RNAi AND FUNCTIONAL CHARACTERIZATION OF GENES INVOLVED IN PLANT-INSECT TRITROPHIC INTERACTIONS

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A tutte le persone che mi vogliono bene

I vostri occhi lucidi sono per me la gioia più grande…
Grazie per avermi sostenuto nei momenti difficili,
e per aver riso con me nei momenti felici.

INDEX

RIASSUNTO

Le piante essendo degli organismi incapaci di allontanarsi dai pericoli, durante la loro vita sono soggette ad una serie di condizioni avverse. Gli stress biotici ed abiotici riescono ad influenzare la loro fisiologia ed il loro sviluppo favorendo cambiamenti sia a livello dell'espressione genica che dell'alterazione del metabolismo cellulare. Nel settore agricolo stress biotici ed abiotici possono causare una riduzione della produttività delle piante, che può arrivare fino alla perdita totale del raccolto a seconda della tipologia di coltura e dello stress applicato al sistema. Gli stress biotici sono legati alla presenza di altri organismi quali macrorganismi, come insetti ed erbivori, e microrganismi, come batteri e funghi. Gli agenti patogeni possono interagire con le piante a seguito di un contatto fisico utilizzando differenti strategie. Per esempio, alcuni agenti patogeni quali particolari classi di batteri proliferano negli spazi intracellulari penetrando nei tessuti fogliari attraverso ferite e lesioni, mentre agenti patogeni quali funghi possono invadere i tessuti estendendo ife negli spazi intercellulari.
Per quanto riguarda gli insetti, questi organismi hanno evoluto molte strategie per superare i meccanismi di difesa della pianta per nutrirsi, crescere e riprodursi.
I meccanismi di difesa delle piante contro gli insetti possono essere diretti o indiretti. Si definiscono meccanismi di difesa diretti quei meccanismi che prevedono degli adattamenti morfologici, fisiologici e biochimici della pianta come ad esempio la presenza di spine, tricomi, cere o resine, al fine di impedire il contatto degli insetti con la superficie della foglia oppure la produzione di sostanze come metaboliti tossici composti volatili repellenti (HIPVs), tossine, inibitori di proteasi, essudati in modo da ostacolare il processo nutritivo degli insetti stessi.
I meccanismi di difesa indiretti invece coinvolgono l'azione di altri organismi viventi, come i nemici naturali degli insetti (parassitoidi e/o predatori). Questi meccanismi sono mediati dal rilascio di miscele di sostanze volatili (VOC) prodotte dalle piante danneggiate (Arimura et al., 2009). Le categorie di molecole presenti nella miscela di volatili sono in comune tra le diverse specie vegetali, come composti C6 quali aldeidi, alcoli, esteri denominati, composti C10 e C15 come terpenoidi e indoli (Parè and Tomlinson, 1999). L'interazione insetto-insetto è molto comune in natura, tra queste interazioni si può citare quella insetto-parassitoide. I parassitoidi sono degli organismi viventi che instaurano con le loro vittime un rapporto trofico affine dal punto di vista tassonomico al parassitismo (parassiti entomofagi). Questi organismi infatti vivono come parassiti durante lo stadio larvale, che termina con la morte dell'ospite portando alla formazione dell'insetto parassitoide adulto (Poirie et al., 2009).
Lo studio delle interazioni antagoniste insetto-insetto rappresenta una fonte innovativa di geni bersaglio per il controllo degli insetti dannosi. Per esempio, la caratterizzazione e l'analisi funzionale di una proteina codificata dal gene *102* di *Heliothis virescens* ha indicato che questo gene svolge un ruolo chiave nella risposta immunitaria degli insetti (Pennacchio et al., 2003; Pennacchio et al., 2012). Il gene *102* è coinvolto nella localizzazione del processo di melanizzazione della capsula emocitaria, mediato dalla produzione di fibrille amiloidi, intorno ad oggetti estranei ed è essenziale per la formazione della capsula stessa (Di Lelio et al., 2014). Dopo lo stimolo dato dal sistema immunitario, le fibrille amiloidi vengono rilasciate sulla superficie del corpo estraneo, dove formano uno strato che funge da impalcatura molecolare, che promuove la sintesi di melanina e l'avvio del processo di incapsulamento localizzato. Quando larve di *Heliothis virescens* sono attaccate dal parassitoide *Braconidae Toxoneuron nigrigeps* è stata osservata una sotto regolazione del gene *102*, attraverso un meccanismo mediato da un RNA non codificante (rc5'ntTnBV) complementare all'estremità 5' del gene. Studi condotti sull'iniezione per via orale di RNA a doppio filamento (dsRNA) del

gene *102*, in larve mature di lepidotteri (*Spodoptera littoralis*) hanno dimostrato un inibizione del processo di incapsulamento e di melanizzazione di sfere cromatografiche iniettate nell'intestino delle larve (Di Lelio et al., 2014).
Tra gli insetti che causano danni ingenti alle colture agricole è importante citare gli afidi. Gli afidi sono degli insetti fitomizi capaci di alimentarsi dai tessuti vascolari delle piante utilizzando un apparato boccale pungente succhiatore, detto stiletto. Questi insetti riescono a stabilire delle lunghe interazioni con le piante durante le quali sottraggono ingenti quantità di linfa elaborata e fotosintetati. L'attacco degli afidi può causare dei danni diretti, quali l'ingiallimento e la deformazione delle foglie, dovuti alla sottrazione della linfa e delle sostanze vitali, ma anche danni indiretti come la trasmissione di virus e patogeni. A differenza dell'attacco degli insetti erbivori, l'attacco di afidi comporta minimi danni meccanici anche se, la loro prolungata interazione riesce ad influire significativamente sulla fisiologia della pianta con conseguente attivazione di *pathways* metabolici diversi rispetto a quelli attivati a seguito dell'attacco degli insetti masticatori.
Lo scopo del progetto è stato quello di sviluppare nuove conoscenze e strumenti volti alla comprensione dei meccanismi di difesa delle piante al fine di sviluppare nuove metodologie per aumentare la loro resistenza a seguito dell'attacco da parte degli insetti.Per raggiungere questo scopo sono stati sfruttati recenti studi di genomica e trascrittomica riguardanti le interazioni pianta-insetto.
Recentemente, profili trascrizionali relativi a studi di interazione tra piante di pomodoro coltivato (*Solanum lycopersicum*) ed afidi (*Macrosiphum euphorbiae*) hanno messo in evidenza che *NIMIN 2C* (Solyc03g119590.1.1), omologo del modulatore di espressione genica *At-NIMIN-1* (Vlot et al. 2009), è tra i geni più altamente differenzialmente espressi (Coppola et al., 2013).
In *Arabidopsis*, le proteine NIMIN sono in grado di regolare negativamente distinte funzioni di NPR1, elemento fondamentale nel *"cross talk"* tra acido jasmonico (JA) e acido salicilico (SA) (Koornneef and Pieterse, 2008). Attualmente, il ruolo di questi due fitormoni nei meccanismi di difesa attivati a seguito dell'attacco degli afidi non è ancora ben compreso.
Per monitorare i cambiamenti nell'espressione dei geni *NIMIN 2C* (Solyc03g119590.1.1 e Solyc03g119600.1.1) in pomodoro sono stati innanzitutto analizzati, mediante real-time PCR, dati relativi all'*Agilent Tomato microarray*. Tale studio ha messo in evidenza l'attivazione di una risposta dinamica nella pianta a seguito dell'attacco degli afidi; infatti il livello di espressione del gene *NIMIN 2C* (Solyc03g119590.1.1) risulta essere crescente nei tre tempi analizzati (24h, 48h, 96h). L'importanza di questi geni nei meccanismi di difesa risiede nell'azione di regolatori del gene *NPR1* (*NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1*). *NPR1* è considerato un regolatore chiave nell'induzione di geni di difesa, infatti esso è essenziale per l'espressione di geni correlati alla patogenesi (*PR*) e per l'attivazione dei meccanismi di difesa sistemica acquisita (SAR). La proteina NPR1 non ha alcuna regione di legame per il DNA ma interagisce con degli importanti fattori di trascrizione TGA che possiedono una regione ricca di leucine (bZIP), essenziale per l'espressione di geni *PR* e per l'attivazione della SAR. Tipicamente, in una cellula non infetta, NPR1 è localizzato principalmente in forma inattiva oligomerica nel citoplasma. Il complesso oligomerico è tenuto insieme da ponti disolfuro tra residui di cisteina e gli atomi di metalli di transizione. L'incremento della concentrazione dell'acido salicilico a livello intracellulare favorisce la riduzione dei ponti disolfuro e il successivo rilascio della forma attiva monomerica di NPR1 (Hermann et al., 2013). L'equilibrio tra la forma monomerica ed oligomerica è regolato da reazioni di ossido-riduzione. In assenza di

stimoli o di attacchi da patogeni, la reazione di equilibrio è spostata verso la forma oligomerica. L' aumento della concentrazione dell'acido salicilico nella cellula, causata, per esempio, dell'attacco di un patogeno, porta alla riduzione dei ponti disolfurici. NPR1 rilasciato in forma monomerica attiva, viene trasportato nel nucleo dove svolge le sue azioni regolatrici. Nel nucleo, infatti, oligomeri di NPR1 interagiscono con fattori di trascrizione TGA e proteine NIMIN.
In *Arabidopsis*, ci sono quattro geni che codificano per le proteine NIMIN: *NIMIN 1*, *NIMIN 1b*, *NIMIN 2* e *NIMIN 3*. I quattro geni *NIMIN* sono espressi diversamente e codificano per proteine che influiscono in modo diverso sull'espressione dei geni *PR*. Infatti è stato dimostrato che mentre le proteine NIMIN 1b, NIMIN 1 e NIMIN 2 riescono ad interagire con l'estremità C-terminale di NPR1, la proteina NIMIN 3, possiede un dominio differente con il quale è in grado legare l'estremità N-terminale di NPR1 (Hermann et al., 2013). L'allineamento delle sequenze proteiche codificate dai geni *NIMIN* in *Arabidopsis* e la costruzione di una matrice di similarità, ha messo in evidenza che l'indice di similarità tra le queste proteine risulta essere basso.
Per avere ulteriori informazioni riguardo la funzione di questi geni in *Arabidopsis* sono stati condotti differenti studi in banche dati con lo scopo di collegare i loro livelli di espressione a differenti tipologie di tessuto e di stress biotici ed abiotici applicati al sistema. A tal proposito sono stati dunque analizzati i dati provenienti dal *GeneChip Arabidopsis ATH1 Genome Array* per i geni *NIMIN 1* e *NIMIN 3*. Il risultato di tale analisi ha messo in evidenza differenti livelli di espressione dei due geni nei vari tessuti e nei i differenti stadi di sviluppo della pianta analizzati. I due geni risultano, inoltre, essere differenzialmente regolati anche a seguito di stress biotici come presenza di funghi e batteri oppure variazioni nei livelli di concentrazione di ormoni, presenza di elicitori ma a seguito di applicazioni al sistema di stress abiotici quali la variazione del fotoperiodo, della temperatura e delle concentrazioni di CO_2. Inoltre i due geni (*NIMIN 1* e *NIMIN 3*) risultano essere connessi ad un network genetico differente. I geni *NIMIN* quindi risultano essere associati e probabilmente regolati da reti molecolari differenti. Un'analisi di co-espressione ha, inoltre, messo in risalto la possibilità di legame tra i membri della famiglia dei geni *NIMIN* ed importanti geni legati alla risposta contro gli stress.
Per quanto riguarda i geni *NIMIN* in pomodoro, una ricerca sul *Sol Genomics database* ha messo in evidenza l'esistenza di due geni *NIMIN 2C* nella specie *Solanum licopersicum* Solyc03g119590.1.1 e Solyc03g119600.1.1. L'allineamento tra le sequenze proteiche NIMIN 2C in pomodoro ha rivelato che la percentuale di identità di sequenza tra le proteine risulta essere maggiore rispetto a quella esistente tra le proteine NIMIN in *Arabidopsis*. La locazione dei due geni *NIMIN 2C* in pomodoro sullo stesso cromosoma (Solyc03g119590.1.1: SL2.50ch03: 68154784.68155119, Solyc03g119600.1.1: SL2.50ch03: 68159889.68160152) dà, inoltre, spazio all'ipotesi di un possibile evento di duplicazione che ha portato alla formazione dei due geni.
Sono stati analizzati, quindi, dati disponibili sul sito web *Sol Genomics* per verificare i differenti livelli di espressione dei geni *NIMIN 2C* in pomodoro nei differenti tessuti ed a seconda di differenti stress applicati alla pianta. Anche in questo caso le condizioni analizzate riguardano differenti tipi di stress biotici o abiotici; nelle varie condizioni analizzate è stato trovato che il gene Solyc03g119600.1.1 risulta essere sotto regolato rispetto Solyc03g119590.1.1.
Per convalidare ed estendere i risultati ottenuti dall'analisi *in silico*, sono stati analizzati i livelli di espressione di pomodoro *Solanum lycopersicum* cv. "Red Setter" dei due geni *NIMIN 2C* in differenti tessuti vegetali ed a seguito di differenti stress biotici ed abiotici applicati al sistema. Per monitorare i livelli di espressione dei due geni nei

diversi tessuti, sono state condotte delle analisi su foglie, fiori, steli e radici di piante di pomodoro adulte.
Per quanto riguarda l'analisi dei livelli di espressione dei due geni a seguito di stress applicati al sistema, sono stati monitorati i livelli di espressione a diversi tempi dopo l'attacco di funghi patogeni seguito di ferite. Per tutte le condizioni analizzate sono stati riscontrati dei differenti livelli di espressione nei due geni.
Dato che l'interazione tra le proteine NIMIN ed NPR1, confermata in *Arabidopsis* (Hermann et al., 2013), non è stata mai verificata in pomodoro, è stato reputato interessante approfondire lo studio di tale interazione mediante un'analisi di *Bimolecular Fluorescence Complementation* (BiFC) (Grefen and Blatt, 2012). Per verificare l'interazione tra le proteine NIMIN 2C (Solyc03g119590.1.1 e Solyc03g119600.1.1) ed NPR1, le sequenze codificanti delle tre proteine sono state inserite in vettori di espressione pBiFCt-2in1 in collaborazione con Dott.ssa Chaban (Università di Tubinga, Germania). I vettori pBiFCt-2in1 costituiscono su un sistema *MultiSite Gateway* che si basa sul sistema di ricombinazione sito-specifica del batteriofago λ.
Esperimenti di fluorescenza a complementazione biomolecolare (BiFC) rendono possibile la visualizzazione delle interazioni proteina-proteina nelle cellule vegetali viventi. Per verificare l'interazione tra le proteine sono state agro-infiltrate piante di *Nicotiana bentamiana* di cinque settimane. Tutte le analisi di microscopia sono state condotte a 48h e 72h dalla trasformazione mediante agro-infiltrazione.
L'interazione tra le due proteine NIMIN 2C di pomodoro e la proteina NPR1 è stata dunque osservata per i costrutti pBiFCt-590NPR1-NN (NPR1-YFP N-terminale; NIMIN 590-YFP N-terminale*)*, pBiFCt-600NPR1-NN (NPR1-YFP N-terminale*;* NIMIN 600-YFP N-terminale)*.* Per verificare la dipendenza dell'attivazione dei geni collegati alle risposte di difesa della pianta indotti dall'acido salicilico e dall'interazione tra le proteine NIMIN ed NPR1, sono stati analizzati campioni fogliari di piante trasformate mediante agro-infiltrazione a differenti tempi a seguito di ferita. L'analisi condotta mediante microscopia ad epi-fluorescenza e microscopia confocale ha evidenziato una maggiore interazione tra le due proteine dopo un'ora dalla prima ferita della foglia. L'analisi dei tessuti fogliari mediante microscopio confocale ha reso evidente che l'interazione tra le due proteine NIMIN 2C ed NPR1 avviene a livello nucleare. Sono state condotte anche delle analisi su tessuti fogliari agro-infiltrati a seguito dell'applicazione di acido salicilico. Tali analisi dovranno essere sviluppate in maggiore dettaglio ma risultati preliminari hanno dimostrato che l'applicazione di acido salicilico riesce ad attivare con maggiore rapidità l'interazione tra le due proteine; buoni livelli di interazione si verificano anche a tempo zero dalla ferita.
Il risultato dell'analisi bioinformatica, il profilo di espressione genico ed i risultati BiFC, hanno indicato che i due geni *NIMIN 2C* risultano espressi entrambi in differenti tessuti e che le loro proteine interagiscono con NPR1 risultando dunque coinvolti nei meccanismi di difesa.
Al fine di studiare il ruolo dei geni *NIMIN 2C* nell'interazione pomodoro-afidi ed al fine di condurre una più completa analisi funzionale del gene, è stato clonato un costrutto per la produzione di RNA a doppio filamento (dsRNA) volti al silenziamento di entrambi i geni in piante di pomodoro genotipo Red Setter. Gli esperimenti di clonaggio sono iniziati con l'inserimento della sequenza del gene *NIMIN 2C* (Solyc03g119590.1.1) all'interno del vettore pGEM T mediante la strategia del *T/A cloning*. Questo vettore è stato utilizzato come vettore *shuttle* per il trasferimento della sequenza in un sistema di clonaggio *Gateway*.

Per la formazione del vettore di espressione è stato utilizzato come vettore di destinazione pHellsgate 12. La scelta del vettore è stata effettuata tenendo presente la necessità di dover inserire i due frammenti nei due opposti orientamenti, intervallati da un introne. E' stato dunque prodotto un vettore di espressione pHellsgateNiminAll (pHNA) contenente il frammento nei due differenti orientamenti. Dopo l'ottenimento del clone di espressione è stata iniziata la trasformazione stabile di piante di pomodoro del genotipo Red Setter. Questi esperimenti sono stati effettuati in collaborazione con il Dr. Pasquale Chiaiese (Università Federico II, Napoli). La trasformazione è stata realizzata mediante la co-coltivazione di espianti cotiledonali di pomodoro ed una sospensione di agrobatterio precedentemente trasformato. La selezione è stata possibile mediante l'utilizzo di agenti selettivi (antibiotici). La caratterizzazione delle piante putative transgeniche è stata effettuata mediante reazioni di amplificazione su estratti di DNA fogliare utilizzando specifici *primers*, e la successiva analisi dei livelli di espressione del gene *NIMIN 2C* (Solyc03g119600.1.1). Quest'ultima analisi è stata condotta su cDNA sintetizzato a partire dall'RNA estratto da piante risultate positive alla reazione di amplificazione condotta su estratti di DNA fogliare. Tali analisi hanno portato alla caratterizzazione di due linee transgeniche TGA RA e TGA RB.
La caratterizzazione di tali linee è stata condotta utilizzando come controllo piante ottenute dalla rigenerazione di espianti di pomodoro non co-coltivati. L'analisi dei livelli di espressione ha messo in evidenza una sotto regolazione del gene *NIMIN 2C* (Solyc03g119600.1.1) nelle piante transgeniche rispetto a quelle controllo. Su queste piante saranno dunque effettuati ulteriori studi e saggi biologici volti alla caratterizzazione delle risposte di difesa delle piante a seguito dell'attacco degli afidi.
La tecnica di RNAi può essere utilizzata anche per sopprimere geni essenziali per lo sviluppo e la sopravvivenza dei parassiti producendo *in planta* appropriati dsRNA.
A tal proposito dunque è stato clonato un frammento del gene *102* utilizzando un sistema di clonaggio *Gateway*. Anche in questo caso è stato utilizzato come vettore di destinazione il vettore pHellsgate 12. Il vettore (clone di espressione) ottenuto mediante le reazioni di ricombinazione è stato denominato pHellsgate102 (pH102). Per la trasformazione stabile, condotta in collaborazione con il Dr. Pasquale Chiaiese (Università Federico II, Napoli) sono stati utilizzati degli espianti provenienti foglie di piante di tabacco (*Nicotiana tabacum)* co-coltivati con sospensioni di agrobatterio precedentemente trasformato. Sono state ottenute differenti linee transgeniche che sono state poi caratterizzate mediante reazioni di amplificazione. Queste piante saranno poi utilizzate per i saggi biologici con larve di *Spodoptera littoralis*. Le larve saranno dunque alimentate con il tessuto fogliare prodotto da queste piante per verificare se la produzione di dsRNA del gene *102* in pianta ha lo stesso effetto di sotto regolazione del gene *102* verificato attraverso precedenti esperimenti, riportati in letteratura, in cui i dsRNA sono stati introdotti in larve di *Spodoptera littoralis* attraverso ingestione orale (Di Lelio et al., 2014).

