bacterial-Resistance Locus in Tomato. *Molecular Plant-Microbe Interactions* **6(1)**: 26-34.

McDowell, J. M., Cuzick, A., Can, C., Beynon, J., Dangl, J. L., & Holub, E. B. (2000) Downy mildew (*Peronospora parasitica*) resistance genes in Arabidopsis vary in functional requirements for *NDR1, EDS1, NPR1* and salicylic acid accumulation. *Plant Journal* **22(6)**: 523-529.

Murashige, T. & Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* **15(3)**: 473-&.

Muskett, P. R., Kahn, K., Austin, M. J., Moisan, L. J., Sadanandom, A., Shirasu, K., Jones, J. D. G., & Parker, J. E. (2002) *Arabidopsis RAR1* exerts rate-limiting control of R gene-mediated defenses against multiple pathogens. *Plant Cell* **14(5)**: 979-992.

Mysore, K. S. & Ryu, C. M. (2004) Nonhost resistance: how much do we know? *Trends in Plant Science* **9(2)**: 97-104.

Nicholls,H. (2004) Stopping the rot. *PLoS Biology* **2** (7): 0891-0895.

Nicks, R.E. (1986) Failure of haustorial development as a factor in slow growth and development of *Puccinia hordei* in partially resistantbarley seedlings. *Physiological and Molecular Plant Pathology* **28**: 309-322.

Niks, R. E. (1987). Nonhost Plant-Species As Donors for Resistance to Pathogens with Narrow Host Range .1. Determination of Nonhost Status. *Euphytica* **36(3)**: 841-852.

Oyarzun, P.J., Yanez, J. and Forbes, G.A. (2004) Evidence for Host mediation of preinfection stages of *Phytophthora infestans* on the leaf surface of *Solanum phureja. J. Phytopathology* **152**: 651-657.

Paal, J., Henselewski, H., Muth, J., Meksem, K., Menendez, C. M., Salamini, F., Ballvora, A., & Gebhardt, C. (2004). Molecular cloning of the potato *Gro1-4* gene conferring resistance to pathotype *Ro1* of the root cyst nematode Globodera rostochiensis, based on a candidate gene approach. *Plant Journal* **38(2)**: 285-297.

Pajerowska, K.M., Parker, J.E., and Gebhardt, C. (2005) Potato homologs of *Arabidopsis thaliana* genes functional in defence signalling – identification, genetic mapping and molecular cloning. *MPMI* **18**(10): 1107-1119.

Parker, J. E., Holub, E. B., Frost, L. N., Falk, A., Gunn, N. D., & Daniels, M. J. (1996) Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. *Plant Cell* **8(11)**: 2033-2046.

Peart, J.R., Cook, G., Parker, J.E., and Baulcombe, D. (2002) An *EDS1* orthologue is required for *N*-mediated resistance against tobacco mosaic virus. *The Plant Journal* **29(5)**: 569-579.

Pieterse, C. M. J., Derksen, A. M. C. E., Folders, J., & Govers, F. (1994) Expression of the *Phytophthora Infestans Ipib* and *Ipio* Genes *in Planta* and *In-Vitro. Molecular & General Genetics* **244(3)**: 269-277.

Platt, H. W. (1999) Response of *solanaceous* cultivated plants and weed species to inoculation with A1 or A2 mating time strains of *Phytophthora infestans*. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **21(3)**: 301-307.

Porter, L.D. and Johnson D.A. (2004). Survival of *Phytophthora infestans* in Surface Water. *Phytopathology* **94**: 380-387.

Ratcliff, F., Martin-Hernandez, A. M., & Baulcombe, D. C. (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant Journal* **25(2)**: 237-245.

Rauscher, G.M., Smart, C.D, Simko, I., Bonierbale, M., Mayton, H., Greenland, A. and Fry, W.E. (2006) Characterization and mapping of $R_{Pi\text{-}ber}$, a novel potato late blight resistance from *Solanum berthaultii*. *Theoretical and Applied Genetics* **112(4)**: 674-687

Restrepo, S., Myers, K.L., del Pozo, O., Martin, G.b., Hart, A.l., Buell, C.R., Fry, W.E., and Smart, C.D. (2005) Gene profiling of a compatible interaction between *Phytophthora infestans* and *Solanum tuberosum* suggests a role for carbonic anhydrase. *MPMI* **18(9)**: 913-922.

Ribeiro, O.K. (1978) A sourcebook of the Genus *Phytophthora*. (Ed. Cramer, J.) Germany.

Rivera-Pena, A. (1990) Wild tuber-bearing species of *Solanum* and incidence of *Phytophthora infestans* (Mont.) de Bary on the Western slopes of volcano Nevado de Toluca. 5. Type of resistance to *P. infestans*. *Potato Research* **33**: 479-486.

Robertson, D. (2004) VIGS Vectors for Gene Silencing: many targets, many tools. *Annu. Rev. Plant Biol*. **55**: 495-519.

Roetschi, A., Si-Ammour, A., Belbahri, L., Mauch, F. and Mauch-Mani,B. (2001) Characterization of an *Arabidopsis-Phytophthora* pathosystem: resistance requires a functional *PAD2* gene and is independent on salicylic acid, ethylene and jasmonic acid signalling. *The Plant Journal* **28(3)**: 293-305.

Ros, B., Thümmler, F., and Wenzel, G. (2005) Comparative analysis of *Phytophthora infestans* induced gene expression in potato cultivars with different levels of resistance. *Plant Biology* **7**: 686-693.

Rotenberg, D., Thompson, T.S., German, T.L., and Willis, D.K. (2006) Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *Journal of Virological Methods* **138**: 49-59.

Rusterucci, C., Aviv, D. H., Holt, B. F., Dangl, J. L., & Parker, J. E. (2001) The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathway controlled by *LSD1* in arabidopsis. *Plant Cell* **13(10)**: 2211-2224.

Ryu, C.M., Anand, A., Li Kang, and Mysore, K.S. (2004) Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse *Solanaceous* species. *Plant Journal* **40(2)**: 322-331.

Sasabe, M., Takeuchi, K., Kamoun, S., Ichinose, Y., Govers, F., Toyoda, K., Shiraishi, T., & Yamada, T. (2000). Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture. *European Journal of Biochemistry* **267(16)**: 5005-5013.

Sasaki, Y., Sone, T., Yoshida, S., Yahata, K., Hotta, J., Chesnut, J.D., Honda, T., Imamoto, F. (2004) Evidence for high specificity and efficienc of multiple recombination signals in mixed DNA cloning by the Multisite Gateway system. *Journal of Biotechnology* **107**: 233-243.

Schmidt, D.D., Kessler, A., Kessler, D., Schmidt, S., Lim, M., Gase, K. and Baldwin (2004) *Solanum nigrum*: A model ecological expression system and its tools. *Molecular Ecology* **13**: 981-995.

Shwa, D.S. (1983) The cytogenetics and genetics of Phytophthora. *Phytophthora: Its Biology, Taxonomy, Ecology, and Pathology*. *The American Phytopathological Society, St Paul, MN* 81-94.

Shaw, D.S. (1988) The *Phytophthora* species. *Adv. Plant Pathol*. **6**: 27-51.

Shen, G., Wang, Y.C., Zhang, W.L., and Zheng, X.B. (2005) Development of a PCR assay for the Molecular detection of *Phytophthora boehmeriae* in infected cotton. *J. Phytopathology* **153**: 291-196.

Shimony, C. and Friend, J. (1975) Ultrastructure of the interaction between Phytophthora infestans and leaves of two cultivars of potato (*Solanum tuberosum L.*) orion and majestic. *New Phytol.* **74**: 59-65.

Shirasu, K., Lahaye, T., Tan, M. W., Zhou, F. S., Azevedo, C., & Schulze-Lefert, P. (1999) A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in C-elegans. *Cell* **99(4)**: 355-366.

Sliwka, J. (2004) Genetic factors encoding resistance to late blight caused by Phytophthora infestans (Mont.) de Bary on the potato genetic map. *Cellular & Molecular Biology* Letters **9**: 855-867.

Smith, M. M. & Mccully, M. E. (1978) Enhancing Aniline Blue Fluorescent Staining of Cell-Wall Structures. *Stain Technology* **53(2)**: 79-85.

Song, J. Q., Bradeen, J. M., Naess, S. K., Raasch, J. A., Wielgus, S. M., Haberlach, G. T., Liu, J., Kuang, H. H., Austin-Phillips, S., Buell, C. R., Helgeson, J. P., & Jiang, J. M. (2003) Gene *RB* cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight. *Proceedings of the National Academy of Sciences of the United States of America* **100(16)**: 9128-9133.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C., and Ronald, P. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene *Xa21*. *Science* **270**: 1804–1806.