SUMMARY

The field of plant-insect interactions is currently undergoing a revolution with the availability of the genome sequences and comprehensive Expressed Sequence Tag libraries from both crop plants and agriculturally important insect pests. The study of plant defence mechanisms can lead to the discovery of new strategies for crop protection against insects. To this aim, the first step is the characterization of the genes that are involved in plant defence mechanisms. Aphids are among the most damaging pests in temperate climates. These insects evolved the ability to establish a prolonged feeding site on plant tissues, most likely because of their predicted ability to deceive plant defence mechanisms (Gary et al., 2006; Nierczyk et al., 2008). A recent transcriptomic study indicated that tomato (*Solanum lycopersicum*) has a dynamic transcriptional response following *Macrosiphum euphorbiae* attack (Coppola et al., 2013). In that study, a probe annotated as *NIMIN 2C*, was among the genes with the higher level of expression. Based on the transcriptomic and proteomic data, the authors proposed a model in which *NIMIN 2C* may be an important component of the plant's defence. In *Arabidopsis NIMIN* genes are able to negatively regulate distinct functions of NPR1, a crucial player in the pathway cross talk between jasmonic (JA) and salicylic acid (SA) (Vlot et al., 2009). Currently, the role of these two phytohormones in determining plant resistance to aphids is not well understood. These apparent discrepancies can be explained considering that in a compatible interaction, phloem-feeders may antagonize the innate plant wound responses (essentially mediated by the JA) to make the plant a more suitable host (Weigel et al., 2005). Therefore, the functional characterization of tomato *NIMIN 2C* genes aims to identify essential modulators of plant resistance, ultimately paving the way for the development of new breeding targets and control strategies. To achieve this goal, expression levels of *NIMIM* genes in *Arabidopsis* and tomato plants were studied in different conditions using wet and dry-lab experiments. To check the interaction in tomatoes among *NIMIN 2C* and *NPR1* genes, Bimolecular Fluorescence Complementation (*BiFC*) analysis were performed. Transgenic plants were also produced for the interference RNA-mediated suppression of these genes. Interference (RNAi) is an epigenetic mechanism that has been shown to reduce gene expression of a vast range of living organisms. It is a powerful tool for functional analysis of genes involved in plant defence against biotic stress (Smith et al., 2004). This goal is usually achieved by either the ectopic production or the introduction of double stranded RNA molecules (dsRNA), in order to phenocopy the loss of function of specific target genes. RNAi can be also used to suppress genes essential for pest development, survival, or parasitism by producing *in planta* appropriate dsRNA. Recently, a gene involved in insect immune response (named *102*) has been isolated and characterized in a phytopaghous lepidoptera (Falabella et al., 2012). Preliminary available data indicate that RNAi-mediated silencing of the *102* gene generate a lethal phenotype during embryonic development and, at larval stages, immunosuppression and an increase in susceptibility to stress (Pennacchio et al., 2003; Pennacchio et al., 2012; Di Lelio et al., 2014). *Toxoneuron nigriceps* is a parasitoid of larvae of different species. During oviposition, the parasitoid injects a polydnavirus into the host together with eggs. This polydnavirus is responsible for the suppression of the host immune system. A non-coding RNA (rc5'ntTnBV) coded by the polydnavirus was isolated from parasitized larvae. This RNA has a region complementary to the untranslated 5 ' region of *102* gene. For this reason the *102* represents a suitable target gene to be suppressed producing dsRNAs to indirectly increase plant resistance to specific lepidoptera.

1. INTRODUCTION

Plants are very likely to face adverse conditions during their lifetime. Stresses have significant effects on plant physiology and development because plants are sessile organisms (Boyer, 1982). Plants are primary producers in many ecosystems and therefore, they are attacked by different biotic predators and pathogens. Biotic stresses include macro-organisms, such as insects and herbivores, and microorganisms, such as bacteria, viruses and fungi. Pathogens can interact with plants by an initial physical contact, even if they use different strategies. For example, many pathogenic bacteria proliferate in intracellular spaces after entering into plant tissues through injuries, wounds, whereas pathogenic fungi invade tissues by extending hyphae into the intercellular spaces. Insects have also evolved many strategies to overcome plant defence mechanisms to feed, grow and reproduce.

1.1 Plant defence against insects

The field of plant-insect interactions is currently undergoing a revolution with the availability of the first genome sequences as well as comprehensive Expressed Sequence Tag libraries of crop plants and agriculturally important insect pests.
Several strategies are being used to exploit this wealth of information considering that these interactions can be beneficial as well as pathogenic. Although in nature plants are exposed to several potential attacks, they are susceptible to only a few. This is due to the elaborate system of defence mechanisms.
Plant defence mechanisms can be divided into constitutive and inducible defences (Wu and Baldwin, 2010). The constitutive defence includes barriers like cell walls, bark, epidermal cuticles, resins, and toxic substances such as saponins, cyanogenic glycosides and glucosinolates, which represent a protection against different types of pathogens (González-Lamothe et al., 2009; Harborne, 1998; Bennet and Wallsgrove, 1994). Generally, a permanent synthesis and the formation of physical barriers impose a metabolic cost so many plants have developed defence strategies that are activated only in the case of an attack (inducible defence), such as insect feeding (Wu and Baldwin, 2010). Inducible defences involve signalling molecules that are able to activate defence mechanisms in the different plant tissues (Gatehouse, 2002) and the production of specific volatile organic compounds (VOCs). The blend of VOCs is a complex mixture, often composed of hundreds of different compounds in different concentrations. The mixture emitted can change according to plant species. It can be different in terms of both quantity and quality. Several factors can affect the type of volatile products: the plant genotype, the development stage, the damaged tissue and the intensity of the damage (Dicke et al., 2009).
The first step after a potential attack is the identification of the biotic stress. Plants have a receptive surface that is able to detect physical and chemical signals of insects and pathogens (Hardham et al., 2010; Hardham et al., 2007).
Plant defence mechanisms can, also, be divided into direct and indirect defences. Defence is called direct if the plant is able to interfere directly with pests through morphological features that can prevent insect coming into contact with the leaf surface, such as spines, resins containing channels, wax crystals, epicuticular wax film, but also chemical defence, such as secondary metabolites, protease inhibitors enzymes (PIs) and anti-nutritive enzymes (Howe and Schaller, 2008). Secondary metabolites include glucosinolates, glycosides, terpenoids, and alkaloids. They act as

toxins, poisons or repellents (Dicke and Van Loon, 2000). Protease inhibitors (PIs) are usually activated in the insects after ingestion of proteins like digestibility-reducing proteins (Zhu-Salzaman et al., 2008), whereas anti-nutritional proteins are able to degrade amino acids necessary for pests' survival (Chen et al., 2005).
Indirect defences involve other living organisms such as natural enemies of the insect pests (parasitoids and/or predators). Indirect defences against insects are mediated by the release of a blend of volatiles produced by damaged plants (Arimura et al., 2009). VOCs facilitate the identification of the attacked plant. The plant has a mutual relationship with pests' natural enemies by providing nourishment (for example, flower nectar) and by producing volatile chemicals that can facilitate the identification of the plant attacked by the insect prey. The categories of molecules present in VOC mixture can be in common among different species of plants. Among VOCs compounds there are C6 compounds such as aldehydes, alcohols, esters denominated "green leaf volatiles", and C10-C15 compounds such as terpenoids and indoles (Parè and Tomlinson, 1999). VOCs are produced as a result of secondary metabolism pathways. Monoterpenes, sesquiterpenes and aromatic compounds are accumulated in significant amounts within specialized structures represented by glands or trichomes, whereas green-leaf volatiles are produced through the rupture of the membrane lipids, and released when leaves are mechanically damaged.
The insect-insect interaction is common in nature and many insects are natural enemies of other insects. Predator insects are those that kill and eat other insects. Parasites live on or in a host; parasitoids (entomophagous parasites) live as parasites during the larval stage but becomes free-living when adult). Generally, parasitoids have a shorter life cycle living on or in a host, which ends with the death of the host when the young parasitoid has completed its larval development (Poirie et al., 2009) leading to the death of their victim (Vinson, 1975; Vinson and Iwantsch, 1980; Godfray, 1994; Quicke, 1997; Pennacchio and Strand, 2006).
Parasitoids can be classified as monophagous, oligophagous or stenophagous and polyphagous, depending on the number of host species that the parasitoid has available to complete its life cycle. Other classifications are possible according to several parameters: for example, the host stage, the number of eggs laid by the female into the host (solitary or gregarious) or the nutritional mode (Godfray, 1994). The study of antagonistic insect-insect interaction represents an innovate source of target genes for pest control (Pennacchio et al., 2003; Pennacchio et al., 2012). For instance, the characterization and functional analysis of a protein encoded by the *102* gene of *Heliothis virescens* indicated that this gene plays a key role in the immune response of the insects. The *102* gene is involved in the localization of the process of melanisation of the hemocyte capsule around the foreign objects and is essential for the formation of the capsule itself. This process is mediated by the production of amyloid fibres. Following immune stimulus, the amyloid fibres are released on the surface of the foreign body. They form a layer that serves as a molecular scaffold, which promotes the synthesis of melanin and localized encapsulation. The braconidae parasitoid *Toxoneuron nigrigeps* can induce a down-regulation of *102* gene in *Heliothis virescens* larvae through a mechanism mediated by a non-coding RNA (rc5'ntTnBV) complementary to its 5'UTR (Pennacchio et al., 2003; Pennacchio et al., 2012).
The oral injection of dsRNA*102* gene into mature lepidoptera larvae (*Spodoptera littoralis*) inhibited encapsulation and melanisation of injected chromatography beads (Di Lelio et al.; 2014).

1.2 Induced plant defence response

1.2.1 Recognition of biotic stress

The mechanism responsible for plant defence response involves the recognition of the type of damage by a pathogen agent (Maffei et al., 2007) and the response. The study of plant defence mechanisms led to the discovery of elicitors, which are synthetic or natural compounds that activate chemical defence in plants after attack (Gómez-Vásquez et al., 2004). There are different types of elicitors according to the species of insect or the kind of pathogens. These molecules can attack to receptor proteins located on plant cell membranes. As for the plant-insect interactions, the elicitors can present a highly conserved structure depending on the type of feedings and the species of insect. Different types of elicitors have been characterized. They include some proteins of the bacterial cell wall, oral secretions like fatty acid-amino acid conjugates (FACS), carbohydrate polymers, lipids and glycopeptides present in both secretions and insect fluids. Elicitors can be plant proteins modified by insect enzymes before being injected into the plant tissues (Howe and Jander, 2008). The elicitors deriving from plant-microbial or plant-pathogen interaction can be classified as microbe-associated molecular pattern (MAMPs) or pathogen-associated molecular patterns (PAMPs) (Jones and Dangl 2006; Zipfel, 2009). The elicitor perception leads to the production of ethylene and some active oxygen species (AOS) as well as the production of molecules to reinforce plant cells. For instance, phenylpropanoid compounds reinforce the cell walls but are also involved in the creation of calluses structures, the synthesis of defence enzyme and the production of pathogenesis-related (PR) proteins (Van Loon et al., 1999). Pattern recognition receptors (PRRs) present on the cell surface are able to sense PAMPs molecules and activate signal transduction cascades. The plant response to pathogen attack results in the synthesis of metabolites that can increase resistance to pests and reduce plant damage. This is an immune response known as PAMP-triggered immunity (PTI) (Walling, 2009). PAMP-triggered immunity (PTI) is characterized by an increase in calcium (Ca^+) and hydrogen (H^+) ions concentration. PTI is the core of plant resistance to host. The rapid influx of calcium and hydrogen lead to the activation of kinase signal-trasduction cascades, the production of reactive oxygen species and the emission of ethylene (ET). MAP kinase signals are coordinated with salicylic acid (SA), jasmonic acid (JA) and ethylene regulated defence-signaling pathways (Glazebrook, 2005; Wang et al., 2008). Successful population of plant pathogens to recover nutrients form colonized plants induce virulent factors or effectors that can interfere with PTI by blocking its essential steps. To counteract the virulence factors, plants evolved resistance (R) protein.

The recognition of some virulence factors by plant resistance proteins activates the effector-triggered immunity (ETI). Generally, ETI and PTI trigger the same kind of response, even if defence responses in ETI happen quickly and last longer than in PTI (Tao et al., 2003; Truman et al., 2006; Tsuda and Katagiri, 2010). To overcome the plant defence system, pathogens have evolved MAMPs or PAMPs molecules. Pathogens produce new elicitors that are able to suppress ETI. These mechanisms lead plants to the development of new defence mechanisms that end with the production of new molecules. This model of plant defence response is known as the zig-zag model (Jones and Dangl, 2006).

According to the zig-zag model (figure 1.1), ETI is typically a pathogen strain-specific because it does not depend directly on PAMPs, whereas PTI, depending on PAMPs

that are highly conserved, is generally triggered by most pathogens. The whole mechanism is involved in specific host resistance (HR).

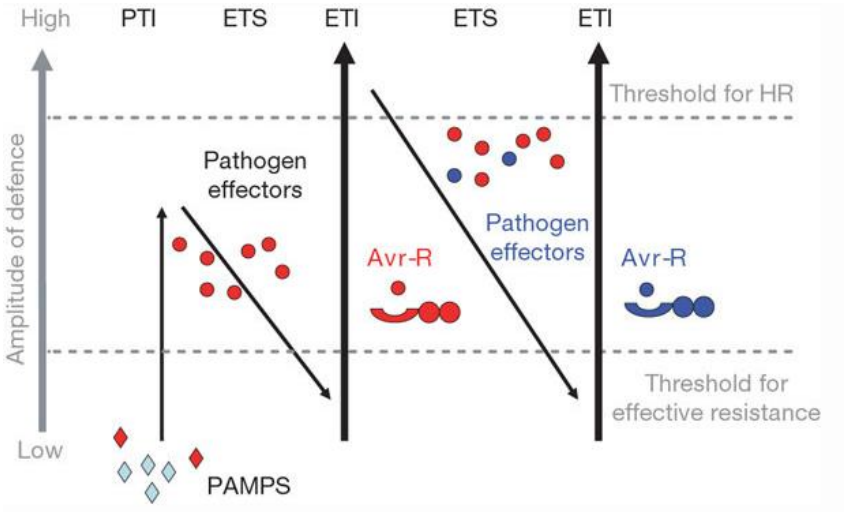

Figure 1.1: Plant immunity system, zig-zag model reproduced from Jones and Dangl (2006).

1.2.2 Chewing insects

There are different ways to classify plant-insect interactions. The most common is based on the nutrition and lifestyle of insects. Insects can be divided into two principal classes: the chewing insects and piercing/sucking ones.

Phytophagous insects are herbivorous insects equipped with jaws able to macerate plant tissues. Their feeding style causes extensive damage to plant tissues, with the activation of defence responses comparable to mechanical damage. Phytophagous insects lead to the production of signals that promote the accumulation of molecules involved in direct and indirect resistance (Mcgurl et al., 1994; Ryan, 2000). The jasmonic acid (JA) pathway plays a central role in the response to chewing-insects (Stotz et al., 2000; Reymond et al., 2000).
After attack, there is also a change in the membrane potential. This effect yields to a series of signals propagated from the site of damage that can induce, at local and systemic levels, the production of molecules inducing the amplification of signal transduction in the plant. These signals generate an increase in cytosolic calcium concentration, resulting in activation of the calcium-sensitive proteins such as calmodulin, calmodulin binding protein and calcium-dependent protein kinase (CDPKs) (Lecourieux, 2006). The increment in cytosolic calcium levels results in a higher production of reactive oxygen species (ROS) and nitric oxide (NO).
In tomatoes and other plant species, this signal transduction cascade leads to the activation of phospholipases (Ryan, 2000). These enzymes hydrolyses phospholipids into polyunsaturated fatty acids (PUFAs) and other lipophilic substances such as linoleic acid and linolenic acid (Narvaez-Vasquez et al., 1999).
Polyunsaturated fatty acids, including linoleic acid, are abundant in chloroplast membranes. The consecutive action of enzymes such as lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), through a series of reactions localized in the chloroplasts, lead to the production of 12-oxo-phytodienoic acids (OPDA). Oxo-phytodienoic acids are then transported to peroxisomes, where

jasmonic acid (figure1.2) is formed by oxophytodienoate reductase 3 (OPR3) followed by three cycles of beta-oxidation (Schaller and Stintzi, 2009).

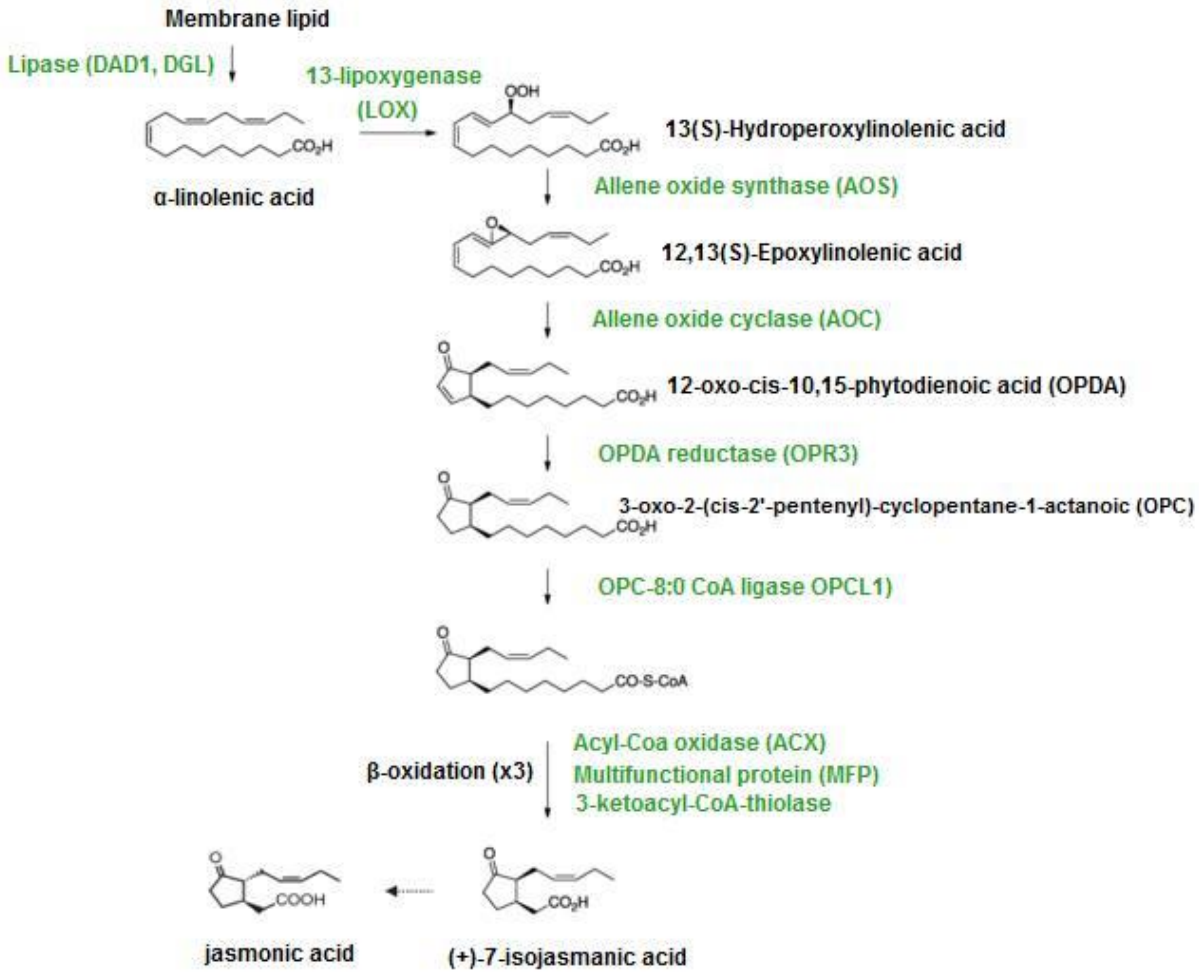

Figure 1.2: Jasmonate biosynthesis. Polyunsaturated fatty acids (PUFAs) are converted in 12-oxo-phytodienoic acid (OPDA) in chloroplast membranes. Oxo-phytodienoic acids are then transported in peroxisomes where jasmonic acid is formed by oxophytodienoate reductase 3 (OPR3) followed by three cycles of β-oxidation. Modified from Plant Hormone Reserch Network (http://hormones.psc.riken.jp/).

The jasmonic acid is related to a network of defensive factors, and increases the production of secondary metabolites and proteins such as inhibitors of digestive enzymes, proteases, saponins (Frost et al., 2008; Howe and Jander, 2008).

In tomato plants, the production of JA is linked to the systemin, the first plant peptide hormone involved in wound defence response in *Solanaceae* family (Pearce et al., 1991). The systemin (Sys) is a peptide of 18 amino acids. Its precursor is a polypeptide of 200 amino acid, the prosystemin (McGurl et al., 1992). Genetic evidence indicated that systemin amplifies the systemic defence signal supporting the jasmonic acid pathway (Lee and Howe, 2003; Li et al., 2002; Schilmiller and Howe, 2005). Studies of the prosystemin gene over expression showed an accumulation of protease inhibitors that determine the degradation of the essential amino acids in chewing insects (Chen

et al., 2005; Mcgurl et al., 1994). The silencing of this gene has shown great susceptibility of plant following larvae attack due to a strong reduction of protease inhibitors (Orozco-Cardenas et al., 1993). Tomato plants over expressing the prosystemin gene are able to defend themselves by different biotic stress (Coppola et al., 2015). Those plants also have a higher tolerance to salt stress conditions (Orsini et al., 2010).

1.2.3 Sucking insects

The phloem-feeder insects are able to feed from plant vascular tissues using stinging-sucking mouthparts. The damage is due not only to the insertion of the mouthparts into plant tissue, but also to the ingest of large amounts of phloem. Infested leaves become yellow, wilted or deformed and sometimes, the infestation can lead to plant death. Some sucking insects are able to inject toxic material into plants. The salivary secretion contains effectors that are able to manipulate host cell processes and promote infestation. Sucking insects can often transmit plant viruses. Among all phloem-feeders, aphids have gained a reputation as notorious pests. Aphids are able to feed from the vascular tissues using the stylet, which allows them to ingest large amounts of fluid rich photosynthates for a period of time ranging from hours to weeks. The aphid is capable of releasing two types of salivary secretions: aqueous and gelatinous. The insect releases the first secretion to taste the cellular environment (Powell et al., 2006; Walling, 2008). The aqueous salivary secretion contains lithic and digestive enzymes such as pectinase, cellulase and amylase that contribute to the formation of the damage site (Walling, 2008; Guerrieri and Digilio, 2008). The aqueous salivary secretion also contains elicitors able to activate the plant defence responses.
The gelatinous salivary secretion is able to create a barrier around the stylet to isolate it from host tissues with the purpose to prevent the recognition and the activation of defence mechanisms (Walling, 2008). The gelatinous salivary secretion contains phenoloxidase, peroxidase, pectinase, β-glucosidase, phospholipids and conjugated carbohydrates (Cherqui et al., 2000). Smith and Boyko (2006) proposed a model in which the active aphid attack triggers two different defence mechanisms. The first is based on the perception of the physical and mechanical damage, caused by stylet penetration in leaf tissues. It leads to the production of signaling molecules and the activation of defence pathways induced after injury or pathogenic agents attack (Kaloshian Walling, 2005). The second one involves a mechanism of interaction known as "gene by gene". According to this model, aphids produce molecules recognized by plant resistance proteins; plant-pathogen interaction is mediated by the interaction of host plant resistance gene product (PR) and a pathogen avirulence gene product (Avr), which codes for a specific elicitor (Flor, 1955).
Most of the R genes encode for transmembrane receptors with highly conserved domains: the high-energy content nucleotide-binding domains (NBD) for binding nucleotides such as ATP and GTP, the leucine rich repeats (LRR) domains that are very frequent in plant receptors, and the leucine zipper domains (LZ) which are important for interactions with nucleic acids.
This type of interaction often triggers a hypersensitive response (HR) causing rapid cell death in the localized site of infection, the depolarization of the membrane, ion fluxes and the activation of cascades of kinases and phosphatases catalysed reaction. This molecular response leads to defence genes activation, first locally (local Acquired Resistance, LAR) then systemically (systemic Acquired Resistance, SAR).
Bos et al., (2011) have identified two proteins in the *M. persicae* saliva, MP10 and

MP42. They are able to induce a response in *N. benthamiana* defence. These two proteins are involved in the development of chlorotic and necrotic areas on the leaves attacked reducing aphid performance. These proteins seem to be involved in tobacco plants resistance mechanism following an insect attack (Bos et al., 2011). The protein elicitor MP10 is able to induce premature senescence, which limits the ability of *M. persicae* aphids to draw nutrients from *Arabidopsis* leaves after attack (Joe and Jyoti, 2013).

1.2.4 Phytopathogenic fungi

Phytopathogenic fungi have evolved different infection modes and nutritional strategies. They are able to invade plants through stomata or wounds created following the action of other biotic or abiotic factors. Phytopathogenic fungi penetrate directly into epidermal cells or extend their hyphae through plant cells (Walling, 2009). Even if fungi have evolved many infection modes, the signalling pathways that govern pathogenicity are highly conserved (Turrà et al., 2014). Most phytopathogenic fungi belong to the Ascomycetes and the Basidiomycetes species. Phytopathogenic fungi are recognised by plants thanks to specific elicitors (PAMPs) such as the fungal wall structures like chitin, glucans or chitosans and metabolites such as protein and polysaccharide delivered by the pathogen.
This recognition encourages the activation of salicylic or jasmonic acid and ethylene defence pathways (Wang et al., 2008). Plant-pathogen fungi interaction is mediated recognition of the avirulence gene (Avr gene) of the fungus by receptors that trigger the activation of the plant resistance gene (R) in plants. This kind of recognition gives rise to the production of ROS and NO, which trigger a hypersensitivity reaction (HR). The hypersensitivity reaction leads to a rapid cell death in the sites of infection, to the depolarization a membrane, to the activation of ion fluxes and defence genes. The defence reaction starts locally (Local Acquired Resistance, LAR), and then at the systemic level (Systemic Acquired Resistance, SAR).
Numerous studies have shown that salicylic acid (SA) plays an important role in establishing both the LAR and the SAR following the attack of pathogens (Summermatter et al., 1995).

1.3 Systemic acquired resistance (SAR)

SAR is a mechanism of induced defence that occurs following a plant exposure to a pathogen (Durrant and Dong, 2004; Mishina and Zeier, 2007). SAR confers a long lasting protection against a broad range of pathogens (Ryals et al., 1996), which is characterized by a reduction in disease symptoms after pathogen infection (Durrant and Dong 2004; Ryals et al., 1996; Sticher et al., 1997). SAR requires the salicylic acid signal. Salicylic acid concentration rises in the phloem after damage (Yalpani et al., 1991), even if systemic signals produced at the infection site are translocated to uninfected parts of the plant (Ross, 1961). The main result of the SAR activation is the accumulation of pathogenesis-related proteins (Durrant and Dong, 2004). Two pathways of SA biosynthesis have been proposed in plants. In the first, SA is produced from isochorismate. In the second, SA is produced from phenylalanine (Wildermuth *et al.,* 2001; Garcion *et al.,* 2008). Genetic studies indicated that the bulk of SA is produced from isochorismate (figure 1.3) (Chen et al., 2009).
The biosynthesis pathway through isochorismate consists of the conversion of chorismic acid into isochorismate. The isochorismate synthase (ICS) and the

isochorismate pyruvate lyase (IPL) catalyze the reaction which lead from chorismate to SA.
The biosynthesis pathway through phenylalanine consists of the conversion of chorismic acid into phenylalanine, the phenylalanine gives rise to trans-cinnamic acid by the action of ammonia lyase (PAL). The trans-cinnamic acid is transformed in benzoic acid through the action of benzoic acid 2-hydroxylase (Chen et al., 2009).

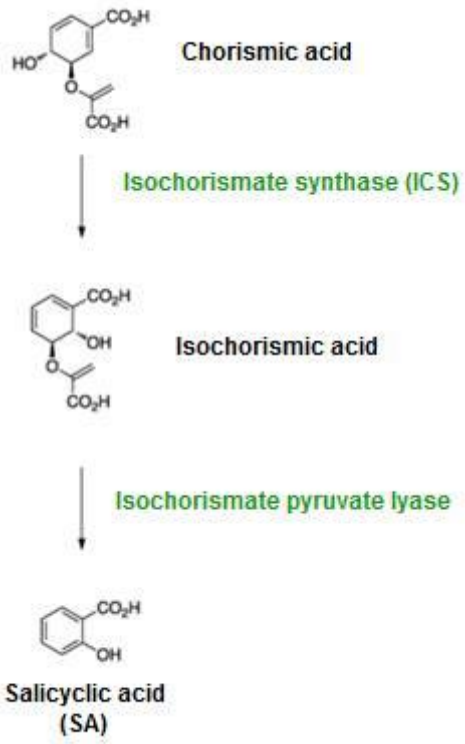

Figure 1.3: SA biosynthesis pathway through isochorismate in *Arabidopsis*. Modified form Plant Hormone Reserch Network (http://hormones.psc.riken.jp/).

Downstream of the salicylic acid, several genes involved in the activation of SAR have been identified. NPR1 (non-expresser of *PR* gene, also known as NIM1, nonimmunity1, and SAI1, salicilic inducible1 acid) is considered a key regulator of the induction of defence genes activated by salicylic acid (Mou et al., 2003; Zhang et al., 2003). The NPR1 gene was first isolated and cloned from *Arabidopsis* (Cao et al., 1997; Ryals et al., 1997). The NPR1 gene encodes a novel 66 kDa protein (Bork et al., 1993). In *Arabidopsis*, rice, tobacco and apple plants was demonstrated that the over expression of this gene is followed by an increase in the resistance to the attack of bacteria and fungi (Chern et al., 2005; Makandar et al., 2006). NPR1 has a nuclear localization signal in the C-terminal region with no apparent biochemical function and two conserved motifs that are involved in the protein-protein interaction. This highlights its function as a regulatory protein (Cao et al., 1997; Ryals et al., 1997). These two domains are an ankyrin repeat domain, located in the central region, and a BTB/POZ (broad-complex, tramtrack, and bric-a-brac/poxvirus, zinc finger) domain at the N-terminal end (Cao et al., 1997; Ryals et al., 1997). The ankyrin repeat domain is able to bind two transcription factors belonging to the TGA family (Zhou et al., 2000). NPR1 protein does not have a region for DNA binding. It has been observed

that the interaction of NPR1 with the transcriptional factor TGA, which possesses a basic region rich of leucine (bZIP), through which DNA molecules are binded, is essential for the expression of *PR* genes and for SAR the activation (Fan and Dong, 2002 Despres et al., 2000; Zhou et al., 2000; Zhang et al., 1999). In plants, different TGA transcription factors have been identified (Riechmann et al., 2000) for example *Arabidopsis* genome encodes ten TGA transcription factors that influence the expression of *PR* genes in different ways. In particular, TGA1, TGA3, TGA4 and TGA6 were identified as positive regulators while TGA2 as a negative regulator of the expression of these genes (Kesarwani et al., 2007).
Mutation in *NPR1* gene may lead to a non-expression of *PR* genes and then to a non-activation of the SAR with a consequent increase in the susceptibility to pathogen infection (Cao et al., 1997). Typically, in a non-infected cell, NPR1 is localized mostly in an oligomeric inactive form especially in cytoplasm, but also in the nucleus. Disulphide bridges between cysteine residues and transition metal atoms hold the oligomeric complex together. The increase of intracellular SA favours the reduction of disulphide bonds by thioredoxin and the subsequent release of an active monomer NPR1. The equilibrium between the monomeric and oligomeric forms can be favoured by the presence of S-nitrosothiol (SNO) and thioredoxin (TRXs) through redox reactions. In the absence of stimuli or pathogen attacks, the formation of the monomers has a very slow kinetic. An increase of SA concentration in the cell due to a pathogen attack, leads to the reduction of cysteine and NPR1 is released in the active monomeric form (Pajerowska-Mukhtar et al., 2013). The monomeric form is transported into the nucleus where it carries out its regulatory functions, including those related to the expression of *PR* genes. In the nucleus, oligomers NPR1 interact positively with the TGA family of transcription factors (figure 1.3). These factors, in turn, promote the *PR1* transcription.

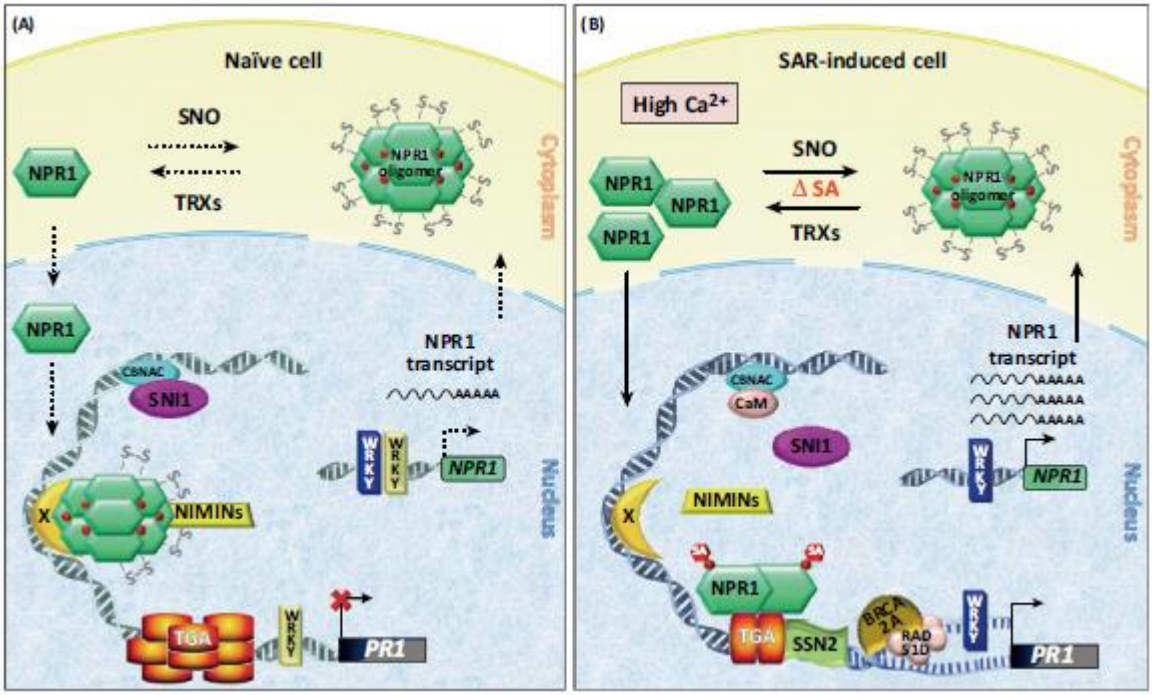

Figure 1.3: A model for the NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1)-mediated salicylic acid (SA) signalling pathway. In uninfected cells (A), the inactive form of NPR1 protein is located in cytoplasm and nucleus as oligomeric and monomeric form. The equilibrium between oligomeric and monomeric form of NPR1 protein can be facilitated by S-nitrosothiol (SNO) and thioredoxins (TRXs). In uninfected cells the amount of monomeric form is lower than oligomeric form, but it can be translocated in nucleous where interact with repressor protein NON-INDUCIBLE IMMUNITY 1 (NIM1)-INTERACTING (NIMIN) to suppress PR1 transcription. In addition, inactive octameric TGA and repressive WRKY transcription factors act to suppress PR1 transcription. In infected cells (B) the increased concentration of salicylic acid swift the equilibrium towards the monomeric form. In the nucleous the active forms of both NPR1 dimer (held together by two molecules of SA) and a TGA dimer interact physically on the PR1 promoter meanwhile the repressor proteins are dissociated. Taken form Pajerowska-Mukhtar et al., 2013.

In the absence of attack, NPR1 is degraded by the proteasome to prevent the activation of defence genes (Spoel et al., 2009). NPR1 interacts not only with TGA transcription factors but also with a group of small proteins call NIMIN proteins (NIM-1-Interacting) (Weigel et al., 2001). NIMIN proteins also interact with NPR1 at different stages of SAR to suppress the induction of *PR* genes and to modulate the defence response.

In *Arabidopsis*, there are four *NIMIN* genes*: NIMIN1*, *NIMIN1b*, *NIMIN2* and *NIMIN3*. The four *NIMIN* genes are expressed differently and encode for proteins that differently affect the expression of *PR* genes (Hermann et al. 2013). *NIMIN1b*, *NIMIN1* and *NIMIN2* have the same binding domain in the N-terminal region by which they are able to bind the C-terminal region of NPR1. NIMIN3, however, possesses another domain by which it is able to bind the N-terminal of NPR1. The expression of NIMIN 1 and NIMIN 2 is strongly induced by SA, whereas NIMIN 3 is expressed constitutively at low levels of expression (Hermann et al., 2013). Studies conducted on *Arabidopsis* plants have shown that NIMIN3 is able to suppress the expression of *PR-1* gene in non-attacked leaves.

Over expression studies of the *NIMIN1* gene showed a suppression in induction of *PR-1* gene and a SA-mediated reduction of the SAR whereas RNAi showed a low

expression of the gene PR-1 following treatment with SA (Weigel et al., 2005). *NIMIN* genes are able to form a ternary complex with TGA and NPR1, repressing or reducing the expression of PR genes (figure 1.4). RT-PCR analysis of *Arabidopsis* plants treated with SA, has highlighted the correlation between the concentration of salicylic acid with the interaction between NIMIN and NPR1 proteins. *NIMIN* genes are both expressed differently and encode for proteins that affect NPR1, a central regulator of the systemic acquired resistance (SAR) differently. *NIMIN* genes expression analysis, in *Arabidopsis* plants, by RT-PCR analysis, revealed that in uninfected cell *NIMIN 3*, whose protein interacts with the N-terminal region of NPR1, is expressed constitutively at a low level, differently from the others Arabidopsis NIMIN proteins that are responsive to SA. The infection can lead to the rise in cellular compartments of salicylic acid concentration. This process can bring to a rapid increase in transcriptional levels of NIMIN 2 protein that interacts with NPR1 instead of NIMIN 3 one. NIMIN 2, which interacts with the N-terminal region of NPR1, is an immediate early SA-induced gene immediately replaced by *NIMIN 1* (N1), which interacts with the C-terminal region of *NPR1*. NIMIN 1 is an early SA-activated and NPR1-dependent gene, which is induced after *NIMIN 2*, but clearly before PR-1. Finally, high levels of SA are able to reduce the stability of NIMIN1-NPR1 complex that is able to promote the expression of PR-1.

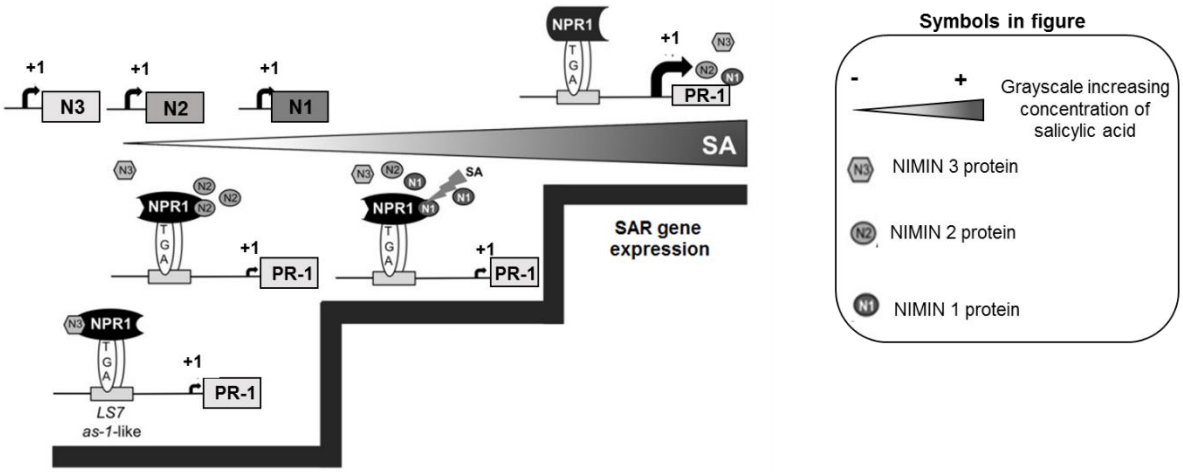

Figure 1.4: Model of interaction between *NIMIN* and *NPR1* genes in response to SA in *Arabidopsis*. *Arabidopsis* NIMIN protein bind differentially to NPR1 in ternary protein complexes including TGA transcription factors. The SA induced expression of PR-1 is mediated by an *as-1*–type promoter *cis* element (*LS7*) that is recognized by TGA factors. TGA factors, TGA2 and TGA3, are the principal contributors to an *LS7* binding activity. NIMIN 3 (N3) protein is expressed constitutively and interacts with the N-terminal region of the NPR1 protein favouring the reduction of the expression of *PR-1* genes. The increase in intracellular concentration of SA leads to the production of the NIMIN 2 (N2) protein that interacts with the C-terminal region of NPR1 this lead to a small activation of the expression of *PR-1* genes. NIMIN 1 (N1) protein, that interacts with the C-terminal region of NPR1, immediately replaces NIMIN 2 protein. *NIMIN 1* is an early SA-activated and NPR1-dependent gene which is induced after *NIMIN 2*, but clearly before *PR-1.* Finally, high levels of SA are able to reduce the stability of NIMIN1-NPR1 complex, which is able to promote the expression of PR-1. Modified from Hermann et al., 2013.