Spielman, L.J., McMaster, B.J. and Fry, W.E. (1989) Dominance and recessiveness at loci for virulence against potato and tomato in *Phytophthora infestans*. *Theor Appl Genet* **77**: 832-838.

Szczerbakowa, A., Maciejewska, U., Zimnoch-Guzowska, E., & Wielgat, B. (2003) Somatic hybrids *Solanum nigrum* (plus) *S-tuberosum*: morphological assessment and verification of hybridity. *Plant Cell Reports* **21(6)**: 577-584.

Takahashi, A., Casais, C., Ichimura, K., & Shirasu, K. (2003) *HSP90* interacts with *RAR1* and *SGT1* and is essential for *RPS2*-mediated disease resistance in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **100(20)**: 11777-11782.

Takemoto, D., Jones, D.A. and Hardham, A.R. (2003) GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by Oomycetes pathogens. *The Plant Journal* **33**: 775-792.

Tani, S., Yatzkan, E., and Judelson, H.S. (2004) Multiple pathway regulate the induction of genes during zoosporogenesis in Phytophthora infestans. *MPMI* **17(3)**: 330-337.

Thurow, C., Schiermeyer, A., Krawczyk, S., Butterbrodt, T., Nickolov, K., and Gatz, C. (2005) Tobacco bZIP transcription factor *TGA2.2* and related factor *TGA2.1* have distinct roles in plant defense responses and plant development. *Plant Journal* **44(1)**: 100-113.

Ton, J. and Mauch-Mani, B. (2004) ß-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *The Plant Journal* **38**: 119-130.

Tornero, P., Merritt, P., Sadanandom, A., Shirasu, K., Innes, R. W., & Dangl, J. L. (2002) *RAR1* and *NDR1* contribute quantitatively to disease resistance in Arabidopsis, and their relative contributions are dependent on the *R* gene assayed. *Plant Cell* **14(5)**: 1005-1015.

Trognitz, B.R. (1998) Inheritance of resistance in potato to lesion expansion and sporulation by *Phytophthora infestans*. *Plant Pathology* **47**: 712-722.

Trognitz, B.R., Orrillo, M., Portal, L., Roman, C., Ramon, P., Perez, S. and Chacon, G. (2001) Evaluation and analysis of reduction of late blight disease in a diploid potato progeny. *Plant Pathology* **50**: 281-191.

Trognitz, B.R., Orrillo, M., Portal, L., Pinedo, H., Ramon, P., and Trognitz, F.C. (2000) Towards understanding the genetic nature of quantitative resistance in field to late blight potato. *Plant breeding and sees science* **44 (2)**: 67-79.

Ueeda, M., Kubota, M., and Nishi, K. (2006) Contribution of jasmonic acid to resistance against *Phytophthora* blight in *Capsicum annuum* cv. SCM334. *Physiological and Molecular Plant Pathology* **67**: 149-154.

Turkensteen, L.J. and Flier, W.G. (2003) Host and non-host resistance against *Phytophthora infestans*, the causal organism of late blight of potatoes and tomatoes. *Eucarpia Leafy Vegetables* 23-24.

Turkensteen, L. J., Flier, W. G., Wanningen, R., & Mulder, A. (2000) Production, survival and infectivity of oospores of *Phytophthora infestans*. *Plant Pathology* **49(6)**: 688-696.

Umaerus, V. (1970) Studies on Field Resistance to *Phytophthora infestans* .5. Mechanisms of Resistance and Applications to Potato Breeding. *Zeitschrift Fur Pflanzenzuchtung* **63(1)**: 1-&.

van der Voort, J. R., van der Vossen, E., Bakker, E., Overmars, H., van Zandroort, P., Hutten, R., Lankhorst, R. K., & Bakker, J. (2000). Two additive QTLs conferring broad-spectrum resistance in potato to *Globodera pallida* are localized on resistance gene clusters. *Theoretical and Applied Genetics* **101(7)**: 1122-1130.

Van der Vossen, E., Sikkema, A., Hekkert, B.L., Gors, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W., and Allefs, S. (2003) An ancient R-gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. *The Plant Journal* **36**: 867-882.

Van der Vossen, E. A. G., van der Voort, J. N. A. M., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D. C., Bakker, J., Stiekema, W. J., & Klein-Lankhorst, R. M. (2000). Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. *Plant Journal* **23(5)**: 567-576.

Vleeshouwers, V.G.A.A., Driesprong, J.D., Kamphuis, L.G., Trudy Torto-Alalibo, Van't Slot, Francine Govers, Visser, R.G.F, Evert Jacobsen and Sophien Kamoun (2006) Agroinfection-based high-throughput screening reveals specific recognition of *INF* elicitins in *Solanum*. *Molecular Plant Pathology* **7(6)**: 499-510.

Vleeshouwers, V.G.A.A, Van Dooijeweert, W., Govers, F., Kamoun, S. and Colon. L.T. (2000) Does basal PR gene expression in Solanum species contribute to non-specific resistance to *Phytophthora infestans*? *Physiological and Molecular Plant Pathology* **57**:35-42.

Vleeshouwers, V.G.A.A, Van Dooijeweert, W., Govers, F., Kamoun, S. and Colon. L.T. (2000) The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans. Planta* **210**: 853-864.

Vleeshouwers, V.G.A.A, Van Dooijeweert, Keizer, L.C.P., Sijpkes, L. (1999) A laboratory assay for Phytophthora infestans resistance in various Solanum species reflects the field situation. *European Journal of Plant Pathology* **105**: 241-250.

Voinnet, O., Rivas, S., Mestre, P., and David Baulcombe (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33**: 949-956.

Waterhouse, P.M. and Helliwell, C.A. (2003) Exploring plant genomes by RNA-induced gene silencing. *Nature Reviews* **4**: 29-38.

Wiermer, M., Feys, B. J., & Parker, J. E. (2005) Plant immunity: the *EDS1* regulatory node. *Current Opinion in Plant Biology*, **8(4)**: 383-389.

Wilson, U. E. & Coffey, M. D. (1980) Cytological Evaluation of General Resistance to Phytophthora-Infestans in Potato Foliage. *Annals of Botany* **45(1)**: 81-&.

Wrobblevski, T., Piskurewicz, U., Tomczak, A., Ochoa, O., and Michelmore, R.W. (2007) Silencing of the major family of *NBS-LRR*-encoding genes in lettuce results in the loss of multiple resistance specificities. *Plant Journal* **51(5)**: 803-818.

Xiao, S. Y., Ellwood, S., Calis, O., Patrick, E., Li, T. X., Coleman, M., & Turner, J. G. (2001) Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by RPW8. *Science* **291(5501)**: 118-120.

Yun, B. W., Atkinson, H. A., Gaborit, C., Greenland, A., Read, N. D., Pallas, J. A., & Loake, G. J. (2003) Loss of actin cytoskeletal function and *EDS1* activity, in combination, severely compromises non-host resistance in Arabidopsis against wheat powdery mildew. *Plant Journal* **34(6)**: 768-777.

Zhang, Y., Dorey, S., Swiderski, M., and Jones, J.D.G. (2004) expression of RPS4 in tobacco induces an AvrRps4-independent HR that requires *EDS1, SGT1* and *HSP90*. *The Plant Journal* **40**: 213-224.

Zimmerli, L., Stein, M., Lipka, V., Schulze-Lefert, P. and Somerville, S. (2004) Host and non-host pathogens elicit different jasmonate/ ethylene responses in Arabidopsis. *The Plant Journal* **40**: 633-646.

Zimnoch-Guzowska, E., Marczewski, W., Lebecka, R., Flis, B., Schafer-Pregl, R., Salamini, F., & Gebhardt, C. 2000. QTL analysis of new sources of resistance to Erwinia carotovora ssp atroseptica in potato done by AFLP, RFLP, and resistance-gene-like markers. *Crop Science* **40(4)**: 1156-1167.

Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F., & Glazebrook, J. (1998) *PAD4* functions upstream from salicylic acid to control defense responses in Arabidopsis. *Plant Cell* **10(6)**: 1021-1030.