1.3.1 The action of jasmonic and salicylic acid in the regulation of defence mechanisms

The transcriptional regulation of defence genes is a fundamental process in the plant response to pathogen and herbivore insects (Vos et al., 2013). The recognition of plant pathogen agents determines the accumulation of signalling molecules such as salicylic acid, jasmonic acid and their derivatives (Pieterse et al., 2012, Kunkel et al., 2002).
Salicilic acid is generally involved in the activation of genes against fungi and biothropy pathogen attacks, while JA is critical for activating genes against herbivores and nectrotrophy pathogens attacks. The defence pathways regulated by SA and JA are involved in complex networks. Other hormones participating in these networks are abscisic acid (ABA) and ethylene are able to act synergistically with the defence responses regulated by JA. Auxins, gibberellins and cytokinins are able to suppress the defence mechanisms mediated by SA and JA favouring plant growth.
The SA and JA signalling pathways are mutually antagonistic, because of their ability to inhibit each other (Kunkel and Brooks, 2002). In this way, plants can prioritize one defence response according to the type of pathogen attack. In the presence of SA, NPR1 was found to be involved in the suppression of defence genes mediated by JA (Spoel et al., 2003). In the absence of NIMIN proteins and with a subsequent over expression of NPR1, in *Arabidopsis* plants an increased susceptibility to many necrotrophic pathogens was observed (Heil and Baldwin, 2002). NIMIN proteins may be indirectly involved in the resource adjustment of defence responses mediated by SA and JA or processes relating to growth and reproduction. Recent studies have also hypothesized that insects such as aphids, have developed mechanisms capable to induce the activation of defence pathways in the host plant mediated by SA and antagonized those mediated by JA (Walling, 2008).
To confirm that jasmonic acid is involved in the activation of defence genes against aphids, Ellis et al., 2002 observed that *Arabidopsis* plants expressing high levels of JA appeared to be more resistant to the attack of aphid compared to wild-type plants.

It was also demonstrated that exogenous applications of methyl jasmonate (MeJA) are able to promote the resistance against aphids in *Arabidopsis* (Ellis et al., 2002). The jasmonic acid promotes accumulation of metabolites such as indole-glucosinolate and camalexina (Ellis et al., 2002; Mewis et al., 2005-2006) which are harmful to aphids.

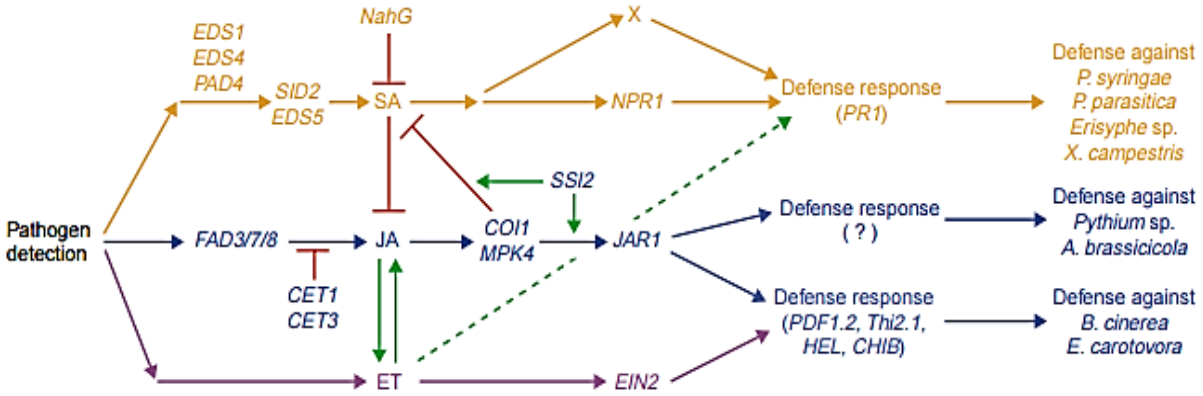

Figure 1.5: SA, JA and ET pathogen defence pathways in *Arabidopsis* thaliana. The figure shows different genes involved in SA, JA and ET biosynthesis. The genes involved in SA biosynthesis such as *SID2, EDS5, ED1, EDS4, PAD4* and other genes involved in SA defence pathway like NPR1. The genes involved in JA biosynthesis are *FAD 3/7/8*, *CET 1/3* whereas *COI*1 and *MPK4* genes are involved in JA defence response. The gene *SSI2 d*ownstream the genes COI1 and *MPK4*. The figure also shows the crosstalk among different SA, JA and ET signalling pathways and some spices that active a specific plant defence pathway in *Arabidopsis*. Reproduced from Kunkel and Brooks, 2002.

Recent studies in *Arabidopsis* plants have also shown that aphids often use the ethylene and salicylic acid mediating defence pathways to interfere with the action of defence mediated by jasmonic acid. This can promote insect infestation on the plant (Joe and Jyoti, 2013).

1.4 RNA interference as a biotechnological approach to increase biotic stress tolerance

The study of physiological and molecular mechanisms of antagonistic plant-insect and insect-insect interaction can provide useful information to develop biotechnological approaches for pest control of pests (Pennacchio et al., 2003; Pennacchio et al., 2012). RNA interference (RNAi) has been shown to reduce the gene expression of a vast range of living organisms. It is a powerful tool for the functional analysis of genes involved in plant defence against biotic stress (Burch-Smith et al., 2004). RNAi is a biological mechanism in which RNA molecules drive the post-transcriptional silencing of genes with homologous sequences. A messenger RNA (mRNA), in the presence of complementary RNA (endogenous or exogenous), form a very stable double-stranded molecule, which is not translated but degraded (Zamore et al., 2000; Filipowicz et al., 2005).
The long dsRNA are cleaved into short RNA of 21-22 nucleotides of "small interfering RNA" (siRNAs) by a dimeric enzyme called Dicer, which belongs to the class RNase family III (Sledz and Williams, 2005). Crystallographic studies show two catalytic domains that can influence the activity of endonuclease (RNase III), a domain NH_2-terminal helicase ATP-dependent and a COOH-terminal with specific sites for binding to dsRNA (Filipowicz et al., 2005) in addition to a PAZ domain (Piwi/Argonaute/Zwille) (Moss, 2001).
The siRNA produced by the Dicer protein joins to RNase complex, RISC (RNA-induced silencing complex), and functions as a guide for sequence-specific mRNA degradation

(Sledz and Williams, 2005). The RNA antisense strand acts as a guide for the mRNA sequence specific degradation (Sledz and Williams, 2005), while the sense strand acts as a guide. The protein complex RISC degrades the mRNA, present in the cytosol, complementary to the RNA antisense fragment associated with the complex itself. If there is pairing between the siRNA and mRNA, a component of the RISC complex (Argonaute protein) is able to operate a cutting on the mRNA. The mRNA cut are rapidly degraded by RNase of the cell itself (Ross et al., 2013). The figure 1.6 report a diagram of the RNAi mechanism.

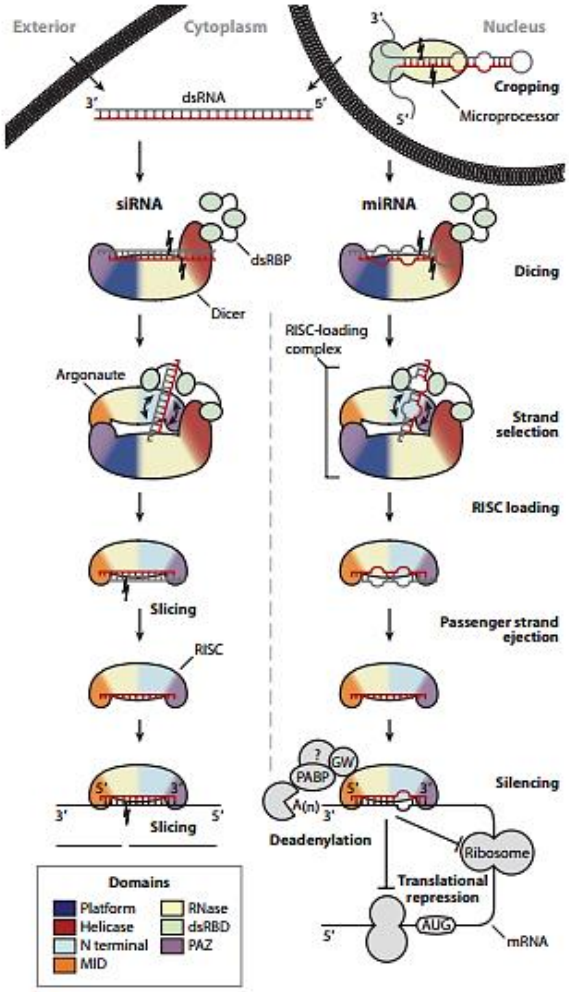

Figure 1.6: RNA interference mechanism. Mechanism of degradation of siRNA may be derived from a viral infection or other exogenous sources, and miRNA derived from the genome. The figure also shows the role of different enzymes such as helicases, dsRNA endonucleases, RNA-dependent RNA polymerase in RNA silencing. Taken from Ross et al., 2013.

1.5 Research objectives

The aim of the project is to develop new knowledge and tools to understand plant defence mechanisms and increase resistance against insect pests, exploiting recent genomic and transcriptomic studies on plant-insect tritrophic interactions. Very recently, a transcriptional profile of a compatible tomato-aphid interaction indicated that the *NIMIN 2C gene*, homologue of the modulator of pathogenesis related gene expression At-NIMIN-1 (Vlot et al. 2009), is among the most highly differentially expressed genes. In *Arabidopsis*, *NIMIN* genes are able to negatively regulate distinct functions of *NPR1*, a crucial player in the pathway cross talk between jasmonic (JA) and salicylic acid (SA) (Koornneef and Pieterse, 2008). Currently, the role of these two phytohormones in determining plant resistance to aphids is not well understood, with conflicting evidence present in the literature. These apparent discrepancies can be explained considering that in a compatible interaction, phloem-feeders may antagonize the innate plant wound responses (essentially mediated by the JA) to make the plant a more suitable host (Weigel et al., 2005).
Therefore, the functional characterization of *NIMIN 2C* aims to identify an essential modulator of plant resistance, ultimately paving the way for the development of new breeding targets and control strategies. To achieve this goal, expression levels of *NIMIM* genes in *Arabidopsis* and tomato plants were studied in different conditions using wet and dry-lab experiments. To check the interaction in tomatoes among *NIMIN 2C* genes and *NPR1*, bimolecular fluorescence complementation (BiFC) analysis was also performed. The bimolecular fluorescence complementation makes the visualization of protein–protein interactions possible in living plant cells. This technique is based on the formation of a fluorescent complex by the interaction of two non-fluorescent fragments of the yellow fluorescent protein (YFP) brought together by the association of interacting proteins fused to these fragments (Schütze et al.,2009). The BiFC vectors used are pBiFCt-2in1 vectors that are available for the transient and stable transformation. These vectors are MultiSite Gateway systems based on bacteriophage λ's site-specific recombination system (Grefen and Blatt, 2012) that enables co-expression of fusion proteins on a cell-by-cell basis from a single plasmid. Transgenic plants were produced for the RNAi-mediated suppression of these genes. RNAi can be also use to suppress genes essential for pest development, survival, or parasitism by producing *in planta* appropriate dsRNA. Recently, a gene involved in the insect immune response (named *102*) has been isolated and characterized in some phytopaghous lepidoptera (Falabella et al., 2012). Preliminary data indicate that RNAi-mediated silencing of the *102* gene generates lethal phenotypes during embryonic development and, at larval stages, immunosuppression and an increase in susceptibility to stress (Prof Pennacchio, UNINA). *Toxoneuron nigriceps* is a parasitoid of larvae of different species. During oviposition, the parasitoid injects a polydnavirus into the host together with eggs. This polydnavirus is responsible for the suppression of the host immune system. A non-coding RNA (rc5'ntTnBV) coded by the polydnavirus was isolated from parasitized larvae. This RNA has a region complementary to the untranslated 5' region of the *102* gene (Pennacchio et al., 2003; Pennacchio et al., 2012). For this reason the *102* gene represents a suitable target gene to be suppressed by plant produced dsRNAs to indirectly increase plant resistance to specific lepidoptera. By using the downregulation of specific genes through RNA interference, the sequence of the *102* gene was cloned in a binary vector and tobacco plants were stably transformed.

2. MATERIALS AND METHODS

2.1 Plasmid isolation

Plasmid isolation was performed using the alkalane lysis protocol or the PureLink Quick Plasmid Miniprep Kit (Invitrogen). A single bacterial colony was inoculeted into 2 ml of Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl in a final volume 950 mL H_2O; pH 7.0) containing the appropriate antibiotic, the culture was incubated overnight at 37°C with vigorous shaking (Sambrook et al., 2001). After the overnight incubation, the bacterial was resuspend by vortexing in 100 µl of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl; pH 8.0, 10 mM EDTA; pH 8.0).
At samples were add 200 µl of freshly prepared Solution II (0.2 M NaOH, 1% SDS).
At samples were add 150 µl of ice-cold Solution III (60 ml potassium acetate 5M, 11.5 ml glacial acetic acid, 28.5 ml H_2O). The samples were mixed by inversion and stored on ice for five minutes. The samples were centrifuged at 12.000x g for five minutes.
The supernatant was incubated with Rnase at 37°C for 30 minutes. The plasmid was precipitated by adding 1/10 volume of sodium acetate, pH 5.2, and 2.5 volumes of cold 100% ethanol. The samples were incubated in -80°C for ten minutes, centrifuged and washed with ethanol 70%. The solution was centrifuged and the pellet dry dissolved in TE buffer (pH 8.0). All the plasmids isolated were quantified by using agarose gels electrophoresis. Recombinant plasmids were controlled by PCR reaction, using primers listed in table 2.1, or by digestion reactions. The sequences were controlled by Sanger sequencing (Bio-Fab Research Company, Rome).

2.2 Polymerase chain reaction (PCR)

DNA samples were amplified by PCR in a final volume of 20 µl containing 0.5 U of Taq DNA polymerase (Promega, Milan, Itay), 1X buffer (Promega, Milan, Italy), 1.5mM $MgCl_2$, 10 mM of dNTP, 20 pmol of primers (forward and reverse).
The amount of DNA used as template goes form 10ng (for amplification of genes or fragments from vectors) to 100 ng (for amplification of a gene or fragment from genomic DNA). PCR colony was used for the screening for recombinants plasmids (Sambrook et al., 2001). The reactions were performed by picking and transferring single colonies into 50µl of sterile water. An incubation at high temperature (about 96°C) was used to break the cells membrane making available plasmid DNA. The samples were than centrifuged at 16000 x g for five minutes and 5µl of the supernatant were used as template.
The amplification conditions are reported in table 2.1. The reactions were carried out in a Gene Amp 2700 PCR cycler (Applied Biosystem). PCR products were verified by agarose gels electrophoresis (Sambrook et al., 2001). Oligonucleotides were obtained from the Invitrogen Life Technology. The table 2.1 reports the primers used for PCR reactions and their amplification conditions.

Table 2.1: List of primers and their amplification conditions. The table show the name of the primers used for amplification (1), sequence of the primers used (2), length of the amplification expected (3), length of the amplification in the empty vector (4), cycle used for amplification (5), number of repetitions (6).

Primers name	Sequence (5'-3')	Amplified length (bp)	Amplified length in empty vector (bp)	PCR cycle	NR
102 gene (AttB1) FW	GGGGACAAGTTTGTACAAAAAAG CAGGCTGAGAATGTGTGGAATG GTCCA	479	NA	95°C for 30s 60°C for 30s 72°C for 120s	25
102 gene (AttB2) RV	GGGGACCACTTTGTACAAGAAAG CTGGGTCATAGACAAACTGGATC TCTC				
102 gene AGRI 51 FW	CAACAACGTCTTCAAAGCAA	630	1561	95°C for 30s 60°C for 30s 72°C for 120s	25
102 gene AGRI 56 RV	CTGGGGTACGGAATTCCTC				
102 gene AGRI 64 FW	CTTGCCTGCAGTTATCATC	727	1669	95°C for 30s 60°C for 30s 72°C for 120s	25
102 gene AGRI 69 RV	AGGCGTCTCGCATATCTCAT				
102 gene AGRI 51 FW	CAACAACGTCTTCAAAGCAAGGG GACCACTTTGTACAAGA	607	NA	95°C for 30s 60°C for 30s 72°C for 120s	25
102 gene AttB2 RV	AAGCTGGGTCATAGACAAACTGG ATCTCTC				
102 gene AGRI64 FW	CTTGCCTGCAGTTATCATC GGGGACAAGTTTGTACAAAA	690	NA	95°C for 30s 60°C for 30s 72°C for 120s	25
102 gene AttB1 RV	AAGCAGGCTGAGAATGTGTGGA ATGGTCCA				
102 gene M13 FW	GTAAAACGACGGCCAG	709	2498	95°C for 30s 55°C for 30s 72°C for 120s	25
102 gene M13 RV	CAGGAAACAGCTATGAC				
EF1α Rt FW	CTCCATTGGGTCGTTTTGCT	101	NA	95°C for 900s 56°C for 60s 72°C for 20s	35
EF1α Rt RV	GGTCACCTTGGCACCAGTTG				

NIMIN590 Rt FW	GCTCAGATTCAGCCGGAAAAC	111	NA	95°C for 900s 56°C for 60s 72°C for 20s	35
NIMIN590 Rt RV	TCTCACGATTCCCCGTTCTCT				
NIMIN590 P1P4 FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCTACTTATGGACGGAGAA	397	NA	95°C for 30s 56°C for 30s 72°C for 180s	30
NIMIN590 P1P4 RV	GGGGACAACTTTGTATAGAAAAGTTGGGTGTTAGTCTCCGCAGTCCGGC				
NIMIN600 Rt FW	GAAGGCGAACACCGTAGTATC	142	NA	95°C for 900s 56°C for 60s 72°C for 20s	35
NIMIN600 Rt RV	ATGGCTATCCTCTACTTCACC				
NIMIN600 P1P4 FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGACCGGCACAAGAAGCG	325	NA	95°C for 30s 56°C for 30s 72°C for 180s	30
NIMIN600 P1P4 RV	GGGGACAACTTTGTATAGAAAAGTTGGGTGTTAATCTCCGCCTTCCGGTAGTG				
NIMINAll AGRI 51 FW	CAACAACGTCTTCAAAGCAA	510	1561	95°C for 30s 54°C for 30s 72°C for 120s	25
NIMINAll AGRI 56 RV	CTGGGGTACGGAATTCCTC				
NIMINAll AGRI 64 FW	CTTGCCTGCAGTTATCATC	607	1669	95°C for 30s 54°C for 30s 72°C for 120s	25
NIMINAll AGRI 69 RV	AGGCGTCTCGCATATCTCAT				
NIMINAll AGRI 51 FW	CAACAACGTCTTCAAAGCAA	472	NA	95°C for 30s 54°C for 30s 72°C for 120s	25
NIMINAll AttB2 RV	GGGGACCACTTTGTACAAGAAAGCTGGGTGAGGTCCAAATCACCTTTTC				
NIMINAll AGRI64 FW	CTTGCCTGCAGTTATCATC	562	NA	95°C for 30s 54°C for 30s 72°C for 120s	25
NIMINAll AttB1 RV	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGACGGAGAAAAGAAGAG				
NIMINAll FW (AttB1)	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGACGGAGAAAAGAAGAG	358	NA	95°C for 30s 54°C for 30s 72°C for 45s	30
NIMINAll RV (AttB2)	GGGGACCACTTTGTACAAGAAAGCTGGGTGAGGTCCAAATCACCTTTTC				

NIMINAll M13 FW	GTAAAACGACGGCCAG	588	2498	95°C for 30s 55°C for 30s 72°C for 120s	25
NIMINAll M13 RV	CAGGAAACAGCTATGAC				
NPR1 P2P3 FW	GGGGACAACTTTGTATAATAAAG TTGTAATGGATAGTAGAACTGCT TTT	1799	NA	95°C for 30s 56°C for 30s 72°C for 120s	30
NPR1 P2P3 RV	GGGGACCACTTTGTACAAGAAAG CTGGGTTCTATTTCCTAAATGGG AGATT				
StbEF FW	AAGCTGCTGAGATGAACAAG	750 (DNA)	NA	95°C for 30s 54°C for 30s 72°C for 45s	30
LeEF RV	TCAAACCAGTAGGGCCAAA	650 (RNA)			

Recombinant plasmids were controlled by PCR reaction, using primers listed in table 2.1, or by digestion reactions. The sequences were controlled by Sanger sequencing (Bio-Fab Research Company, Rome).

2.3 Gateway-Cloning System

2.3.1 Creation of Gateway Entry Clones

BP reactions were performed with 20-40 fmol of PCR products with attB sites, 150 ng of donor vector and TE buffer (10mM Tris HCl, 1mM EDTA; pH 8.0) to a final volume of 8µl. After mixing, 2µl of BP Clonase were added to the mix. The reaction was incubated at room temperature overnight. Subsequently, 1 µl of Proteinase K was added and samples incubated at 37°C for ten minutes to terminate the reaction. The donor vector used are pDonor/Zeo and pDonor221 (pDonor221 P1-P4, pDonor P2-P3). The pDonor/Zeo™ vector (Invitrogen) is used to create a shuttle vector (entry clone) for the destination vector pHellsgate 12 vector. A map of the vector is shown in figure 2.1.