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Figure 38: DNA sequence of pEntry11Amp:

CTAACTACTAAGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGGAAGACTGGGCCTTTCGTTTTAT
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Underlined sequences correspond to the pENTR11 primer sites and the color blue indicates *att*L sites

Figure 39: DNA sequence of pEntry11:

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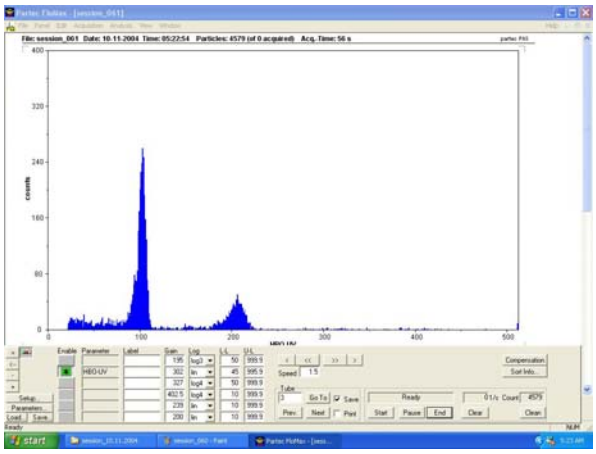
GATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAACTTTTGCCAT
TCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATT
AATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAAC
TGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATA
TGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACA
TTATTCAGATTGGGCCCCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGAT
CCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGC
CGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGT
TCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTG
CTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGAT
AGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAAC
GACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAG
GCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACG
CCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTC
AGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCT
TTTGCTCACATGTT
```

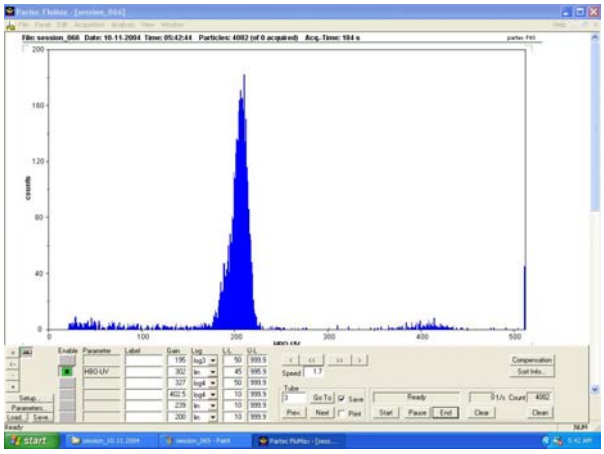
pENTR11 vector sequence was downloaded from Invitrogen's web site 20/8/2006 Ghil

http://bip.weizmann.ac.il/plasmid/txt/180.txt

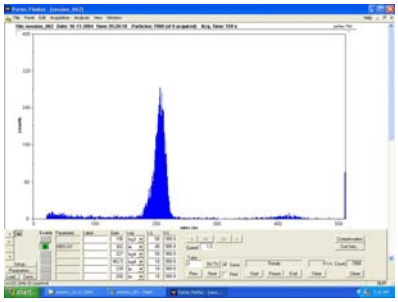
Figure 40: Results from the flow cytometry of *S. nigrum* accessions:



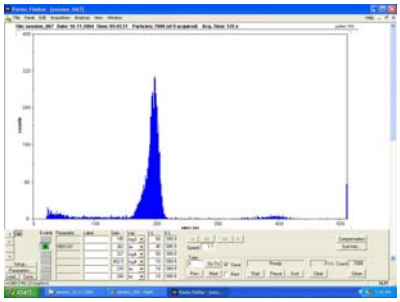
Potato



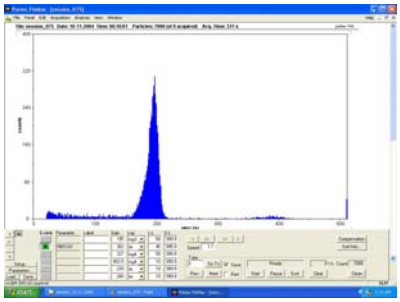
Plant 2



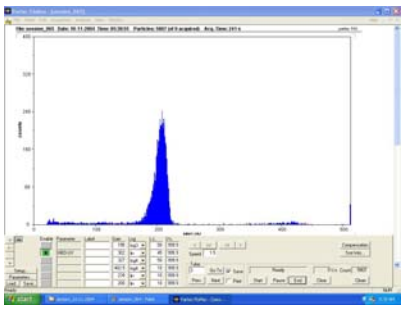
Plant3



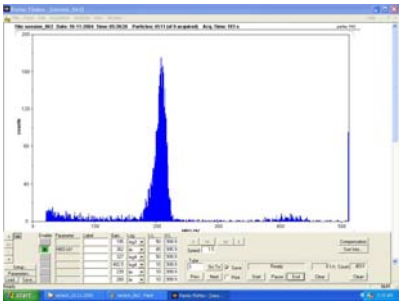
Plant 4



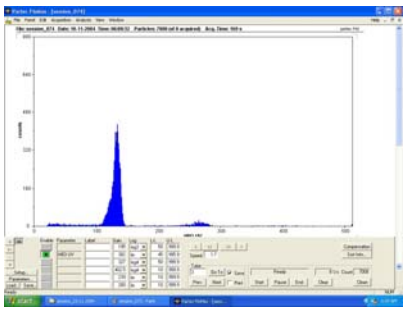
Plant 5



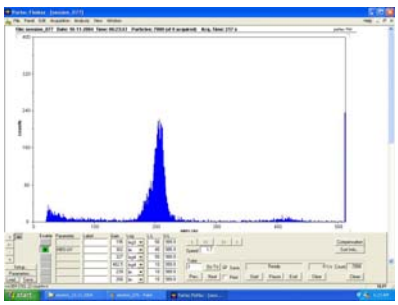
SOL 20/02



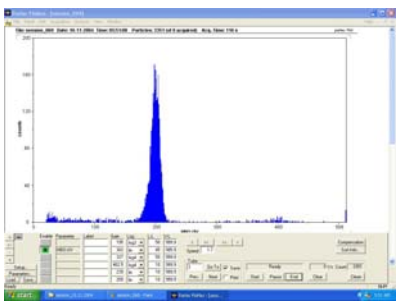
SOL 23/02



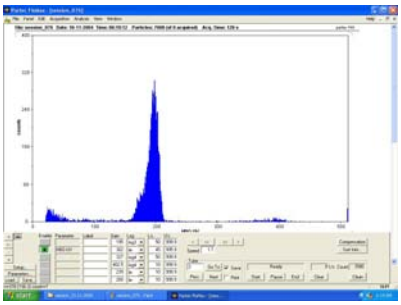
SOL 25/78



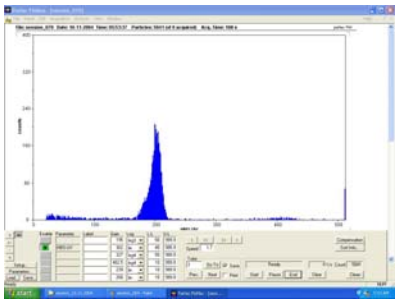
SOL 33/78



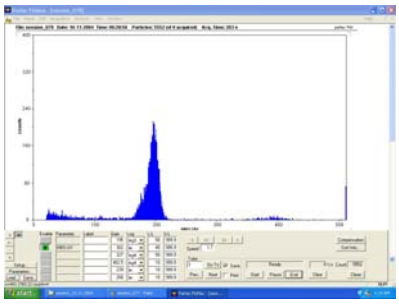
SOL 35/79



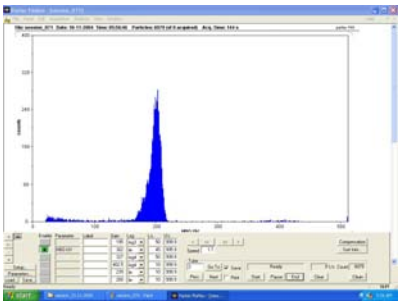
SOL38/01



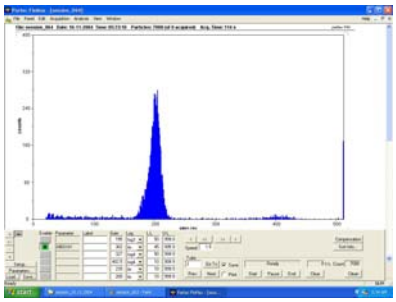
SOL 40/01



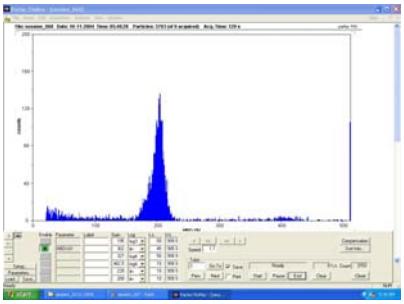
SOL 42/01



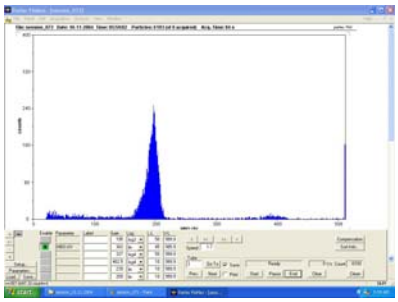
SOL 44/77



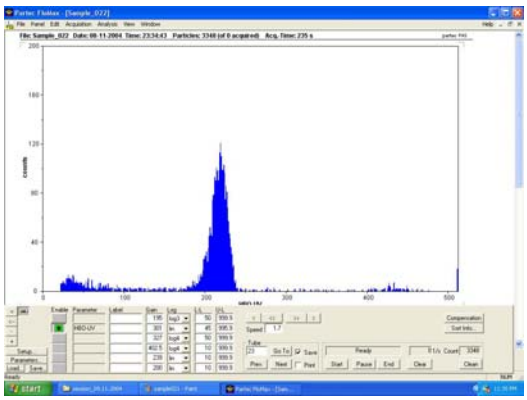
SOL 50/77



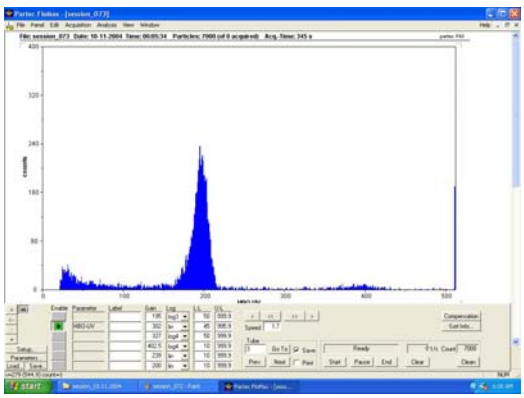
SOL 55/77



S.nigrum nodiflorum

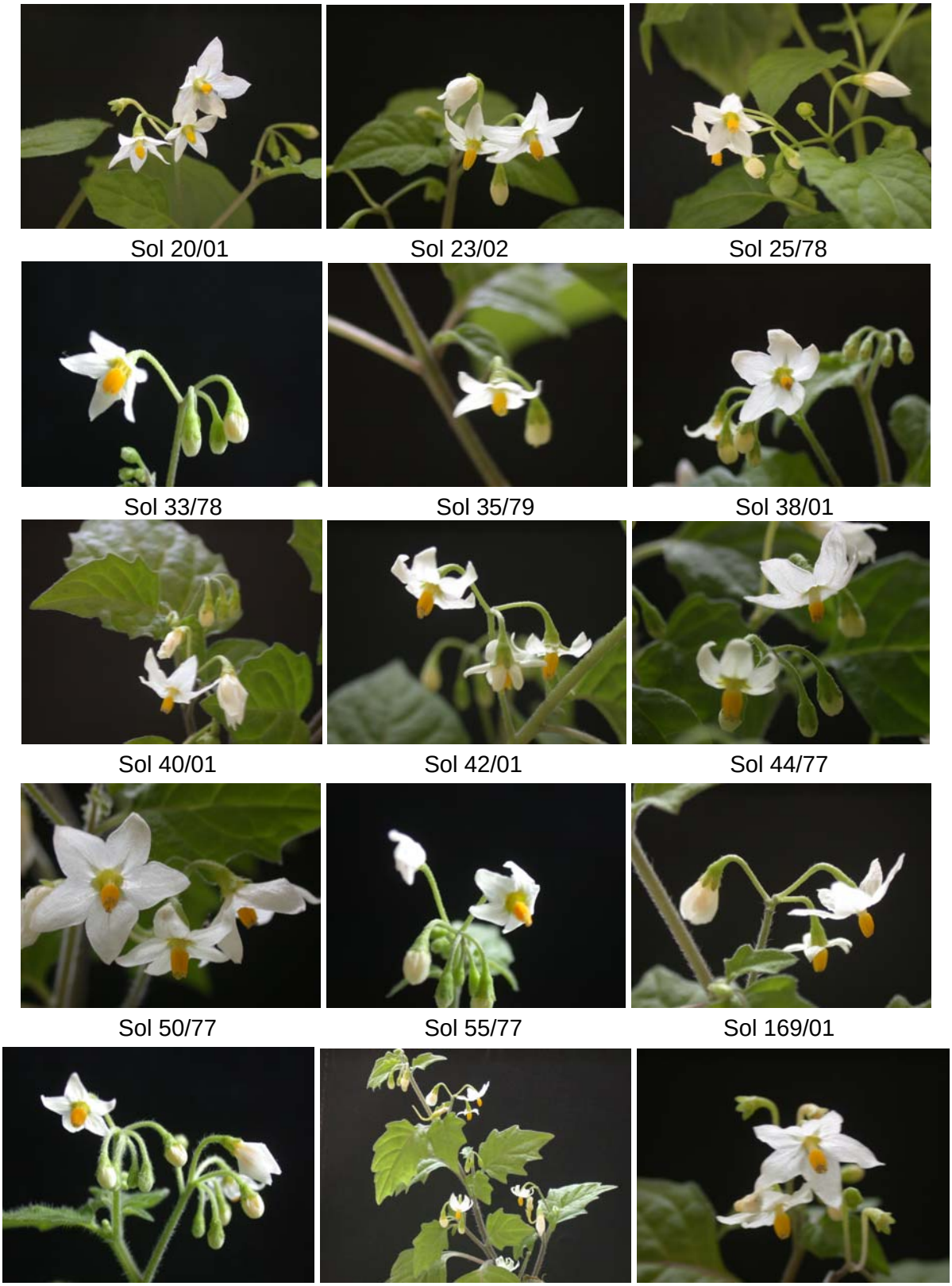


SN18



2003

Figure 41: Flowers of the twenty *S. nigrum* accessions:



Plant 3 Plant 4 Plant 5

S.nigrum nodiflorum SN18

Figure 42: Berries of the twenty *S. nigrum* accessions:

Sol 20/02 Sol 23/02 Sol 25/78

Sol 33/79 Sol 35/79 Sol 38/08

Sol 40/01 Sol 42/01 Sol 44/77

Sol 50/77

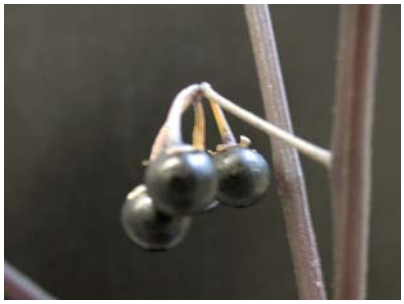
Sol 55/77

Sol 169/01

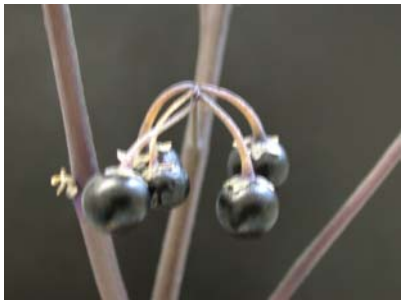
Sol 2003

Sol 2004

Plant 2



Plant3



Plant4

Plant5

S.nigrum nodiflorum

SN18

Figure 43: Some *S. nigrum* accessions 6 weeks after infection with T04:


Sol 20/02


Sol 23/02


Sol 35/79


Sol 38/08


Sol 40/01


Sol 42/01


Sol 44/77


Sol 50/77


Sol 55/77


Sol 169/01


Plant 2


Plant3


Plant5

Figure 44: All *Sn Eds1* sequences alignment:

```
1E              AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTAGGGACAC 59
55E             AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTAGGGACAC 59
8Ec             AAGGTATGAGCTCCCAGATAGCTTTCGAGGGACGAAAGGATTGGATACAACTAGGGACAC 60
49Ec-F          AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTCGGGACAC 59
11Ec-139F       AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTCGGGACAC 59
16Eds1c-r       AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTCGGGACAC 59
83eds1c-F       AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTAGGGACAC 59
19E             AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGATTGGATACAACTAGGGACAC 59
26E             AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTAGGGACAC 59
21E             AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGATTGGATACAACTAGGGACAC 59
47E             AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGATTGGATACAACTAGGGACAC 59
115Ec-rev       AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAAGGACTGGATACAACTAGGGACAC 59
Tomato          AAGGTATGAGCTCCCAGATAGCTT-CGAGGGACGAAGAGACTGGATAGAACTAGGGACAC 59
                ************************ ***********  ** ****** **** *******

1E              AGTTTCGCAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACATGAAGAATG 119
55E             AGTTTCGCAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACATGAAGAATG 119
8Ec             AGTTTCGCAGGCAAGTTGAGCCTTTGGATATAGCAAACTATTACAGGCACATGAAGAATG 120
49Ec-F          AGTTTCGTAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACTTGAAGAATG 119
11Ec-139F       AGTTTCGTAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACTTGAAGAATG 119
16Eds1c-r       AGTTTCGTAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACTTGAAGAATG 119
83eds1c-F       AGTTTCGCAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACATGAAGAATG 119
19E             AGTTTCGCAGGCAAGTTGAGCCTTTGGATATAGCAAACTATTACAGGCACTTGAAGAATG 119
26E             AGTTTCGCAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACATGAAGAATG 119
21E             AGTTTCGCAGGCAAGTTGAGCCTTTGGATATAGCAAACTATTACAGGCACTTGAAGAATG 119
47E             AGTTTCGCAGGCAAGTTGAGCCTTTGGATATAGCAAACTATTACAGGCACTTGAAGAATG 119
115Ec-rev       AGTTTCGCAGGCAAGTTGAGCCCTTGGATATAGCAAACTATTACAGGCACATGAAGAATG 119
Tomato          AGTTTCGCAGGCAAGTTGAGCCCTTGGATATTGCAAACTATTACAGGCACTTGAAGAATG 119
                ******* ************** ******** ****************** *********

1E              AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGAT 179
55E             AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGAT 179
8Ec             AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGAT 180
49Ec-F          AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGCTATCGGTACACACAGAGAT 179
11Ec-139F       AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGAT 179
16Eds1c-r       AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGAT 179
83eds1c-F       AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGTTATCTGTTCACACAGAGAT 179
19E             AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGCTATCGGTACACACAGAGAT 179
26E             AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGCTATCGGTACACACAGAGAT 179
21E             AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGCTATCGGTACACACAGAGAT 179
47E             AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGCTATCGGTACACACAGAGAT 179
115Ec-rev       AAGATACTGGACCTTACATGATCAGGGCTAGGCCTAAGCGCTATCGGTACACACAGAGAT 179
Tomato          AAGATACTGGACCTTACTTGATCAGGGCTAGGCCTAAGCGTTATCGGTTCACACAGAGAT 179
                ***************** ********************** **** ** ***********

1E              GGTTAGAGCATGCTGAGAAAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 239
55E             GGTTAGAGCATGCTGAGAAAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 239
8Ec             GGTTAGAGCATGCTGAGAAAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 240
49Ec-F          GGTTAGAGCATGCTGAGAGAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 239
11Ec-139F       GGTTAGAGCATGCTGAGAAAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 239
16Eds1c-r       GGTTAGAGCATGCTGAGAAAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 239
83eds1c-F       GGTTAGAGCATGCTGAGAAAGTGCAAGCTGGTACGCGTTCCGAGTCTTGTTTCTGGGCAG 239
19E             GGTTAGAGCATGCTGAGAGAGTGCAAGCTGGTGCACGTTCCGAGTCTTGTTTCTGGGCAG 239
26E             GGTTAGAGCATGCTGAGAGAGTGCAAGCTGGTGCGCGTTCCGAGTCTTGTTTCTGGGCAG 239
21E             GGTTAGAGCATGCTGAGAGAGTGCAAGCTGGTGCACGTTCCGAGTCTTGTTTCTGGGCAG 239
47E             GGTTAGAGCATGCTGAGAGAGTGCAAGCTGGTGCACGTTCCGAGTCTTGTTTCTGGGCAG 239
115Ec-rev       GGTTAGAGCATGCTGAGAGAGTGCAAGCTGGTGCACGTTCCGAGTCTTGTTTCTGGGCAG 239
Tomato          GGTTAGAGCATTTTGATAGAGTGCAAGCTGGTGCACGTTCCGAGTCTTGTTTCTGGGCAG 239
                ***********  *** * ************* * *************************

1E              AAGTGGAGGAACTTAGAAACAAGCAATTTGCAGAAGGGCAAAACAGGGTTTTGAGTTTAG 299
55E             AAGTGGAGGAACTTAGAAACAAGCAATTTGCAGAAGGGCAAAACAGGGTTTTGAGTTTAG 299
8Ec             AAGTGGAGGAACTTAGAAACAAGCAATTTGCAGAAGGGCAAAACAGGGTTTTGAGTTTAG 300
49Ec-F          AAGTGGAGGAACTTAGAAACAAGCAATTTGCAGAAGGGCAAAACAGGGTTTTGAGTTTAG 299
11Ec-139F       AAGTGGAGGAACTTAGAAACAAGCCATTTGCAGAAGAACAAAACAGGGTTTTGAGTTTAG 299
16Eds1c-r       AAGTGGAGGAACTTAGAAACAAGCAATTTGCAGAAGGGCAAAACAGGGTTTTGAGTTTAG 299
83eds1c-F       AAGTGGAGGAACTTAGAAACAAGCCATTTGCAGAAGGGCAAAACAGGGTTTTGAGTTTAG 299
19E             AAGTGGAGGAACTAAGAAACAAGCCATTTGCAGAAGAACAAAACAGGATTTTGAGTTTAG 299
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26E             AAGTGGAGGAACTAAGAAACAAGCCATTTGCAGAAGAACAAAACAGGGTTTTGAGTTTAG 299
21E             AAGTGGAGGAACTAAGAAACAAGCCATTTGCAGAAGAACAAAACAGGATTTTGAGTTTAG 299
47E             AAGTGGAGGAACTAAGAAACAAGCCATTTGCAGAAGAACAAAACAGGATTTTGAGTTTAG 299
115Ec-rev       AAGTGGAGGAACTAAGAAACAAGCCATTTGCAGAAGAACAAAACAGGATTTTGAGTTTAG 299
Tomato          AAGTTGAGGAACTAAGAAACAAGCCATTTGCACAAGTGCAAGACAGGGTTTTGAATTTAG 299
                **** ******** ********** ******* ***  *** ***** ****** *****

1E              AAACAACTGCAAGTGGCTGGATCCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
55E             AAACAACTGCAAGTGGCTGGATCCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
8Ec             AAACAACTGCAAGTGGCTGGATCCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 360
49Ec-F          AAACAACTGCAAGTGGCTGGATCCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
11Ec-139F       AAACAGCTGCAAGTGGCTGGATTCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
16Eds1c-r       AAACAACTGCAAGTGGCTGGATCCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
83eds1c-F       AAACAGCTGCAAGTGGCTGGATTCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
19E             AAACAGCTGCAAGTGGCTGGATTCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
26E             AAACAGCTGCAAGTGGCTGGATTCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
21E             AAACAGCTGCAAGTGGCTGGATTCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
47E             AAACAGCTGCAAGTGGCTGGATTCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
115Ec-rev       AAACAGCTGCAAGTGGCTGGATTCAGAGTGGCCTTCTTGGCAATGATGTTTTCTTCCCTG 359
Tomato          AAACAGCTGCAAATGGCTGGATCCAGAGTAGCCTTCTTGGCGATGATATTTTCTTCCCTG 359
                ***** ****** ********* ****** *********** ***** ************

1E              AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGACTGGCATCTTGGG 419
55E             AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGACTGGCATCTTGGG 419
8Ec             AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGACTGGCATCTTGGG 420
49Ec-F          AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGACTGGCATCTTGGG 419
11Ec-139F       AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGACTGGCATCTTGGG 419
16Eds1c-r       AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGATTGGCATCTTGGG 419
83eds1c-F       AGTCTACCTTTGCTAAATGGTGGAAAACACTCCCTACTCAGCACAGATTGGCATCTTGGG 419
19E             AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGATTGGCATCTTGGG 419
26E             AGTCTACCTTTACTAAATGGTGGAAAACACTCCCTACTCAGCACAGATTGGCATCTTGGG 419
21E             AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGATTGGCATCTTGGG 419
47E             AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGATTGGCATCTTGGG 419
115Ec-rev       AGTCTACCTTTACCAAATGGTGGAAAACACTCCCTACTCAGCACAGATTGGCATCTTGGG 419
Tomato          AATCTACCTATACCAAGTGGTGGAAAACACTCCCACCTCAGCACAAACAGGCATCTTGGG 419
                * ******* * * ** *****************  ********* *  ***********