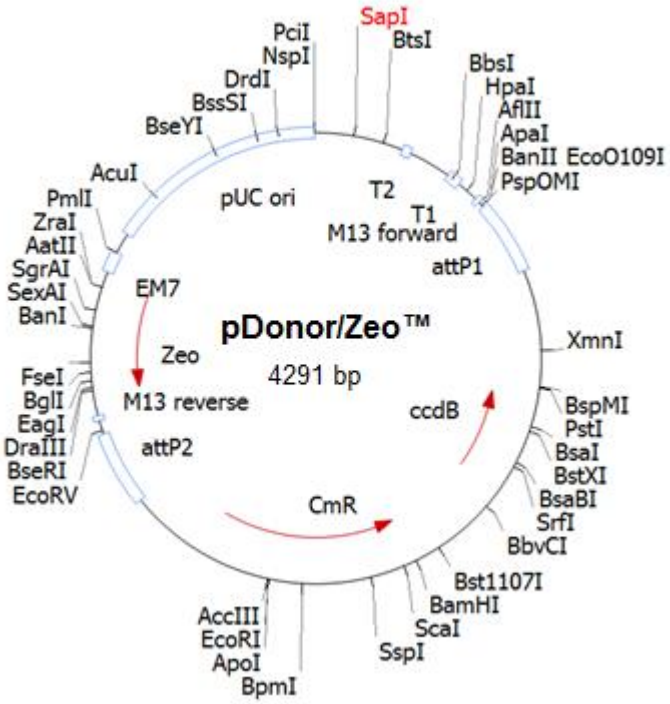

Figure 2.1: Map of the pDonr/Zeo vector (Invitrogen). The important regions are shown on the map in blue rectangles: two recombination sites attP1 - attP2, the origin of replication (pUC origin), the M13 Forward and M13 Reverse priming sites, the rrnB T1 and T2 transcription terminators and the EM7 promoter. The red arrows represent the genes for selection (zeocine resistance and ccdB gene) and *chloramphenicol resistance* gene (*CmR*). Outside there are all the unique restriction sites.

The vectors pDonor™221 P1P4 and the pDonor™ P2P3 vectors (Invitrogen) were used to create shuttle vectors (entry clones) for the destination vector pBiFCt-2in1. The maps of the vectors are shown in figure 2.2.

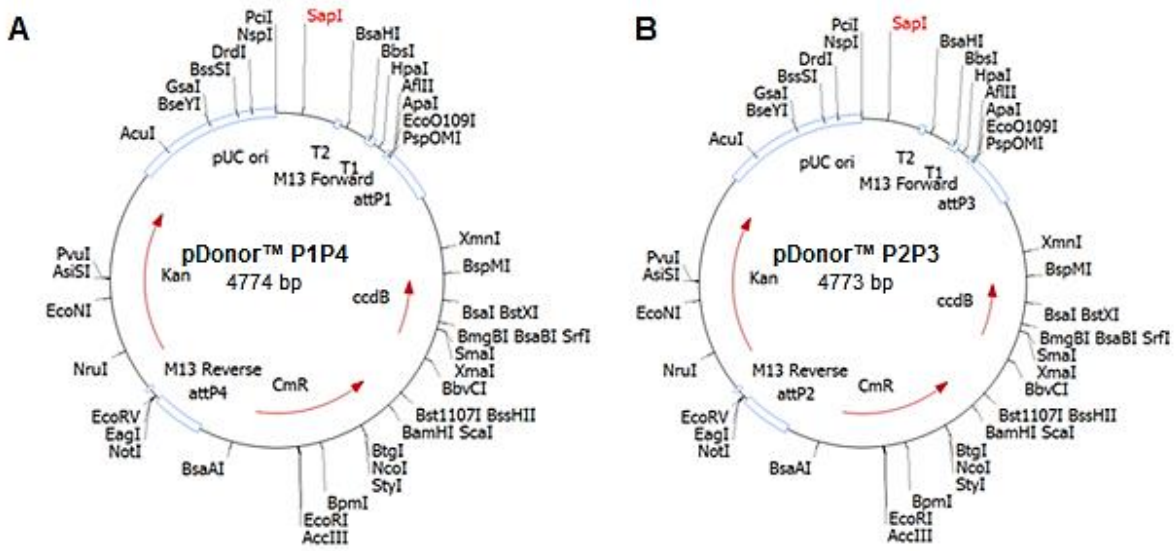

Figure 2.2: Map of the pDonor221 P1P4 (A) and the pDonor P2P3 (B) vectors (Invitrogen). The important regions are shown on the map in blue rectangles: two recombination sites attP (attP1-attP4 sites, for pDonor221 P1P4 and attP1-attP4 sites for pDonor P2P3) the origin of replication (pUC origin), the M13 Forward and M13 Reverse priming sites. The red arrows represent the genes for selection (*kanamicyn resistence* and *ccdB* gene) and *chloramphenicol resistance* gene (*CmR*). Outside there are all the unique restriction sites.

2.3.2 Creation of Gateway Expression Clones

LR reactions were performed using 250 ng of entry clone, 150 ng of destination vector and TE buffer (10 mM Tris HCl, 1 mM EDTA; pH 8.0) to a final volume of 8 µl. After mixing, 2 µl LR Clonase were added to the overnight reaction. The destination vectors used were pHellsgate12 and pBiFCt-2in1 vectors.
The pHellsgate12 vector (CSIRO Plant Industry, Canberra, Australia) is a Gateway adapted vector suitable for high-throughput gene silencing (Helliwell et al., 2002). A map of the vector is shown in figure 2.3.

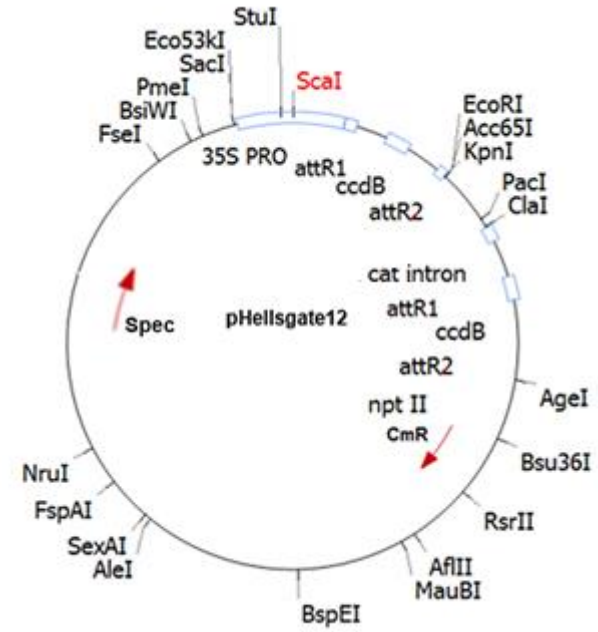

Figure 2.3: Map of the vector pHellsgate12 vector (CSIRO Plant Industry). The vector map reports in blue rectangles important sites like the recombination sites attR1-attR2 in two directions, the intron (cat intron), the CaMV 35S promoter (35S PRO). The vector is provided of red arrows that represent the genes for selection (*spectinomycin resistance*) and *chloramphenicol resistance* gene (*CmR*). Outside there are all the sites for restriction enzymes.

The pBiFCt-2in1 vectors were provided from Dr Christopher Grefen, University of Tuebingen, Germany. They are Gateway-adapted vectors very useful in understanding of protein complexes, gene expression and binary interactions (Grefen and Blatt, 2012).

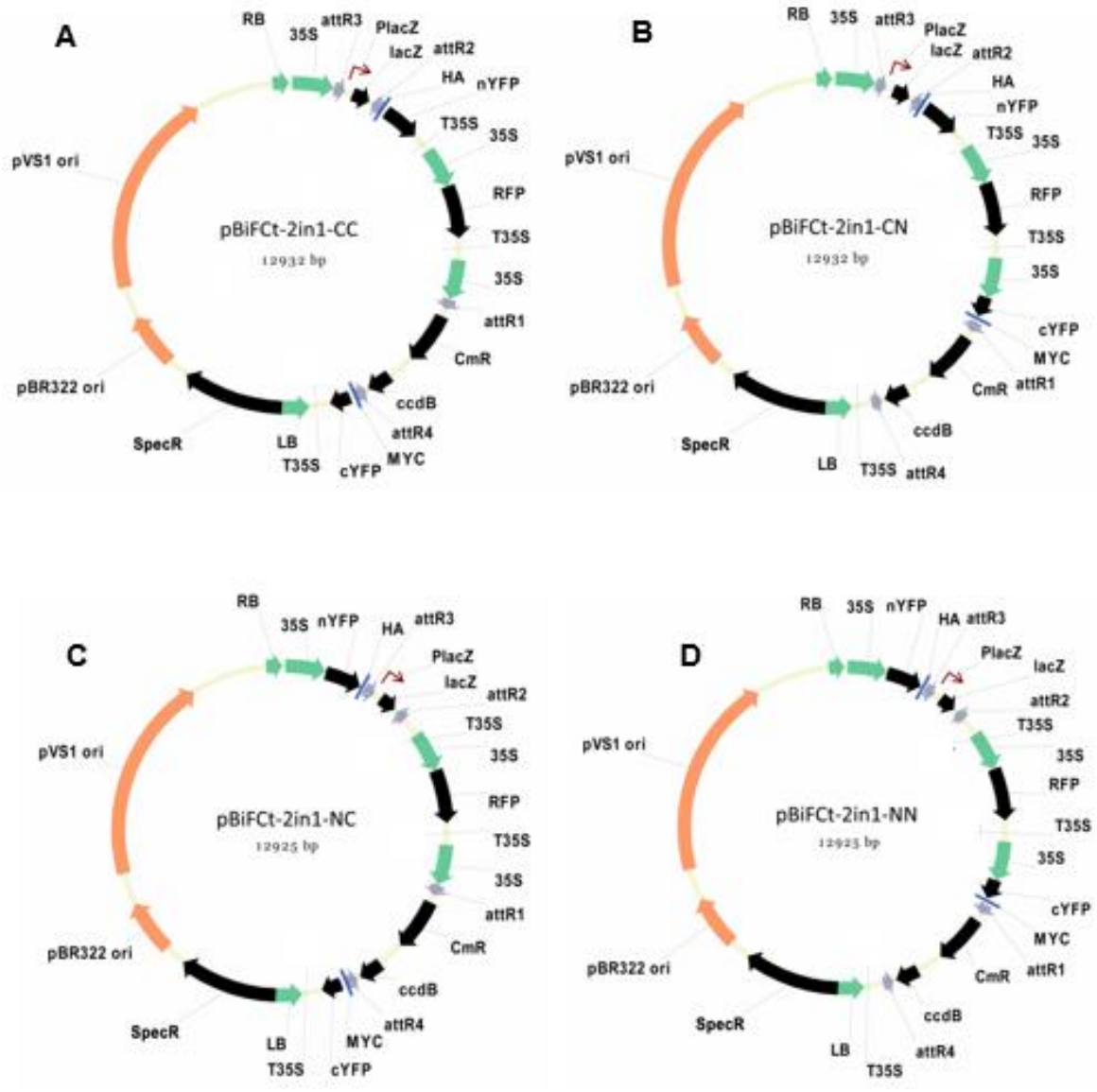

Figure 2.4: Maps of BifC vectors: pBiFCt-2in1-CC (A), pBiFCt-2in1-CN (B), pBiFCt-2in1-NC (C), pBiFCt-2in1-NN (D). The maps report, between blue arrows, the two recombination sites attR (attR1-attR4 sites and attP2-attP3). The pink arrows represents the origin of replication in *Escherichia coli* (pBR322 ori) and in *Agrobacterium tumefaciens* (pVS1 ori). The black arrows represent the genes for selection (spectinomycin resistance and ccdB), the *chloramphenicol resistance* gene *(CmR)*, the lacZ codon-optimized for *E. coli*, the C-terminal region of the YFP (cYFP: 156–239 amino acids) and the N-terminal region of the YFP (nYFP: 1-155 amino acid). The green arrows represent the right border of T-DNA (RB), the left border of T-DNA (LB), the 35s RNA CaMV promoter (35S). The vertical blue lines represent the sites for c-Myc or hemagglutinin (HA) epitope tag. Reproduced from http://www.zmbp.uni-tuebingen.de/dev-genetics/grefen/resources.html.

2.4 Bacterial transformation

Transformation of plasmid DNA into *E.coli* was performed using the heat shock method or electroporation (Sambrook et al., 2001). For heat-shock transformation a plasmid (10 ng) or a ligation product (2 µl) were added to 50µl of bacterial competent cells (e.g. INVαF'). Following a short incubation in ice, a rapid heat shock at 42°C for 45 sec ensured the transformation.
Electroporation was used to transform available competent bacteria cells (e.g. DH5α). Competent cells (50µl) were mixed with 2µl of ligation product. The electric shock was performed by the application to mixture of an electric potential of 2.5kW for five milliseconds. Following transformation, the mixture of *E.coli* cells was placed back into ice and 250 µl SOC *medium* (2 % tryptone, 0.5 % yeast extract,10 mM NaCl, 2.5 mM KCl, 10 mM MgCl, 10 mM MgSO4 e 20 mM glucose) were added. Mixtures were then incubated at 37°C for 30 minutes with agitation (150 rpm).
Agrobacterium tumefaciens transformation was performed using electroporation.
Dr. Pasquale Chiaiese (University of Federico II, Naples) provided the Agrobacterium tumefaciens strain LBA4404. Competent cells (50 µl) were mixed with the plasmid (1 µg). The electric shock was performed by the application to mixture of an electric potential of 1.6kW for approximately five milliseconds. After electric shock, 800 µL of YEP (10 g l^{-1} of peptone, 5 g l^{-1} of yeast extract, 10 g l^{-1} NaCl) were added, and cells were incubated at 28°C for one hour at 200 rpm.

2.5 Tomato genetic transformation

2.5.1 Co-Culture of tomato explants with *Agrobacterium tumefaciens*

These experiments were done in collaboration with Dr Pasquale Chiaiese (University of Federico II, Naples).
For sterilization, seeds were placed in a tube with 70% ethanol for one minute. Seeds were then stirred for ten min with a solution of 2% commercial bleach and finally washed for five times with a large amount of sterile bidistilled water. Sterilized seeds were sown on MS30 solid medium (30 g l^{-1} sucrose, 4.4 g l^{-1} of Murashige and Skoog salts with vitamins, 8 g l^{-1} of agar; pH 5.8) and left to germinate in a growth chamber at 24°C with a photoperiod of 16 hours of light and eight hours dark (16L:8D).
Cotyledons were cut into small pieces of about 5mm and positioned with adaxial side facing on the medium (MS30 supplemented with 0.4mg l^{-1} indole-3-acetic acid *(*IAA) and 0.5mg l^{-1} zeatin). Plates were incubated in a growth chamber at 24°C (16L:8D) for two days. Four days before co-cultivation a single colony of recombinant *Agrobacterium tumefacien*s LBA4404 was inoculated in 5 ml of YEP liquid with 100mg l^{-1} of spectinomycin and 50mg l^{-1} rifampicin and incubated at 28°C in the dark for 48 hours shaken at 180 rpm.
Two days after 1ml of Agrobacterium colture was added to 20 ml of selective liquid YEP (100mg l^{-1} of spectinomycin and 50mg l^{-1} rifampicin) and incubated at 28°C in the dark for 48 hours at 180 rpm. The colture was then diluted to an 0.1 OD_{600} liquid MS30 with 345 mg l^{-1} acetosyringone (4'-Hydroxy-3',5'-dimethoxyacetophenone) and then incubated for two to three hours in the dark at 28°C at 180 rpm.
Approximately ten cotyledons were not co-cultivated with Agrobacterium and left on plates with solid MS30 containing 0.4 mg l^{-1} IAA and 1 mg l^{-1} zeatin as regeneration control. The remaining cotyledons and the bacteria solution were mixed by gentle shaking for 30 minutes. Subsequently, the cotyledons explant were co-cultivated for

48h on solid MS30 plates. Two days after cotyledons explant were transferred in MS30 solid plate containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} IAA, 100 mg l^{-1} claforan and 50 mg l^{-1} kanamycin to favour the differentiation and incubated in a growth chamber at 24°C (16L:8D). Every two weeks, explants that were survived and have produced callus were transferred onto fresh f MS30 medium containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} IAA, 100mg l^{-1} claforan and 50 mg l^{-1} kanamycin.

2.5.2 Rooting of shoots

The shoots, obtained after about four to six weeks, were transferred into plates containing MS30 solid supplemented with 50 mg l^{-1} kanamycin and 100 mg l^{-1} claforan and transferred to fresh substrate every 20-30 days. After several cycles of selection, shoots were transferred into plates containing the MS30 medium.

2.6 Tobacco stable transformation (*Nicotiana tabacum*)

2.6.1 Co-culture of tobacco explants with *Agrobacterium tumefaciens*

These experiments were done in collaboration with Dr Pasquale Chiaiese (University of Federico II, Naples).
Tobacco seeds were sterilized as described in 2.5.1. Sterilized seeds were placed in plate containing MS30 solid at a distance of approximately 0.5 cm and left to germinate in a growth chamber at 24°C (16L:8D).
For tobacco genetic transformation fully expanded true leaves were cut into 0.6 to 0.8 cm squares. Explants were co-cultivated in 30ml of MS30 with acetosyringone containing recombinant *Agrobacterium tumefaciens* LBA4404 diluted to a final concentration of 0.1 OD_{600}. The steps for the preparation of the solution of *Agrobacterium* in MS30 liquid medium and procedures used for the experiment are as described for transformation of tomato (2.5.1)**.**
Subsequently, the cotyledons explant were co-cultivated for 48 hours on solid MS30 plates. Ten cotyledons of cut leaves were not co-cultivated with *Agrobacterium* but left on plates of solid MS30 containing 0.4mg l^{-1} IAA and 1mg l^{-1} zeatin as a control. Two days after leaves explant were transferred in MS30 solid plate containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} IAA, 100 mg l^{-1} claforan and 50 mg l^{-1} kanamycin and incubated in a growth chamber at 24°C (16L:8D). Every two weeks, explants were transferred onto MS30 medium containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} IAA, 100 mg l^{-1} claforan and 50 mg l^{-1} kanamycin.

2.6.2 Rooting of shoots

Shoots, obtained after about four to six weeks, were transferred into plates containing MS30 solid supplemented with 50 mg l^{-1} kanamycin and 100 mg l^{-1} claforan to a further selection and they were transferred onto fresh substrate every 20-30 days. After several cycles of selection to facilitate the further growth of the sprout and the induction of rooting the shoots were transferred into plates containing only MS30 solid. The shoots were transferred onto fresh substrate every 20-30 days.

2.7 Tobacco transient transformation

These experiments were carried out at University of Tuebingen (ZMBP) in collaboration with Dr. Christina Chaban**.**
Single colony of *Agrobacterium t.* (LBA4404) transformed were inoculated in 1ml of fresh YEB medium with selective agents and incubated overnight under shaking at 28°C. An *Agrobacterium t.* strain (GV3101/pMP90) containing the p19 protein of tomato bushy stunt virus was used to suppress gene silencing in transformed tobacco leaves (Danielson and Pezacki, 2013, Voinnet et al., 2003). To achieve this aim a single colony of p19 *Agrobacterium t*. transformed was, also, inoculated in 2ml of fresh YEB medium with selective agents and incubated overnight under shaking at 28°C.
Next morning the liquid culture was diluited with fresh YEB medium, the volume of medium added was twice the initial volume of the inoculum and incubated for four hours at 28°C. The cultures were then centrifuged at 4,000×g for 15 minutes, the pellet was resuspend in AS medium (333 µl of 3 M $MgCl_2$, 1ml of 1 M MES-KOH buffer; pH 5.6, 100 µl of 150 mM acetosyringone and H_2O to a final volume of 100 ml) and diluted to a final concentration of 1 OD_{600}. The cells were incubated at room temperature in this solution for one-two hours before the agro-infiltration.
Well-watered four to five weeks old *Nicotiana benthamiana* plants were used for agro-infiltration. Working suspensions were prepared by mixing appropriate clones; for 2ml of working solution were used 600µl of p19 at 1OD_{600} and 1.4ml of one expression clone (plasmids pBiFCt-590NPR1-CC, pBiFCt-590NPR1-CN, pBiFCt-590NPR1-NC, pBiFCt-590NPR1-NN, pBiFCt-600NPR1-CN, pBiFCt-600NPR1-NC, pBiFCt-600NPR1-NN and pBiFCt-600NPR1-CC) at 1 OD_{600}. Three leaves of each plant were infiltrated using a sterile disposable syringe.

2.8 Bimolecular Fluorescence Complementation (BiFC) analysis

The Bimolecular Fluorescence Complementation (BiFC) experiments were performed in the epidermal cell layer of the abaxial leaf surface two to three days after infiltration to verify the interaction between the proteins. Epi-fluorescence analysis were performed by Nikon eclipse 90*i* microscope. The magnification used for the great part of samples is 20 X magnification. The optimal excitation wavelengths for YFP are in the range of 490–515 nm; the maximal emission intensity is observed in the range of 520–560 nm (figure 2.5). As for RFP, it possesses bright fluorescence with excitation/emission maxima at 555 and 584 nm (figure 2.5).