1E              TATCAGGGAAAATATCTTCTTAGCGCCTTGCGCCCTAATATTTTGAGAAGGATGATATCC 479
55E             TATCAGGGAAAATATCTTCTTAGCGCCTTGCGCCCTAATATTTTGAGAAGGATGATATCC 479
8Ec             TATCAGGGAAAATATCTTCTTAGCGCCTTGCGCCCTAATATTTTGAGAAGGATGATATCC 480
49Ec-F          TATCAGGGAAAATATCTTCTTAGCGCCTTGCGCCCTAATATTTTGAGAAGGATGATATCC 479
11Ec-139F       TATCAGGGAAAATATCTTCTTAGCGCCTTGCGCCCTAATATTTTGATAAAGATGGTATCC 479
16Eds1c-r       TATCAGGGAAAATAACTTTTTAGCGCGTTGCACCCTAATGTTTTGATAAAGATGGTATTC 479
83eds1c-F       TATCAGGGAAAATAACTTTTTAGCGCGTTGCACCCTAATGTTTTGATAAAGATGGTATCC 479
19E             TATCAGGGAAAATAACTTTTTAGCGCGTTGCACCCTAATGTTTTGATAAAGATGGTATTC 479
26E             TATCAGGGAAAATAACTTTTTAGCGCGTTGCACCCTAATGTTTTGATAAAGATGGTATTC 479
21E             TATCAGGGCAAATAACTTCTTAGCGCGTTGCGCCCTAATATTTTGATAAAGATGGTATCC 479
47E             TATCAGGGCAAATAACTTCTTAGCGCGTTGCGCCCTAATATTTTGATAAAGATGGTATCC 479
115Ec-rev       TATCAGGGCAAATATCTTCTTAGCGCGTTGCGCCCTAATATTTTGATAAAGATGGTATCC 479
Tomato          TGTCAAGGAAAATAACTCCTTAGCGCCTTGCGCCCTAATATCTTGATAAAGATGGTATCC 479
                * *** ** ***** **  ******* **** ******* * **** ** **** *** *

1E                AAGCCAACTTGCAGACACCTAAAACATTCCACCACATATATTCCCATGCCCC 531
55E               AAGCCAACTTGCAGACACCTAAAACATTCCACCACATATATTCCCATGCCCC 531
8Ec               AAGCCAACTTGCAGACACCTAAAACATTCCACCACATATATTCCCATGCCCC 532
49Ec-F            AAGCCAACTTGCAGACACCTAAAATATTCCACCACGTATGTTCCCATGCCCC 531
11Ec-139F         GAGCTAACTTGCAGACACCTAAAATATTCCACCACATATATTCCCATGCCCC 531
16Eds1c-r         GAGCCAACTTGCAGACACCTCAAACATACCAGCACATATATTCCCATGCCCC 531
83eds1c-F         GAGCTAACTTGCAGACACCTAAAATATTCCACCACATATATTCCCATGCCCC 531
19E               GAGCCAACTTGCAGACACCTCAAACATACCAGCACGTATATTCCCATGCCCC 531
26E               GAGCCAACTTGCAGACACCTCAAACATACCAGCACGTATATTCCCATGCCCC 531
21E               GAGCTAACTTGCAGACACCTAAAATATTCCACCACATATATTCCCATGCCCC 531
47E               GAGCTAACTTGCAGACACCTAAAATATTCCACCACATATATTCCCATGCCCC 531
115Ec-rev         GAGCTAACTTGCAGACACCTAAAATATTCCACCACATATGTTCCCATGCCCC 531
Tomato            CAGCCAACTCACAGACACCTATAACATTCCACCACATATGTTGCAATGCCCC 531
                   *** ****  *********  ** ** *** *** *** ** * *******
```

Figure 45: All *Sn Ndr1* sequences alignment:

```
65Ndr1          CTCGACGAATTCATGGCCCTTCCATTCCGCCACCAGCCAAAACCTACCACCGTAGCCGCG 60
154NDR1C_new    -TCGACGAATTCATGGCCCTTCCATTCCGCCACCAGCCAAAACCTACCACCGTAGCCGCG 59
4Ndr1           CTCGACGAATTCATGGCCCTTCCATTCCGCCACCAGCCAAAACCTACCACCGTAGCCGCG 60
                ************************************************************

65Ndr1c         GTGGTGGTAGTTGTTGCAACCCATGTAGTTGTCTCTTCAATTGTCTTTGTACTTGTATTT 120
154NDR1C_new    GTGGTGGTAGTTGTTGCAACCCATGTAGTTGTCTCTTCAATTGTCTTTGTACTTGTATTT 119
4Ndr1           GTGGTGGTAGTTGTTGCAACCCATGTAGTTGTCTCTTCAATTGTCTTTGTACTTGTATTT 120
                ************************************************************

65Ndr1c         GCCAAATTATCTTCACCCTTGTCATCATCCTCGGAGTCATTGCATTAGTCCTCTGGCTTG 180
154NDR1C_new    GCCAAATTATCTTCACCCTTGTCATCATCCTCGGAGTCATTGCATTAGTCCTCTGGCTTG 179
4Ndr1           GCCAAATTATCTTCACCCTTGTCATCATCCTCGGAGTCATTGCATTAGTCCTCTGGCTTG 180
                ************************************************************

65Ndr1c         TTCTACGTCCTAACAAAGTCAAATTTTACGTGACAGATGCCACGTTGACACAATTCGATT 240
154NDR1C_new    TTCTACGTCCTAACAAAGTCAAATTTTACGTGACAGATGCCACGTTGACACAATTCGATT 239
4Ndr1           TTCTACGTCCTAACAAAGTCAAATTTTACGTGACAGATGCCACGTTGACACAATTCGATT 240
                ************************************************************

65Ndr1c         ATTCCTCAACAAACAACACCCTCTACTACGATCTAGCCCTCAACATGACCATTAGGAACC 300
154NDR1C_new    ATTCCTCAACAAACAACACCCTCTACTACGATCTAGCCCTCAACATGACCATTAGGAACC 299
4Ndr1           ATTCCTCAACAAACAACACCCTCTACTACGATCTAGCCCTCAACATGACCATTAGGAACC 300
                ************************************************************

65Ndr1c         CCAATAAACGCGTTGGGATCTACTACGATTCAATTGAAGTGAGAGCTATGTATGGTGGCC 360
154NDR1C_new    CCAATAAACGCGTTGGGATCTACTACGATTCAATTGAAGTGAGAGCTATGTATGGTGGCC 359
4Ndr1           CCAATAAACGCGTTGGGATCTACTACGATTCAATTGAAGTGAGAGCTATGTATGGTGGCC 360
                ************************************************************

65Ndr1c         AGAGATTTGCTAGTCAAAATTTGGAACCGTTTTATCAGGGTCATAAAAATACTAGCAGTT 420
154NDR1C_new    AGAGATTTGCTAGTCAAAATTTGGAACCGTTTTATCAGGGTCATAAAAATACTAGCAGTT 419
4Ndr1           AGAGATTTGCTAGTCAAAATTTGGAACCGTTTTATCAGGGTCATAAAAATACTAGCAGTT 420
                ************************************************************

65Ndr1c         TGCATCCGGCGTTTAAAGGACAGAGTTTGGTTCTATTGGGAGATAGAGAGAAATCAAATT 480
154NDR1C_new    TGCATCCGGCGTTTAAAGGACAGAGTTTGGTTCTATTGGGAGATAGAGAGAAATCAAATT 479
4Ndr1           TGCATCCGGTGTTTAAAGGACAGAGTTTGGTTCTATTGGGAGATAGAGAGAAATCAAATT 480
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65Ndr1c         ACAATAATGAGAAGAATTTAGGGGCTCGAGCGCGCG 516
154NDR1C_new    ACAATAATGAGAAGAATTTAGGGGCTCGAGCGCGCG 515
4Ndr1           ACAATAATGAGAAGAATTTAGGGGCTCGAGCGCGCG 516
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Figure 46: All *SnRar1* sequences alignment:

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13Rar1-F        GTGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 60
66R             -TGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 59
72R             GTGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 60
63Rar1-rev      GTGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 60
17Rar1-rev      GTGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 60
54R             -TGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 59
21R-F           GTGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 60
15Rar1c-rev     -GTGCCATCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 59
39R             ------------GGTGCNTGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 48
37Rar1-rev      GTGCCATCCTTTGGTGCATGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 60
5R              GTGCCATCCTTTGGTGCAGGAGGTATGCTCATGAACTCGTCAAATTCTTTGACGTGAAT 60
                             *****  ****************************************

13Rar1-F        ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 120
66R             ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 119
72R             ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 120
63Rar1-rev      ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 120
17Rar1-rev      ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 120
54R             ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 119
21R-F           ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 120
15Rar1c-rev     ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 119
39R             ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 108
37Rar1-rev      ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 120
5R              ATCACAGCATTTCCATCCTCTCATCCGGTCATGGAAGATAGCGGGGCCAGGATGGTAACT 120
                ************************************************************

13Rar1-F        GCAAGCAGTGTCGTGATTTTCCTTTTCTGTAAAGGTCTTACCGCAGCCCTTGTTTTTACA 180
66R             GCAAGCAGTGTCGTGATTTTCCTTTTCTGTAAAGGTCTTACCGCAGCCCTTGTTTTTACA 179
72R             GCAAGCAGTGTCGTGATTTTCCTTTTCTGTAAAGGTCTTACCGCAGCCCTTGTTTTTACA 180
63Rar1-rev      GCAAGCAGTGTCGTGATTTTCCTTTTCTGTAAAGGTCTTACCGCAGCCCTTGTTTTTACA 180
17Rar1-rev      GCAGGCAGTGTCATGATTTTCCTTTTCTGTAAACGTCTTACCACAGCCCTTGTTTTTACA 180
54R             GCAGGCAGTGTCATGATTTTCCTTTTCTGTAAACGTCTTACCACAGCCCTTGTTTTTACA 179
21R-F           GCAGGCAGTGTCATGATTTTCCTTTTCTGTAAACGTCTTACCACAGCCCTTGTTTTTACA 180
15Rar1c-rev     GCAAGCAGTGTCATGATTTTCCTTTTCTTTAAAGGTCTTACCACAGCCCTTATTTTTACA 179
39R             GCAAGCAGTGTCATGATTTTCCTTTTCTTTAAAGGTCTTACCACAGCCCTTATTTTTACA 168
37Rar1-rev      GCAAGCAGTGTCATGATTTTCCTTTTCTTTAAAGGTCTTACCACAGCCCTTATTTTTACA 180
5R              GCAAGCAGTGTCATGATTTTCCTTTTCTTTAAAGGTCTTACCACAGCCCTTATTTTTACA 180
                *** ******** *************** **** ******** ******** ********

13Rar1-F        AATTTGGGGCTCGTTGATATCAATTTTCTTTTTCACTGGAGCCGGATGGTCTTGCTTTAC 240
66R             AATTTGGGGCTCGTTGATATCAATTTTCTTTTTCACTGGAGCCGGATGGTCTTGCTTTAC 239
72R             AATTTGGGGCTCGTTGATATCAATTTTCTTTTTCACTGGAGCCGGATGGTCTTGCTTTAC 240
63Rar1-rev      AATTTGGGGCTCGTTGATATCAATTTTCTTTTTCACTGGAGCCGGATGGTCTTGCTTTAC 240
17Rar1-rev      AATTTGGGGCTCGTTAATATCAATTTTCTTCTTCACCGGGGCCGGATGGTCTTGCTTTAC 240
54R             AATTTGGGGCTCGTTAATATCAATTTTCTTCTTCACCGGGGCCGGATGGTCTTGCTTTAC 239
21R-F           AATTTGGGGCTCGTTAATATCAATTTTCTTCTTCACCGGGGCCGGATGGTCTTGCTTTAC 240
15Rar1c-rev     AATTTGAGGCTCGTTAATATCAATTTTCTTCTTCACTGGGGCCGGATGGTCTTGCTTTAC 239
39R             AATTTGAGGCTCGTTAATATCAATTTTCTTCTTCACTGGGGCCGGATGGTCTTGCTTTAC 228
37Rar1-rev      AATTTGAGGCTCGTTAATATCAATTTTCTTCTTCACTGGGGCCGGATGGTCTTGCTTTAC 240
5R              AATTTGAGGCTCGTTAATATCAATTTTCTTCTTCACTGGGGCCGGATGGTCTTGCTTTAC 240
                ****** ******** ************** ***** ** ********************

13Rar1-F        ATCTGTATTGCTCTCAGAAGGTACAGATGCTACTGTATTTGATGCCTTTTGAATTGCTTC 300
66R             ATCTGTATTGCTCTCAGAAGGTACAGATGCTACTGTATTTGATGCCTTTTGAATTGCTTC 299
72R             ATCTGTATTGCTCTCAGAAGGTACAGATGCTACTGTATTTGATGCCTTTTGAATTGCTTC 300
63Rar1-rev      ATCTGTATTGCTCTCAGAAGGTACAGATGCTACTGTATTTGATGCCTTTTGAATTGCTTC 300
17Rar1-rev      ATCTGTATTGCCCTCAGAAGGTACAGATGTTACTGTATTAGATGCTTTTGGAATTGCTTC 300
54R             ATCTGTATTGCCCTCAGAAGGTACAGATGTTACTGTATTAGATGCTTTTGGAATTGCTTC 299
21R-F           ATCTGTATTGCCCTCAGAAGGTACAGATGTTACTGTATTAGATGCTTTTGGAATTGCTTC 300
15Rar1c-rev     ATCTGTATTGCTCTCAGAAGGTACAGATGTTACTGTATTTGATGCTTTCGGAATTGCTTC 299
39R             ATCTGTATTGCTCTCAGAAGGTACAGATGTTACTGTATTTGATGCTTTCGGAATTGCTTC 288
37Rar1-rev      ATCTGTATTGCTCTCAGAAGGTACAGATGTTACTGTATTTGATGCTTTCGGAATTGCTTC 300
5R              ATCTGTATTGCTCTCAGAAGGTACAGATGTTACTGTATTTGATGCTTTCGGAATTGCTTC 300
                *********** ***************** ********* ***** **  **********

13Rar1-F        TCTGGGTTGTGAACCGTGATCAGAACAAAAAAATCCCTGGCGGCNTCTAGGACAGGCTTC 360
66R             TCTGGGTTGTGAACCGTGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 359
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72R             TCTGGGTTGTGAACCGTGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 360
63Rar1-rev      TCTGGGTTGTGAACCGTGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 360
17Rar1-rev      TCTGGGTTGTGAACCGTGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 360
54R             TCTGGGTTGTGAACCGTGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 359
21R-F           TCTGGGTTGTGAACCGTGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 360
15Rar1c-rev     CCTGGGTTGTGAACCATGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 359
39R             CCTGGGTTGTGAACCATGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 348
37Rar1-rev      CCTGGGTTGTGAACCATGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 360
5R              CCTGGGTTGTGAACCATGATCAGAACAAAAAAATCCCTGGCGGCATCTAGGACAGGCTTC 360
                ************** ***************************** ***************

13Rar1-F        TTTCGGTGATGCATTGGTCGTAGGAGCTGGCACAGGCACAGGCCACAGGAATTGCTTTAT 420
66R             TTTCGGTGATGCATTGGTCGTAGGAGCTGGCACAGGCACAGGC-ACAGGAATTGCTTTAT 418
72R             TTTCGGTGATGCATTGGTCGTAGGAGCTGGCACAGGCACAGGC-ACAGGAATTGCTTTAT 419
63Rar1-rev      TTTCGGTGATGCATTGGTCGTAGGAGCTGGCACAGGCACAGGC-ACAGGAATTGCTTTAT 419
17Rar1-rev      TTTCGGTGATGCATTGGTCATAGGAGCTGCCACAGGCGCAGGC-GCAGGAATTGCTTTAT 419
54R             TTTCGGTGATGCATTGGTCATAGGAGCTGCCACAGGCGCAGGC-GCAGGAATTGCTTTAT 418
21R-F           TTTCGGTGATGCATTGGTCATAGGAGCTGCCACAGGCGCAGGC-GCAGGAATTGCTTTAT 419
15Rar1c-rev     TTTCGCTGATGCATTGGTCATAGGAGCTGCCACAGGCGCAGGC-GCATGAATTGCTTTAT 418
39R             TTTCGCTGATGCATTGGTCATAGGAGCTGCCACAGGCGCAGGC-GCATGAATTGCTTTAT 407
37Rar1-rev      TTTCGCTGATGCATTGGTCATAGGAGCTGCCACAGGCGCAGGC-GCATGAATTGCTTTAT 419
5R              TTTCGCTGATGCATTGGTCATAGGAGCTGCCACAGGCGCAGGC-GCATGAATTGCTTTAT 419
                ***** ************* ********* ******* *****  ** ************