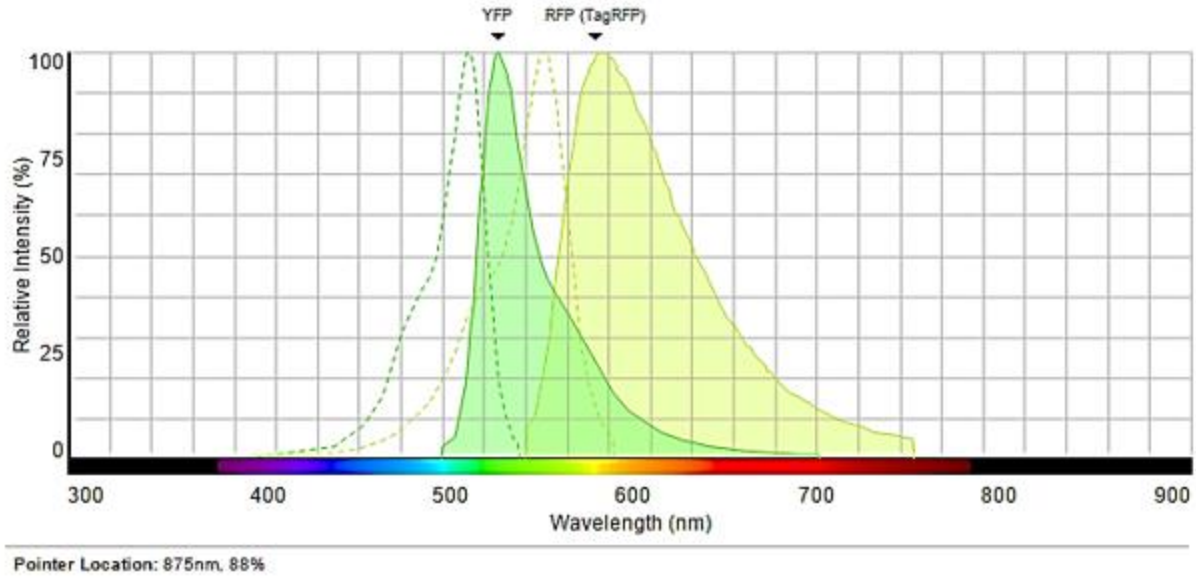

Figure 2.5: Absorption (dashed lane) and emission (continuous lane) spectrum of RFP and YFP proteins reproduced from Thermo Fischer Scientific SpectraViewer.

The confocal microscopy analysis was performed by *Leica SP2 confocal* microscope. The table 2.2 shows the parameters for imaging acquisition. The magnification used was 63 X.

Table 2.2: Parameters for imaging.

Fluorescence protein	Excitation wavelength	Dichroic transition	Emission filter
YFP	514nm	520nm	525LP
RFP	561nm	575nm	575LP

2.9 Western blot analysis

Two leaf discs were homogenized in liquid nitrogen by using glass beads. After addition of a denaturing buffer (150–200 µl of hot SDS-sample buffer), the samples were incubated for five minutes at 95°C. The samples were centrifuged five min at 10000 x g to remove cell fragments. Than 15 µl of the supernatant were used for SDS-PAGE experiment. For protein separation 12.5% polyacrylamide gel was used: 8.3 ml Acrylamide/Bis (29.2% Acrylamide, 0.8% Bisacrylamide), 5.0 ml S-buffer (1.5 M Tris; pH 8.8), 6.4 ml H_2O, 200 µl of 10% SDS, 150 µl of ammonium persulfate (APS), 12 µl tetramethylethylenediamine (TEMED). Collection gel used was compound of 1.3 ml Acrylamide/Bis (29.2% Acrylamide, 0.8% Bisacrylamide), 2.0 ml C-buffer (0.5M Tris; pH 6.8), 3.2 ml H_2O, 80 µl of 10% SDS, 80 µl APS, 12 µl TEMED.
The gel was run at 15 mA in running buffer (30 g Tris, 144 g glycine, 15 g SDS and H_2O to a final volume of 1 l).
Western blot transfer was carried out using standard protocols (Sambrook et al.,2001) for the detection for subsequent detection of fusion proteins by both anti-HA and anti-c-myc antibodies according to the sample. Membrane was incubated for two-three hours at room temperature blocking buffer with continuous shaking. The blocking buffer was antibody dependent; for c-myc detection was used Tris-buffer saline (10x TBS-buffer: 0.5 M Tris- HCl; pH 7.4, 1.5M NaCl) + 1 % albumin bovine serum (BSA), whereas for HA detection TBS buffer containing 0.1% Tween-20 (TBS-T buffer).
The primary antibody used were anti-HA from rat (1:1000) (Roche) and anti-c-myc from mouse (1:1000) (Roche). The incubation of membrane in primary antibody solutions was for two hours. The primary antibodies were then removed by three washes for five minutes in TBS-T buffer. The membranes were then incubated with specific secondary antibodies for one hour at room temperature (anti-rat-AP 1:5000 for anti-HA (Sigma) and anti-mouse-AP 1:5000 for anti-c-myc (Biorad). The membranes were than washed three times for ten min with TBS-T and equilibrated shortly in staining buffer A (100 mM Tris; pH9.5, 100 mM NaCl, 5 mM $MgCl_2$), and then incubated in staining solution. The staining solution was made of 66 µl of NBT solution (50 mg ml^{-1} nitro-blue tetrazolium chloride (NBT) in 70% of dimethyformamid), 33 µl BCIP solution (50 mg ml^{-1} 5-bromo-4-chloro-3'-indolyl-phosphate p-toluidine (BCIP) in 100% of dimethyformamid) and 10 ml staining buffer A. The reaction was stopped by rinsing the membrane twice in water.

2.10 Analysis of gene expression

2.10.1 RNA isolation and quantification

Total RNA was prepared from leaves by a phenol/chloroform extraction and a lithium chloride precipitation. Leaves of three-four weeks old-plants were cut and immediately frozen in liquid nitrogen. Approximately 0.5 g of leaves were powdered in liquid nitrogen using mortars and pestles. To the leaf powder were added 750 µl of RNA extraction buffer (100 mM Tris-HCl; pH 8.5, 100 mM NaCl, 20 mM EDTA; pH 8.0 and 1% SDS) and 750 µl phenol/chloroform 1:1, the samples were immediately vortexed and centrifuged at 12000xg at 4°C for five minutes. Phenol/chloroform extraction was repeated two times on the aqueous phase and then a chloroform extraction was carried out in the same conditions. Nucleic acids precipitation was obtained by adding 750 µl of isopropanol, incubation in ice for five minutes. The samples were then centrifuged at 4°C for ten minutes. The pellet was solubilised in 400 µl of DEPC-treated water (Sambrook et al., 2001). RNA was precipitated by adding 400 µl of 4 M LiCl. Samples were centrifuged at 12000xg at room temperature for 20 minutes, and the pellet was solubilized in 400 µl of DEPC-treated water. RNA was precipitated with 0.1 volume of 3 mM sodium acetate (pH 7.2) and two volumes of ethanol. The samples were incubated at -80°C for ten minutes and then centrifuged for ten minutes at maximum speed at 4°C. The pellet was dissolved in 42 µl DEPC-treated water. Quality control and integrity estimation of the RNA extracted was performed both by the evaluation of absorption curves in a Nanodrop spectrophotomether (Thermo Scientific) of and by electrophoresis analysis of denatured sample in 1.2% agarose gel (Sambrook et al., 2001).

2.10.2 First strand cDNA synthesis protocols

Two micrograms of RNA were treated with DNase I (Invitrogen). The reaction was performed by addiction to RNA solution of 1 U of DNase I (Invitrogen) and 1µl of 1X DNase I Buffer (200 mM Tris-HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl) and DEPC-treated water to 10 µl. The tubes were incubated for 15 minutes at room temperature. The DNase was deactivated by the addition of 1 µl of 25 mM EDTA solution to the reaction mixture and subsequent incubation at 65°C for ten minutes. The synthesis of the first cDNA strand was performed according to the Invitrogen's instruction. The amplification of the constitutively expressed *EF1-α* gene was used to check the efficiency of synthesis of the first cDNA strand. The primers (table 2.1) were designed to span an intron to make possible the distinction of the amplification on the genomic DNA (750 bp) and of the cDNA (650 bp). The amplification reaction was set up using 1µl of each sample according to the conditions described in section 2.2.

2.10.3 Real-Time PCR

Real-time PCR reactions were set up in a final volume of 20 µl, cDNA samples (diluted 1:20) were used for each reaction, to which were added 10µl of 2X QuantiFast SYBR Green PCR Master Mix (Qiagen), primers to a final concentration of and 0.3 µM and sterile MilliQ water to a final volume of 20 µl.
The analysis was performed by 7900HT Fast Real-Time PCR System (Applied Biosystems). The cycle of amplification is shown in table 2.1 and has been preceded by an initial step of denaturation (95°C for ten minutes).
For each conditions, three biological and technical replicates were analysed.
The values obtained were then analysed by the Sequence Detection System software (Applied Biosystems), to calculate RQ (Relative Quantification) values with $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The RQ values were obtained using the endogenous gene *EF1-α*.

3. RESULTS

A previous transcriptomic study, carried out in the host laboratory, indicated that tomato plants have a dynamic transcriptional response following *Macrosiphum euphorbiae* attack. In that study, a probe annotated as *NIMIN 2C* (Solyc03g119590.1.1), was among the genes with the higher level of expression (Coppola et al., 2013). Based on the transcriptomic and proteomic data, the authors proposed a model in which *NIMIN 2C* may be an important component of the plant defence to aphids. For this reason, I first evaluated at different time-points of *Macrosiphum euphorbiae* aphids infestation the expression level of the *NIMIN 2C* present in the Agilent Tomato microarray using an independent technique (real-time PCR). The real-time results indicated that this gene is highly overexpressed during all phases of the infestation analysed (figure 3.1). The data confirmed the involvement of this gene in aphid response.

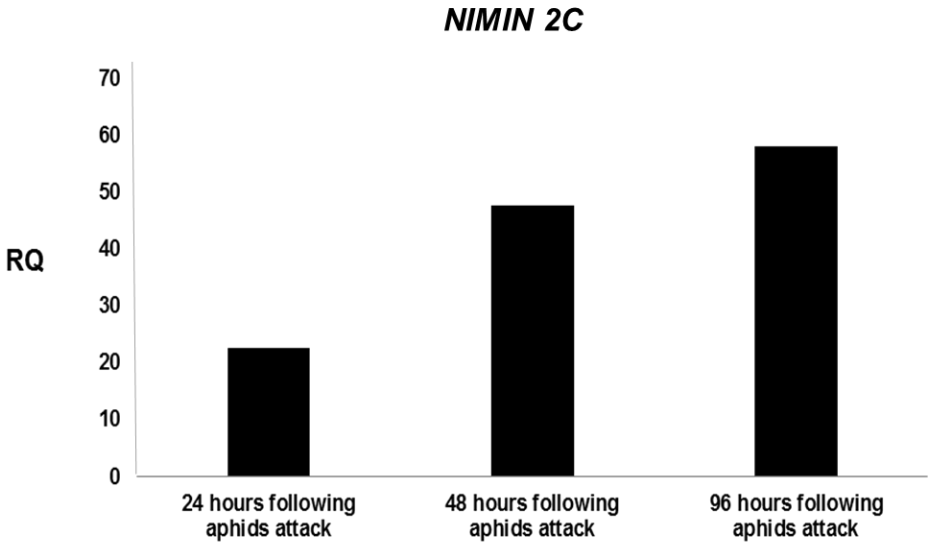

Figure 3.1: Analysis of *NIMIN 2C* gene expression by real-time PCR performed on leaves after 24h, 48h and 96h from *Macrosiphum euphorbiae* attack. For each condition, *NIMIN* expression level in attacked plants is different from the control plants at same time point (t-test; $p < 0.01$).

3.1 Bioinformatic analysis of *NIMIN* genes in *Arabidopsis*

NIMIN proteins interact with NPR1 in different plant species such as *Arabidopsis*, tobacco and rice (Weigel et al., 2001; Zwicker et al., 2007; Maier et al., 2011; Chern et al., 2005, 2012). This suggests that NIMIN proteins are conserved elements of the SAR pathway. *Arabidopsis* NIMIN proteins affect NPR1 differently. They act in a strictly consecutive and SA-regulated manner on the SA sensor protein NPR1 to monitor the progressing threat by pathogens and to promote appropriate defence gene activation at distinct stage of SAR (Hermann et al., 2013). While the role of NIMIN proteins in *Arabidopsis*-fungal interaction has already been described, I wanted to analyse if NIMIN function could be described with a more general role, being related to other biotic stresses.

For this reason, I carried out a bioinformatics analysis on the *NIMIN* genes in *Arabidospis*. According to the current release of the *Arabidopsis* genome (TAIR10), this species has four *NIMIN* members (table 3.1).

Table 3.1: *NIMIN* genes in *Arabidopsis*.

Locus	Gene Model	Other Name (Type)	Coordinates
AT1 g02450	AT1 g02450.1	NIM1-INTERACTING 1; *NIMIN 1*	497976-498516 bp
AT3 g25882	AT3 g25882.1	NIM1-INTERACTING 2; *NIMIN 2*	9470417-9470950 bp
AT1 g09415	AT1 g09415.1	NIM1-INTERACTING 3; *NIMIN 3*	3037754-3038408 bp
AT4 g01895	AT4 g01895.1	NIMIN-1-related; *NIMIN 1b*	819957-820379 bp

An alignment of the protein sequences indicated that the similarity among the members is limited (figure 3.2).

```
CLUSTAL O(1.2.1) multiple sequence alignment


AT3G25882.1      --------MNNS---LKKEERVEEDNGKSDGNRGKPSTEVVRTVTEEEVDEFFKILRRVH
AT1G09415.1      -------------------------MDRDRKR---VKMEKEDDEEEKMEKLYTVLKNAR
AT1G02450.1      MYPKQFSLYNYSLETMSKDENVESKETIRVDKR---VREDEEEEEEKKIDTFFKLIKHYQ
AT4G01895.1      ---------------MNQE--EEKTENKRINEI---DEDEEEELENKKMDMFFNLIKNYQ
                                                :         .   ::::: ::.:::. :

AT3G25882.1      VATRTVA----KVNGGVAEGELPSKKRKRSQNLGL---RNSLDCNGVR------------
AT1G09415.1      EMRKYVNSSMEK-----KR--QEEEERARVRRFPSFQPEDFIFMNKAEANN---IEKAAN
AT1G02450.1      EARKRRREELAENSGVVRR--KSNGGERSGIVVPAFQPEDFSQCRTG-LKPPLMFVSDHK
AT4G01895.1      DAKKRRRQYLTQDSGDVAS--MPTKR-SDYSIVPVFRAEDFSHCMDLNLKPSNSIISTKN
                    :       :                     .     .:

AT3G25882.1      --------------------DGEFDEINRVGLQGLGLDLNCKPEPDSVSLSL
AT1G09415.1      ES------------SSASNEYDGSKEKQEGSETNVCLDLNLSL---------
AT1G02450.1      EENTKVEQEEDQ--TE-------------ERNEDKALDLNLAL---------
AT4G01895.1      QEEEKQEEEEEEDDEEEEDDDEGEEEVEKVMRKDNGLDLNLAL---------
                                                  ****
```

Figure 3.2: Multiple alignment of the *Arabidopsis* NIMIN Proteins. Single dots (.) indicate conservation between groups of weakly similar properties-scoring =< 0.5 in the Gonnet PAM 250 matrix. Double dots (:) indicate conservation between groups of strongly similar properties-scoring > 0.5 in the Gonnet PAM 250 matrix. Asterisk (*) indicates positions which have a conserved residue.

The percentage of similarity between *Arabidospsis* NIMIN proteins is showed in table 3.2.

Table 3.2: Percentage of identity between NIMIN proteins in *Arabidospsis.*

	AT3 g25882.1; NIMIN 2	**AT1 g09415.1; NIMIN 3**	**AT4 g01895; NIMIN 1b**	**AT1 g02450.1; NIMIN 1**
AT3 g25882.1; NIMIN 2	100	17.05	20.79	21.43
AT1 g09415.1; NIMIN 3		100	21.62	27.55
AT4 g01895; NIMIN 1b			100	42.74
AT1 g02450.1; NIMIN 1				100

The low percentage of identity between the NIMIN proteins could justify different interactions between these proteins and NPR1.

Publicly available data were analysed to check whether *NIMIN* genes are differently regulated and how they are involved in different defence responses. To increase consistency, the work was carried out considering only the most diffused microarray format for *A. thaliana*, the GeneChip *Arabidopsis* ATH1 Genome Array. This array was designed in collaboration with TIGR and contains more than 22,500 probe sets representing approximately 24,000 genes. In this array, a probe for *NIMIN 1b* and *NIMIN 2* were not presented. The first analysis was carried out to understand how strongly the genes of interest are expressed across different stages of development, from germination to senescence.
Figure 3.3 reports the level of expression of the two genes taken from 9848 arrays. For each gene, the expression value for a given stage of development represents the average of the absolute expression (the microarray signal) of all samples annotated as such, including treatments and conditions that may alter its level of expression. The data indicated that *NIMIN 1* and *NIMIN 3* are expressed in all the different stages and tissue analysed. According to the Pearson coefficient calculation, the correlation between the absolute values of expression of the two genes was 0.54 and not significant (p=0.10; two sides), essentially because of the differences in the early developmental stages. However, the two genes have a comparable expression level in mature leaves (developed rosette).

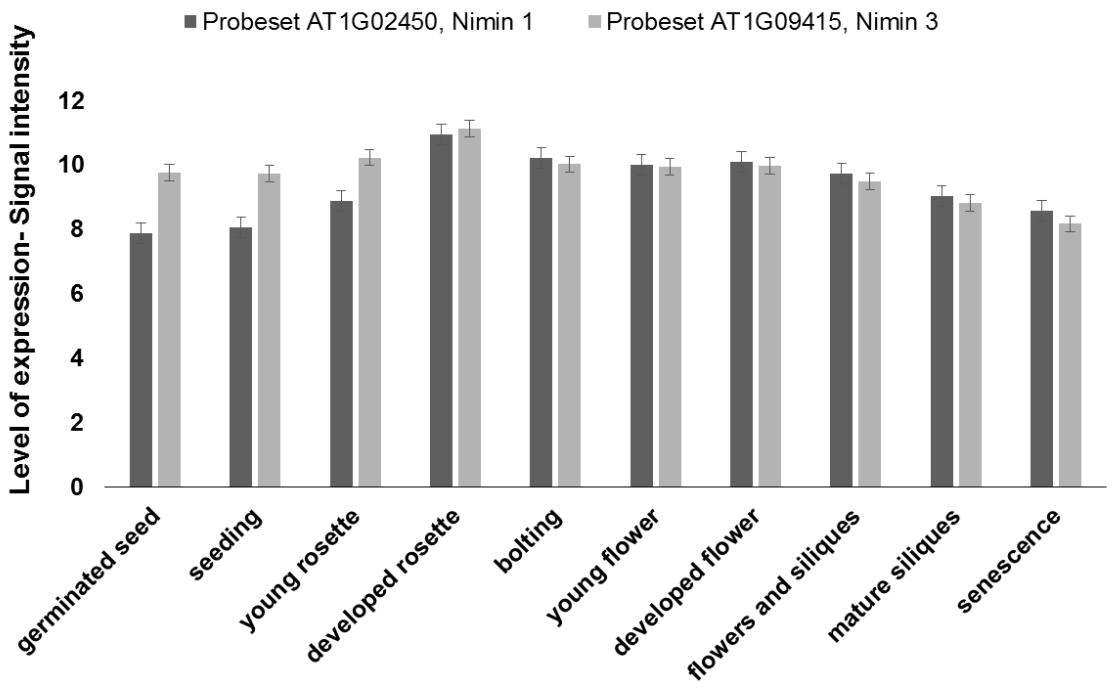

Figure 3.3: Level of expression of *NIMIN 1* and *NIMIN 3* genes. The graph displays the average (± standard deviation) of the standardized signal intensity of the microarray data for *NIMIN 1* and *NIMIN 3*. Signals up to ten represent a low level of expression, from ten to 12.5 a medium level (equal to the interquartile range, IQR) and above 12.5, a high level of expression

Subsequently, the conditions that most strongly affect the expression of the genes of interests were identified by analyzing published data of relative level of expression of *NIMIN 3* and *NIMIN 1*. This analysis was carried out only on data relative to the leaf tissue (at the developed rosette stage; 2538 microarray experiments), consistent with the overall aim of the analysis (i.e. the elucidation of the function of these genes in plant-biotic stress interactions). I then excluded experimental data that were pertinent to the comparison of different genotypes. The conditions tested were the presence of hormones, elicitors, biotic stress such as the presence of fungi and bacteria, abiotic stress such as the variation of photoperiod, light quality and intensity, but also cold and hot stress and the variation of CO_2 concentrations. As for the hormones, data coming from samples treated with different concentration of salicylic acid and 1-Naphthaleneacetic acid were analysed. The effects of elicitor tested were peptide (FLG22) (Denoux et al., 2008) and harpin elicitor protein (HrpZ). The data analysed were also from experiments related to the presence of fungi such as from *Pseudomonas avellanae*, as *Blumeria graminis* and Golovinomyces orontii and bacteria like *Pseudomonas syringae* and *Escherichia coli* at different time point from infestation. The conditions tested clearly indicated a differential regulation of the two genes. The conditions where *NIMIN 1* gene expression was highly affected were essentially related to the presence of salicylic acid, biotic stress (mainly fungal or bacterial pathogens) or the presence of elicitors. In the same conditions, the level of expression of *NIMIN 3* was not greatly affected. Overall, the data indicated that in *Arabidopsis*, members of the *NIMIN* gene family respond to stress in a different fashion implied a possible differential regulation.