13Rar1-F         TTTTGGTAACAGGTGGCTTTGCGATCACTGGTTTTTCTGTTGTGTGCTTTCCT 473
66R              TTTTGGTAACAGGTGGCTTTGCGATCACTGGTTTTTCTGTTGTGTGCTTTCCT 471
72R              TTTTGGTAACAGGTGGCTTTGCGATCACTGGTTTTTCTGTTGTGTGCTTTCCT 472
63Rar1-rev       TTTTGGTAACAGGTGGCTTTGCGATCACTGGTTTTTCTGTTGTGTGCTTTCCT 472
17Rar1-rev       TTTTGGTAACAGGTGGCTTTGCGATCACTGGTTTTTCTGTTGTGTGCTTTCCT 472
54R              TTTTGGTAACAGGTGGCTTTGCGATCACCGGGTTT------------------ 453
21R-F            TTTTGGTAACAGGTGGCTTTGCGATCACTGGTTTTTCTGTTGTGTGCTTTCCT 472
15Rar1c-rev      TTATGGTAACAGGTGGCTTTGCAATCACTGGTTTTTCTGTTGTGTGCTTTCCT 471
39R              TTATGGTAACAGATGGCTTTGCAATCACTGGTTTTTC-GTTGTGTGCTTTCCT 459
37Rar1-rev       TTATGGTAACAGGTGGCTTTGCAATCACTGGTTTTTCTGTTGTGTGCTTTCCT 472
5R               TTATGGTAATAGGTGGCTTTGCAATCACTGGTTTTTCTGTTGTGTGCTTTCCT 472
                 ** ****** ** ********* ***** ** ***
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Figure 47: All *SnTga2.2* sequences alignment:

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21Tga22-rev      CGGGAATTCGACATATTCAGGATAAAAGGGGATGCAGCGAAGGCTGACGTTTTTCACATA 60
94TGA22-rev      CGGGAATTCGACATATTCAGGATAAAAGGGGATGCAGCGAAGGCTGACGTTTTTCACATA 60
67Tga22-F        CGGGAATTCGACATATTCAGGATAAAAGGGGATGCAGCGAAGGCTGACGTTTTTCACATA 60
113Tga22-F       CGGGAATTCGACATATTCAGGATAAAAGGGGATGCAGCGAAGGCTGACGTTTTTCACATA 60
99Tga22c-rev     CGGGAATTCGACATATTCAGGATAAAAGGGGATGCAGCGAAGGCTGACGTTTTTCACATA 60
33TGA22-139F     CGGGAATTCGACATATTCAGGATAAAAGGGGATGCAGCGAAGGCTGACGTTTTTCACATA 60
13Tga22-F        CGGGAATTCGACATATTCAGGATAAA-GGGGATGCAGCGAAGGCTGACGTTTTTCACATA 59
39Tga22-rev      CGGGAATTCGACAC-TTCAGGATAAAAAGGGATGCAGCGAAGGCTGACGTTTTTCACATA 59
                 *************  ***********  ********************************

21Tga22-rev      TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 120
94TGA22-rev      TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 120
67Tga22-F        TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 120
113Tga22-F       TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 120
99Tga22c-rev     TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 120
33TGA22-139F     TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 120
13Tga22-F        TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 119
39Tga22-rev      TTGTCGGGCATGTGGAAAACTCCTGCAGAAAGATGCTTCTTGTGGCTTGGTGGATTCCGT 119
                 ************************************************************

21Tga22-rev      TCGTCTGAACTCCTCAAGCTGCTCATTAATCAGTTGGAGCCTTTAACCGAACAACAATTA 180
94TGA22-rev      TCGTCTGAACTCCTCAAGCTGCTCATTAATCAGTTGGAGCCTTTAACCGAACAACAATTA 180
67Tga22-F        TCGTCTGAACTCCTTAAGCTGCTCATCAACCAGTTGGAGCCTTTAACCGAACAACAATTA 180
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113Tga22-F        TCGTCTGAACTCCTCAAGCTGCTCATTAATCAGTTGGAGCCTTTAACCGAACAACAATTA 180
99Tga22c-rev      TCGTCTGAACTCCTCAAGCTGCTCATTAATCAGTTGGAGCCTTTAACCGAACAACAATTA 180
33TGA22-139F      TCGTCTGAACTCCTCAAGCTGCTCATTAATCAGTTGGAGCCTTTAACCGAACAACAATTA 180
13Tga22-F         TCGTCTGAACTCCTCAAGCTGCTCATTAATCAGTTGGAGCCTTTAACCGAACAACAATTA 179
39Tga22-rev       TCGTCTGAACTCCTCAAGCTGCTCATTAATCAGTTGGAGCCTTTAACCGAACAACAATTA 179
                  ************** *********** ** ******************************

21Tga22-rev       TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 240
94TGA22-rev       TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 240
67Tga22-F         TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 240
113Tga22-F        TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 240
99Tga22c-rev      TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 240
33TGA22-139F      TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 240
13Tga22-F         TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 239
39Tga22-rev       TTGGCGATCAACAACTTACAACAGTCATCCCAACAGGCGGAGGATGCTTTATCCCAGGGA 239
                  ************************************************************

21Tga22-rev       ATGGAGGCATTGCAGCAGTCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 300
94TGA22-rev       ATGGAGGCATTGCAGCAGTCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 300
67Tga22-F         ATGGAGGCATTGCAGCAGTCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 300
113Tga22-F        ATGGAGGCATTGCAGCAGTCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 300
99Tga22c-rev      ATGGAGGCATTGCAGCAGTCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 300
33TGA22-139F      ATGGAGGCATTGCAGCAGTCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 300
13Tga22-F         ATGGAGGCATTGCAGCAGTCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 299
39Tga22-rev       ATGGAGGCATTGCGGCAGNCTTTGGCTGAGACTCTCGCAGGGTCTCTTGGACCTTCAGGT 299
                  ************* **** ****************************************

21Tga22-rev       TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 360
94TGA22-rev       TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 360
67Tga22-F         TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 360
113Tga22-F        TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 360
99Tga22c-rev      TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 360
33TGA22-139F      TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 360
13Tga22-F         TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 359
39Tga22-rev       TCCTCTGGGAATGTTGCAAATTATATGGGTCAAATGGCCATGGCAATGGGGAAGCTTGGA 359
                  ************************************************************

21Tga22-rev       ACTCTTGAGGGCTTCATACGGCAGGCTGATAATCTTCGGCAACAGACATTGCAGCAAATG 420
94TGA22-rev       ACTCTTGAGGGCTTCATACGGCAGGCTGATAATCTTCGGCAACAGACATTGCAGCAAATG 420
67Tga22-F         ACTCTTGAGGGCTTCATATGGCAGGCTGATAATCTTCGGCAACAGACATTGCAGCAAATG 420
113Tga22-F        ACTCTTGAGGGCTTCATACGGCAGGCTGATAATCTTCGGCAACAGACATTGCAGCAAATG 420
99Tga22c-rev      ACTCTTGAGGGCTTCATACGGCAGGCTGATAATCTTCGGCAACAGACATTGCAGCAAATG 420
33TGA22-139F      ACTCTTGAGGGCTTCATACGGCAGGCTGATAACCTTCGGCAACAGACATTGCAGCAAATG 420
13Tga22-F         ACTCTTGAGGGCTTCATACGGCAGGCTGATAACCTTCGGCAACAGACATTGCAGCAAATG 419
39Tga22-rev       ACTCTTGAGGGCTTCATACGGCAGGCTGATAACCTTCGGCAACAGACATTGCAGCAAATG 419
                  ****************** ************* ***************************

21Tga22-rev       CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 480
94TGA22-rev       CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 480
67Tga22-F         CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 480
113Tga22-F        CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 480
99Tga22c-rev      CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 480
33TGA22-139F      CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 480
13Tga22-F         CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 479
39Tga22-rev       CATCGCATATTGACAACTCGCCAATCAGCTCGTGCTCTTCTTGCTATCAGTGATTATTTC 479
                  ************************************************************
21Tga22-rev       TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCCGG-AATAGGTACCCCG- 538
94TGA22-rev       TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCCGGGAATAGGTACCCCG- 539
67Tga22-F         TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCCGGGAATAGGTACCCCG- 539
113Tga22-F        TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCCGGGAATAGGTACCCCG- 539
99Tga22c-rev      TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCGGG--ATAGGTACCCCG- 537
33TGA22-139F      TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCGGG-AATAGGTACCCCG- 538
13Tga22-F         TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCCGGGAATAGGTACCCCG- 538
39Tga22-rev       TCTCGGCTTCGAGCACTGAGCTCTCTCTGGCTTGCTCGCCCCCGGGAATAGGTACCCCGG 539
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