To identify genes that have the most similar profile to the two target genes of interest across the dataset of interest (the perturbations that significantly affected *NIMIN* genes expression), I ran a co-expression analysis. This analysis was based on the Pearson correlation coefficient measure of similarity between genes based on log2-scaled expression data (with a cut-off of 100 sequences). Briefly, this analysis indicated that the Gene Ontology of the two gene list is different. The main ten genes correlated with *NIMIN 3* expression are those genes coding for: DNA glycosilase superfamily protein (score 0.70), S-domain-1 13 (score 0.70), cis-trans isomerase family protein (score 0.69), S-adenosyl-L-methionin-dependent methyltransferase (score 0.65), inositol monophosphase family protein (score 0.64), transcriptional factors B3 family protein (score 0.64), BR enhaunced expression two (score 0.64), DHFS-FPGS homolog D (score 0.63), photosystem II 5 kD protein score (0.63), plant Tudor like RNA-binding protein (score 0.63). The table 3.3 shows the main 30 genes correlated with *NIMIN 3* expression.

Table 3.4: Description of the most correlated genes (top 30) correlated with *NIMIN 3.*

	Gene	Score	Description
1	AT1G80850	0,7	DNA glycosylase supertamily protein
2	AT1G11350	0,7	s-domain-1 13
3	AT1G73655	0,69	cis-trans isomerase family protein
4	AT1G23360	0,65	S·adenosyl-L-methionine-dependent methyltransferase
5	AT1G43670	0,64	Inositol monophosphatase family protein
6	AT4G00260	0,64	AT4G00260, Transcriptional factor B3 family protein
7	AT4G36540	0,64	BR enhanced expression 2
8	AT3G55630	0,63	DHFS·FPGS homolog D
9	AT1G51400	0,63	Photosystem II 5 kD protein
10	AT5G20030	0,63	Plant Tudor-like RNA-binding protein
11	AT3G23080	0,63	Polyketide cyclase/dehydrase
12	AT5G16030	0,62	Unknown protein; *Arabidopsis thaliana* protein
13	AT5G66520	0,62	Tetratricopeptide repeat (TPR)·like superfamily protein
14	AT4G13020	0,62	Protein kinase superfamily protein
15	AT1G54820	0,62	Protein kinase superfamily protein
16	AT5G63180	0,62	Pectin lyase-like superfamily protein
17	AT5G15310	0,62	Myb domain protein 16
18	AT1G77400	0,62	Contains InterPro DOMAIN/s
19	AT3G48490	0,61	Unknown protein
20	AT1G10360	0,61	Glutathione S-transferase TAU 18
21	AT2G35410	0,61	RNA-binding (RRM/RBD/RNP motifs) family protein
22	AT1G21560	0,61	Unknown protein; *Arabidopsis thaliana* protein
23	AT3G47560	0,6	Alpha/beta-Hydrolases superfamily protein
24	AT1G65010	0,6	Plant protein of unknown function (DUF827)
25	AT1G53230	0,6	Teosinte branched 1
26	AT2G05160	0,6	CCCH-type zinc finger family protein with RNA-binding
27	AT2G46710	0,6	Rho GTPase activating protein with PAK·box/P21·R
28	AT5G27950	0,6	P·loop containing nucleoside triphosphate hydrolase .
29	AT2G34620	0,6	Mitochondrial transcription termination factor family
30	AT1G08980	0,59	Amidase 1

The main genes correlated with *NIMIN 1* expression are those genes coding for: WRKY DNA-binding protein 38 (score 0.85), LYS/HIS transporter 7(score 0.82), WRKY DNA-binding protein 54 (score 0.82), calcium-dependent lipid-binding (score 0.79), protein kinase superfamily protein (score 0.79), MAP kinase two (score 0.79), protein phosphatase 2C family protein (score 0.78), calmodulin binding protein-like (score 0.77), tetratricopeptide repeat (TRP)-likr superfamily protein (score 0.77), WRKY DNA-binding protein 70 (score 0.77). The table 3.4 shows the main 30 genes correlated with *NIMIN 1* expression.

Table 3.4: Description of the most correlated genes (top 30) correlated with *NIMIN 1.*

	Gene	Score	Description
1	AT5G22570	0.85	WRKY DNA-binding protein 38
2	AT4G35180	0.82	LYS/HIS transporter 7
3	AT2G40750	0.82	WRKY DNA-binding protein 54
4	AT3G62780	0.79	Calcium-dependent lipid-binding (CaLB domain)
5	AT3G57700	0.79	Protein kinase superfamily protein
6	AT4G29810	0.79	MAP kinase 2
7	AT5G37480	0.78	unknown protein
8	AT1034750	0.78	Protein phosphatase 2C family protein
9	AT1073805	0.77	Calmodulin binding protein-like
10	AT3G09490	0.77	Tetratricopeptide repeat (TPR)-like superfamily protein
11	AT3G56400	0.77	WRKY DNA-binding protein 70
12	AT3G09010	0.77	Protein kinase superfamily protein
13	AT4G26070	0.76	MAP kinase/ ERK kinase 1
14	AT4G23610	0.76	Late embryogenesis abundant (LEA) hydroxyprolin
15	AT1023840	0.76	unknown protein
16	AT5G19930	0.75	Protein of unknown function DUF92, transmembrane
17	AT5G44820	0.75	Nucleotide-diphospho-sugar transferase family protein
18	AT4G19660	0.75	NPR1-like protein 4
19	AT5G45000	0.75	Disease resistance protein (TIR-NBS-LRR class)
20	AT1G15790	0.75	unknown protein; *Arabidopsis thaliana* protein
21	AT5G62770	0.74	Protein of unknown function (DUF1645)
22	AT2G26400	0.74	Acireductone dioxygenase 3
23	AT5G52760	0.74	Copper transport protein family
24	AT1001560	0.74	MAP kinase 11
25	AT1064280	0.74	Regulatory protein (NPR1)
26	AT1073805	0.74	Calmodulin binding protein-like
27	AT4G10500	0.73	2-oxoglutarate(20G) and Fe(ll)-dependent oxygenase
28	AT4G23320	0.73	Cysteine-rich RLK (RECEPTOR-like protein kinase)
29	AT3G28480	0.73	Oxoglutarate/iron-dependent oxygenase
30	AT1G16670	0.73	Protein kinase superfamily protein

The figure 3.4 show the circular plots in which, the target gene (A: *NIMIN 1*; B: *NIMIN 3*) is in the center, and correlated genes are displayed around it at a distance corresponding to their correlation score (i.e.: the closer they are to the center, the higher their correlation with the target gene across the selected dataset).

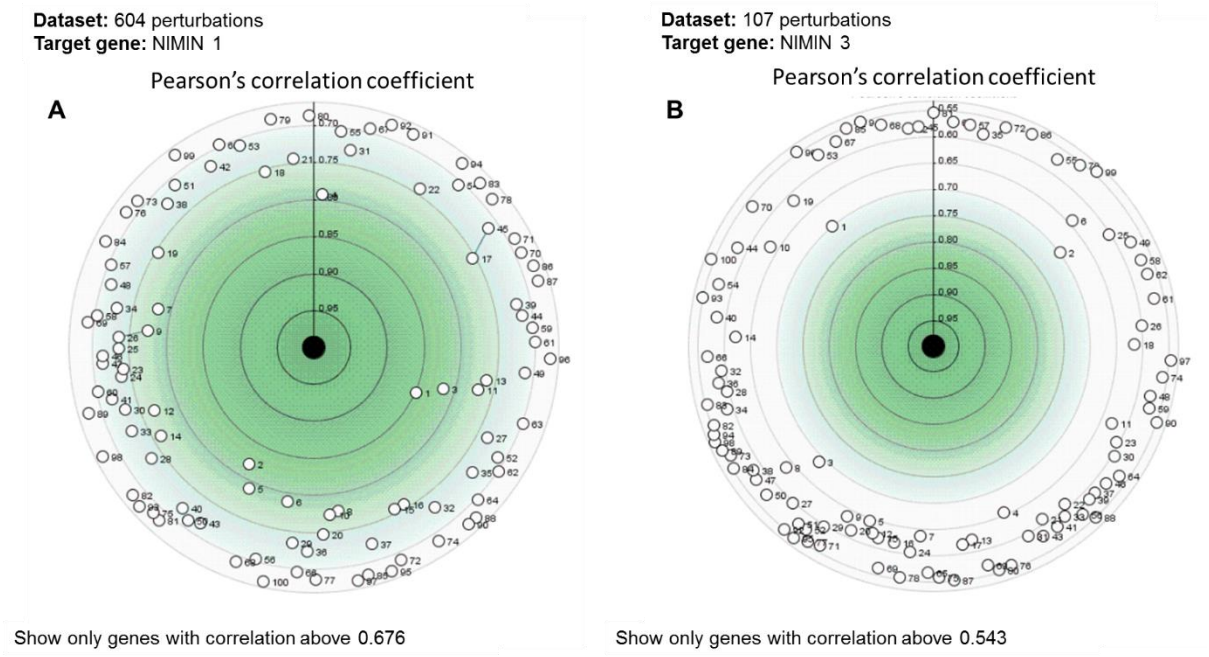

Figure 3.4: Co-expression analysis. Circular plots of the top 100 genes whose expression correlated with *NIMIN* genes in selected conditions (see text for details) (A: *NIMIN 1*, B: *NIMIN 3*). Each white dot represent a gene. The green color intensity is related to the Pearson coefficient, and it is provided to easy visual comparison between the two plots. The main ten genes correlated with *NIMIN 3* expression (table 3.3) are those genes coding for: DNA glycosilase superfamily protein (score 0.70), S-domain-1 13 (score 0.70), cis-trans isomerase family (score 0.69), S-adenosyl-L-methionin-dependent methyltransferase (score 0.65), inositol monophosphase family protein (score 0.64), transcriptional factors B3 family protein (score 0.64), BR enhaunced expression two (score 0.64), DHFS-FPGS homolog D (score 0.63), photosystem II 5 kD protein score (0.63), plant Tudor like RNA-binding protein (score 0.63). The main ten genes correlated with *NIMIN 1* (table 3.4) expression are those genes coding for: WRKY DNA-binding protein 38 (score 0.85), LYS/HIS transporter 7(score 0.82), WRKY DNA-binding protein 54 (score 0.82), calcium-dependent lipid-binding (score 0.79), protein kinase superfamily protein (score 0.79), MAP kinase two (score 0.79), protein phosphatase 2C family protein (score 0.78), calmodulin binding protein-like (score 0.77), tetratricopeptide repeat (TRP)-likr superfamily protein (score 0.77), WRKY DNA-binding protein 70 (score 0.77).

Interestingly among the top seven genes co-ex pressed with *NIMIN* genes there are many involved in plant defence response.

Therefore, it can be summarized that in the model species *Arabidopsis*, members of the *NIMIN* gene family differ in the way they respond to stress. *NIMIN* genes are likely to be associated and probably regulated by different molecular network furthermore the co-expression analysis strengthen the possible link between the members of the *NIMIN* gene family, with important genes related to the response against stress like WRKY transcription factors. So, *NIMIN* genes in *Arabidopsis* seems to be involved in the response against biotic and abiotic stress.

3.2 Bioinformatic analysis of *NIMIN 2C* genes in *Solanum lycopersicum*

Arabidopsis NIMIN proteins sequences were used for a search, using the software Standard Protein-Protein BLAST (blastp) to find similar sequences in protein databases. The analysis did not show any hit for the NIMIN 1-related protein. Sequences similar to Arabidopsis NIMIN 1 and NIMIN 3 where found only for very high e-value (>e10-3) (not shown). Only for the *Arabidopsis* NIMIN 2 protein sequence were two similar protein sequences obtained. These proteins are NIM1-INTERACTING2 of *Nicotiana tomentosiformis* and NIM1-INTERACTING 2-like of *Solanum tuberosum*, respectively, which showed a percentage of identity of 36% and 37% with the *Arabidopsis* NIMIN 2 protein sequence.
A phylogenetic tree was built to show the relationships among the *Arabidopsis* NIMIN 2 protein and homologues in different plant species. The algorithm Fast Minimum Evolution (Desper and Gascuel, 2004) and Grishin distance were used to develop a phylogenetic tree, shown in figure 3.5.
The tree illustrates that *NIMIN* genes are present in a vast number of species and genera. The analysis showed the presence of four NIMIN proteins in the species *Brassica rape* and *Brassica napus*, one in the specie *Solanum lycopersicum*.

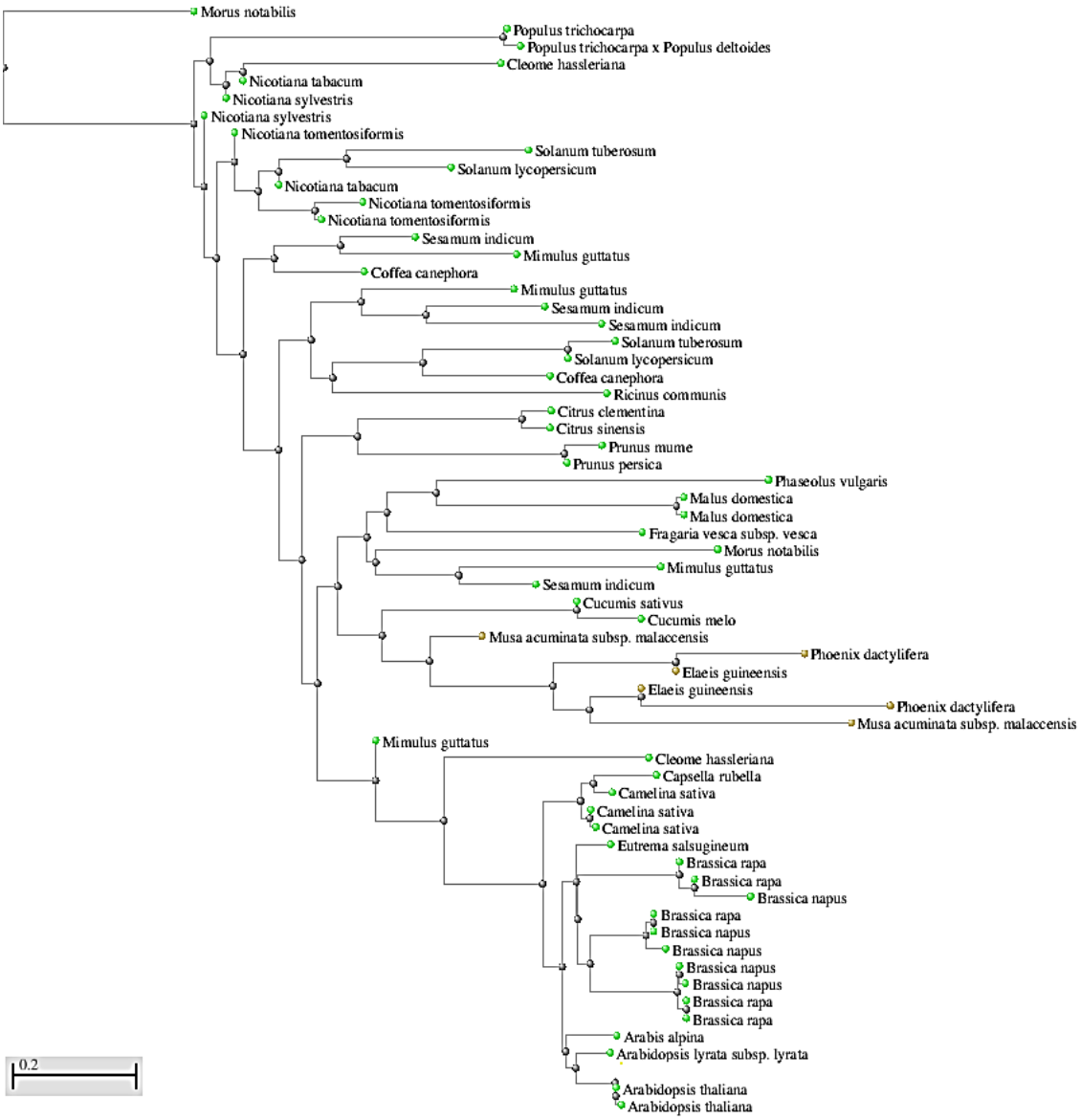

Figure 3.5: Phylogenetic tree based on AtNIMIN2 (AT3 g25882) homologues proteins.
The algorithm Fast Minimum Evolution (Desper and Gascuel, 2004) and Grishin distance were used to develop a phylogenetic tree. The tree is drawn to scale, with branch lengths in the same unit as those of evolutionary distance used to develop the phylogenetic tree.

As for *NIMIN* genes in tomato plants, I extended the search in Sol Genomics database. The recent annotation of the tomato genome (2.40 build) indicated the presence of two *NIMIN 2C*: *Solyc03g119590.1.1* and *Solyc03g119600.1.1*, both on chromosome III (Solyc03g119590 location SL2.50ch03:68154784.68155119, Solyc03g119600 location SL2.50ch03:68159889.68160152) (table 3.5).

Table 3.5: *NIMIN* 2C genes in *Solanum lycopersicum.*

Locus	Gene Model	Other Name (Type)
Solyc03g119590	Solyc03g119590.1	NIMIN 2C protein (AHRD V1 ***-A0FJY4_TOBAC)
Solyc03g11960	Solyc03g119600.1	NIMIN 2C protein (AHRD V1 ***-A0FJY4_TOBAC)

The sequence of Solyc03g119590 proteins was used for a similarity search, this analysis indicated that the most similar protein is the NtNIMIN 2C from tobacco (*Nicotiana tabacum*), from which the automatic annotation of the tomato genes was taken. I aligned the Solyc03g119590 and Solyc03g11960 sequence proteins (figure 3.6). This analysis indicated that the similarity among the members is limited.

```
CLUSTAL O(1.2.1) multiple sequence alignment


Solyc03g119590.1.1      MLLMDGEKKRKRTSIVAGDRSNDD------VKPTVKEEEPPSEAEVNEFFAILRRMHMAV
Solyc03g119600.1.1      ---MDRHKKRKRTD-NGGDRSRHERESSVKANTVVSKPPPPSEAEVNEFFAILRRMNVAV
                           ** .******.  .****..:      .: .*.:  ****************.:**

Solyc03g119590.1.1      KYLQRNAQIQPENVNAHGSKLTASPAGVNGDATGQKRERGIVRKGDLDLNTLPDCGD-
Solyc03g119600.1.1      KYLQKNAQIGEVEDS-------------------------HKRVDLDLNTLPEGGD*
                        ****:****   : .                          :: ********: **
```

Figure 3.6: NIMIN 2C proteins sequence alignment in *Solanum lycopersicum*. Single dots (.) indicate conservation between groups of weakly similar properties-scoring =< 0.5 in the Gonnet PAM 250 matrix. Double dots (:) indicate conservation between groups of strongly similar properties-scoring > 0.5 in the Gonnet PAM 250 matrix. Asterisk (*) indicates positions which have a single, fully conserved residue.

Comparing the results obtained for *Arabidopsis* NIMIN proteins alignment with those obtained for tomato NIMIN 2C proteins alignment it is evident that the identity between the protein product of Solyc03g119590.1.1 and Solyc03g119600.1.1 is 65.43%, higher than the one in *Arabidopsis*.
Taking into account also the close tomato *NIMIN 2C* genomic location, it is possible to speculate that these two sequences derive from a duplication event.
Available NGS-data of the Sol Genomics website were analysed to understand how strongly the genes of interest are expressed across different tissues. The expression level was indicated in RPKM, figure 3.7, to compare the variation of the two different genes (rather than the level of expression). The data were normalized using the Z-

score. In absolute terms (i.e. considering the raw RPKM values), the level of expression of Solyc03g119600.1.1 is higher.

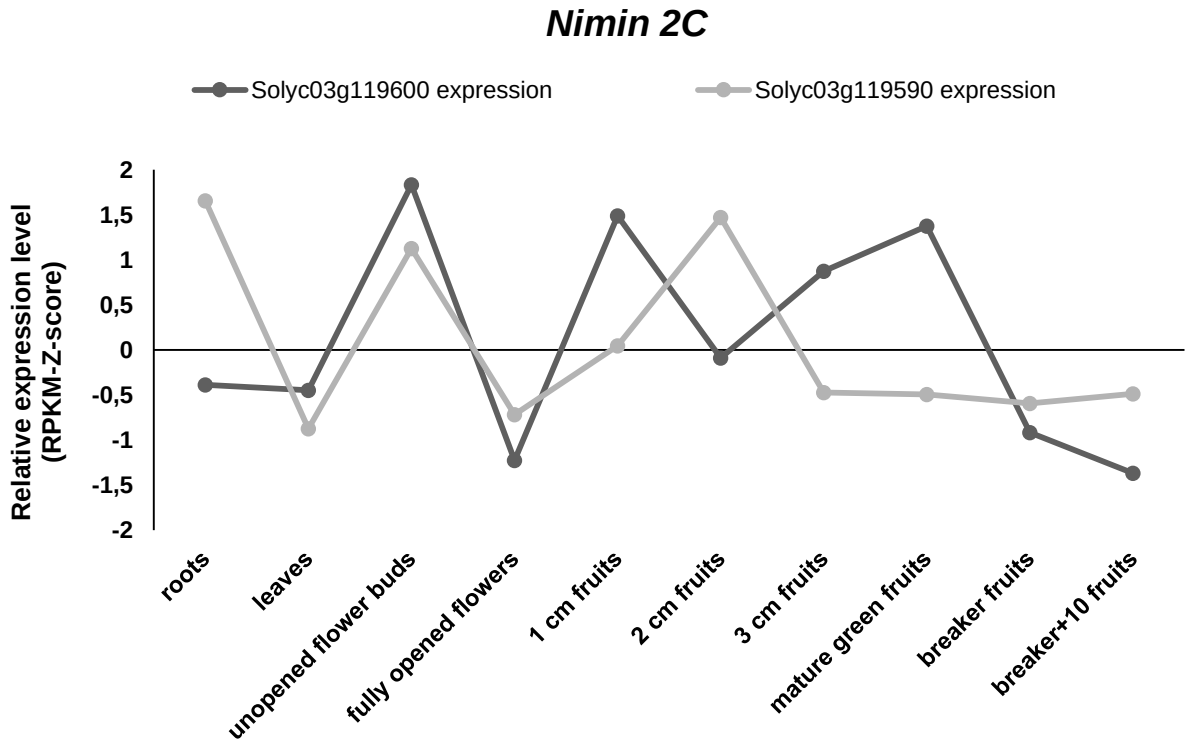

Figure 3.7: Variation in the level of expression of Solyc03g119590.1.1 and Solyc03g119600.1.1 in different tomato tissues. The level of expression is the normalised RPKM value across different tissue for each gene.

To infer about the differential response of the two tomato genes in relation to biotic stress, the expression profile of two tomato *NIMIN 2C* genes were analysed using publicly available NGS-data deriving from the tomato genome project.

The figure 3.8 shows normalized expression levels of Solyc03g119600.1.1 and Solyc03g119590.1.1. The normalization of the expression level (RPKM) was performed only in relation to each condition, to allow for a comparison of the expression between genes and between conditions. However, there is a significant correlation between the level of expression of the two genes. The Pearson correlation was 0.93 and significant ($p<0.01$). The conditions analysed are the presence of the protein flagellin 22 (flg22) or flagellin II-28 (flgII-28) but also the presence of lipopolysaccarides (LPS), peptidoglycan (PGN), cold shock protein 22 (csp22) and finally some lanes of *Pseudomonas syringae like* DC3000ΔhrcQ-UΔfliC and DC3000ΔAvrPtoΔAvrPtoB. The analysis was conducted at 30 minutes and six hours from the application of stress for the majority of the conditions analysed.

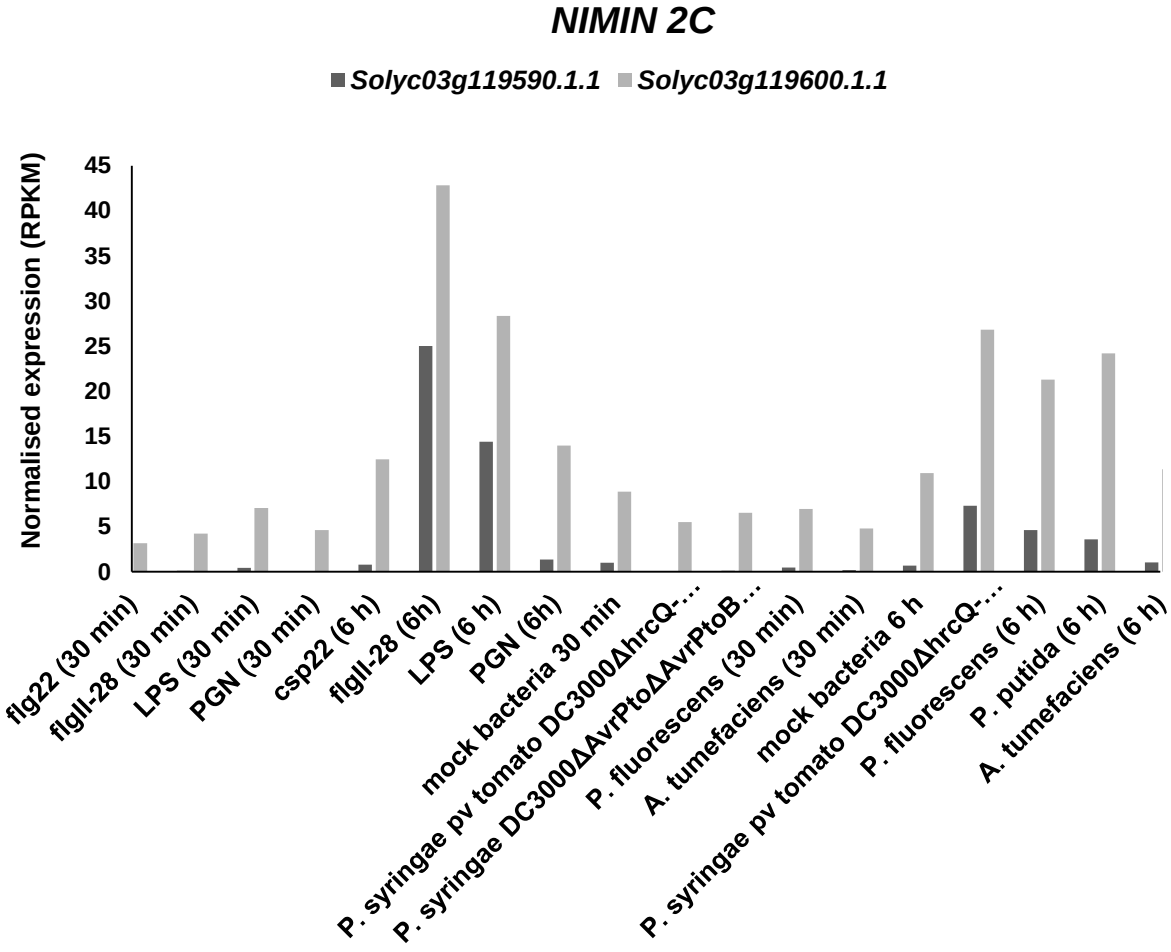

Figure 3.8: Normalized expression level of Solyc03g119600.1.1 and Solyc03g119590.1.1. The graph displays the level of expression expressed in RPKM of two tomato *NIMIN* genes (Solyc03g119600.1.1 and Solyc03g119590.1.1). For each of the conditions investigated such as the presence of the protein flagellin 22 (flg22) or flagellin II-28 (flgII-28), the presence of lipopolysaccarides (LPS), peptidoglycan (PGN), cold shock protein 22 (csp22) and finally some lane of *Pseudomonas syringae like* DC3000ΔhrcQ-UΔfliC and DC3000ΔAvrPtoΔAvrPtoB. The analysis was conducted at 30 minutes and six hours from the application of stress for the majority of the conditions analysed.

The data confirmed that, in absolute term (RPKM) the level of expression of Solyc03g119590.1.1 is in all conditions lower than Solyc03g119600.1.1.

3.3 Expression analisysis of *NIMIN 2C* genes in tomato

To validate and extend the results obtained by the *in silico* analysis, expression levels of tomato *Solanum lycopersicum* cv. “Red Setter” *NIMIN 2C* genes in different plant tissue and after biotic stress were analysed.
Concerning the expression levels in different plant tissues, RNA extraction was performed from leaves, fully opened flowers, stems and roots of tomato adult plants. The biological material was harvested from healthy and undamaged plants grown in controlled conditions. The figure 3.9 displays an example of RNA separation on agarose gel.

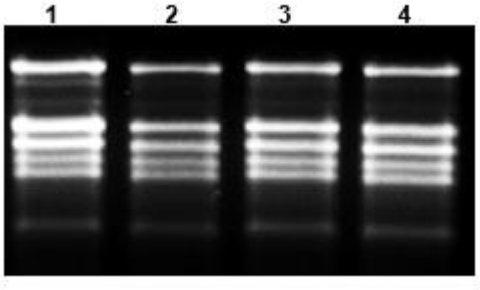

Figure 3.9: RNA separations. Agarose gel electrophoresis of 2 µg of denatured RNA extracted from samples of leaves (1-4).

The cDNA was amplified using the primers StbEF L-Fw and LeEF Rv (table 2.1). They were designed to span an intron of the tomato *EF1-α* gene. Such amplification is also able to evaluate the presence of any DNA contamination in the sample. The figure 3.10 shows an electrophoresis analysis of cDNA samples amplified by specific primers to target *EF1-α* gene.

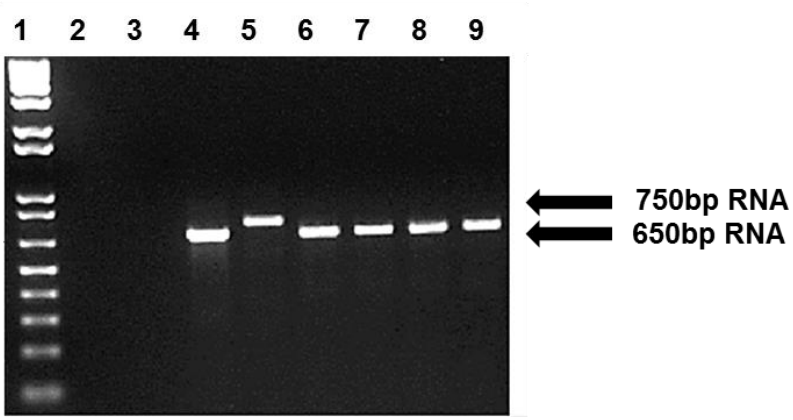

Figure 3.10: DNA fragment analysis on agarose gel electrophoresis of cDNA samples amplified using primers specific for the gene *EF1-α*. Lane 1: 1kb plus ladder (Invitrogen), lane 2: PCR negative control, lane 3: negative control of cDNA synthesis, RT mix without the template, lane 4: positive control, previously amplified cDNA, lane 5: positive control of PCR, amplification of genomic DNA of tomato, lane 6-10: cDNA samples.

The analysis of gene expression was carried out by Real Time RT-PCR. The leaf tissue was used as a calibrator. The data sets obtained were analysed with one-way ANOVA using the Duncan test to check for significant differences between means. The alpha

level was set at 5% (Bewick et al., 2004). The results of the relative quantification of the *NMIN 2C* genes (*NIMIN 590* and *NIMIN 600*) is shown in figure 3.11.

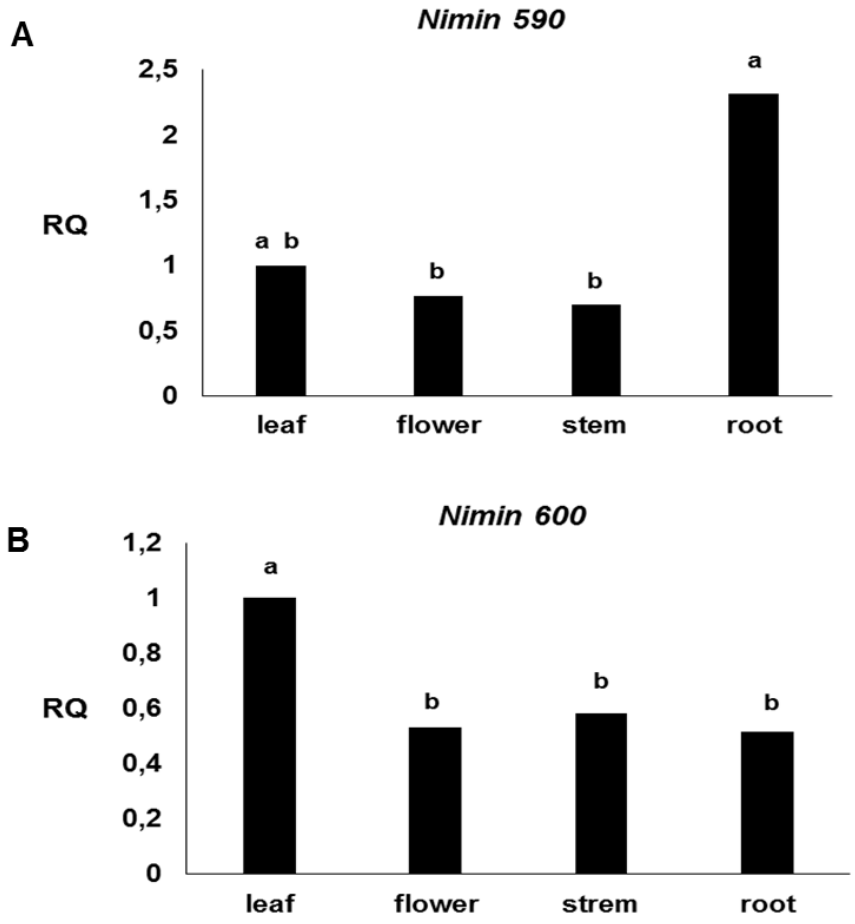

Figure 3.11: *NIMIN 2C* genes (A: *NIMIN 590*, B: *NIMIN 600*) expression levels in different tissues of tomato plant (Red Setter).
The analysis was carried out using a comparative Ct method, the endogenous control was *EF1-α* gene. The tissue that was used as calibrator is the leaf. Letters indicate significant differences between samples according to Duncan test; $p < 0.05$.

The next step was to verify the response of the two *NIMIN 2C* genes to biotic and abiotic stress. For wounding, leaves of tomato plants belonging to the genotype *Red Setter* were damaged with a scissor clamp. The samples were collected 24 and 48 hours after the damage. The RNA extraction was performed from wounded and distal leaves. Undamaged leaves at time 0 were used as control. To validate the wounding effect, I monitored the expression of the protease inhibitors genes (figure 3.12).

The analysis of gene expression was carried out by Real Time RT-PCR, the leaf tissue was used as a calibrator. The data sets obtained were analysed with one-way ANOVA using the Duncan test to check for significant differences between means. The alpha level was set at 5%.

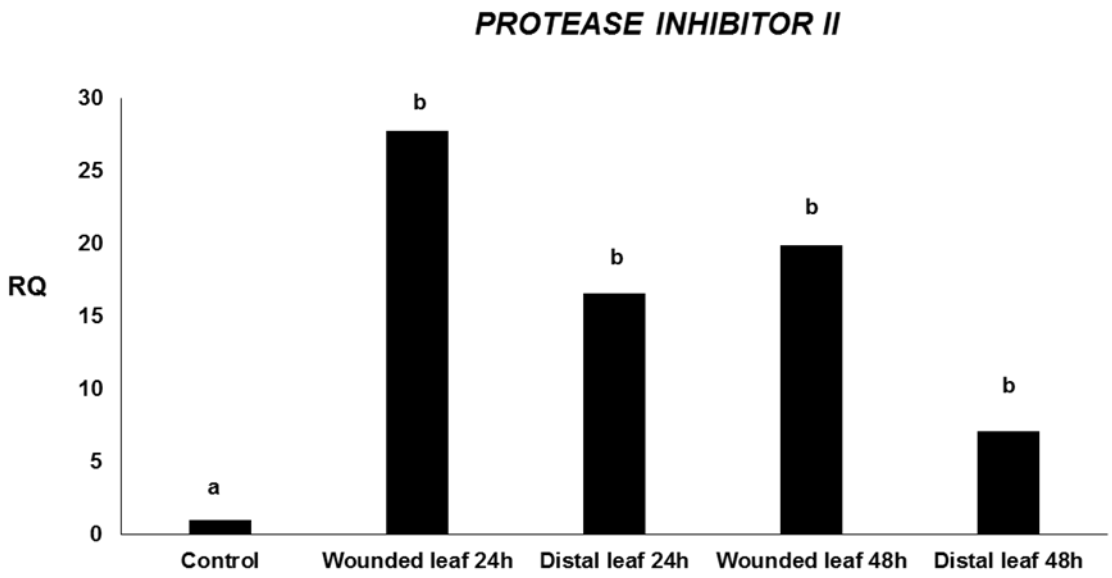

Figure 3.12: *PROTEASE INHIBITOR II* expression levels in different tissues of tomato plant (Red Setter). The analysis was carried out using a comparative Ct method; the endogenous control was *EF1-α* gene. The tissue that was used as a calibrator is a healthy leaf. Tomato plants belonging to the genotype Red Setter were damaged with a scissor clamp to reproduce the process of insect chewing. The samples were collected at time point 24 and 48 hours after the damage. Letters indicate significant differences between samples according to Duncan test; $p < 0.05$.

The figure 3.13 shows a different stress response of the two *NIMIN 2C* genes in response to wounding. While the *NIMIN 590* was strongly induced following wounding, *NIMIN 600* did not show any significant difference.

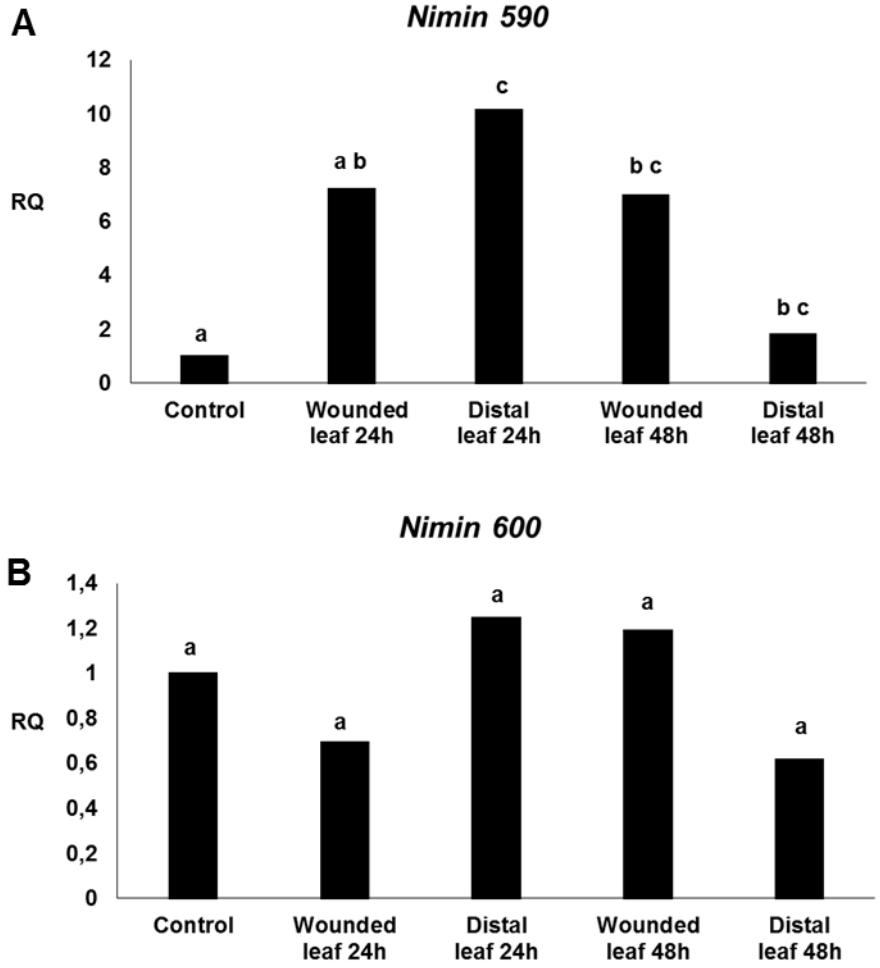

Figure 3.13: *NIMIN 2C* (A: *NIMIN 590*, B: *NIMIN 600*) genes expression levels in different tissues of tomato plant (Red Setter). The analysis was carried out using a comparative Ct method, the endogenous control was *EF1-α* gene. Healthy leaf was used as calibrator. Tomato plants belonging to the genotype Red Setter were damaged with a scissor clamp to reproduce the process of insect chewing. The samples were collected at time point 24-48 hours after the damages. Letters indicate significant differences between samples according to Duncan test; $p < 0.05$.

It was also performed an analysis of *NIMIN 2C* genes expression after 48h and 96h from the attack of the fungus *Botrytis cinerea* in proximal and distal leaves. The expression analysis indicated higher levels of *NIMIN 2C* genes expression in both the leaves attacked by the fungus and the distal 96 hours from the attack (figure 3.14). Also in this case the leaf was used as a calibrator.

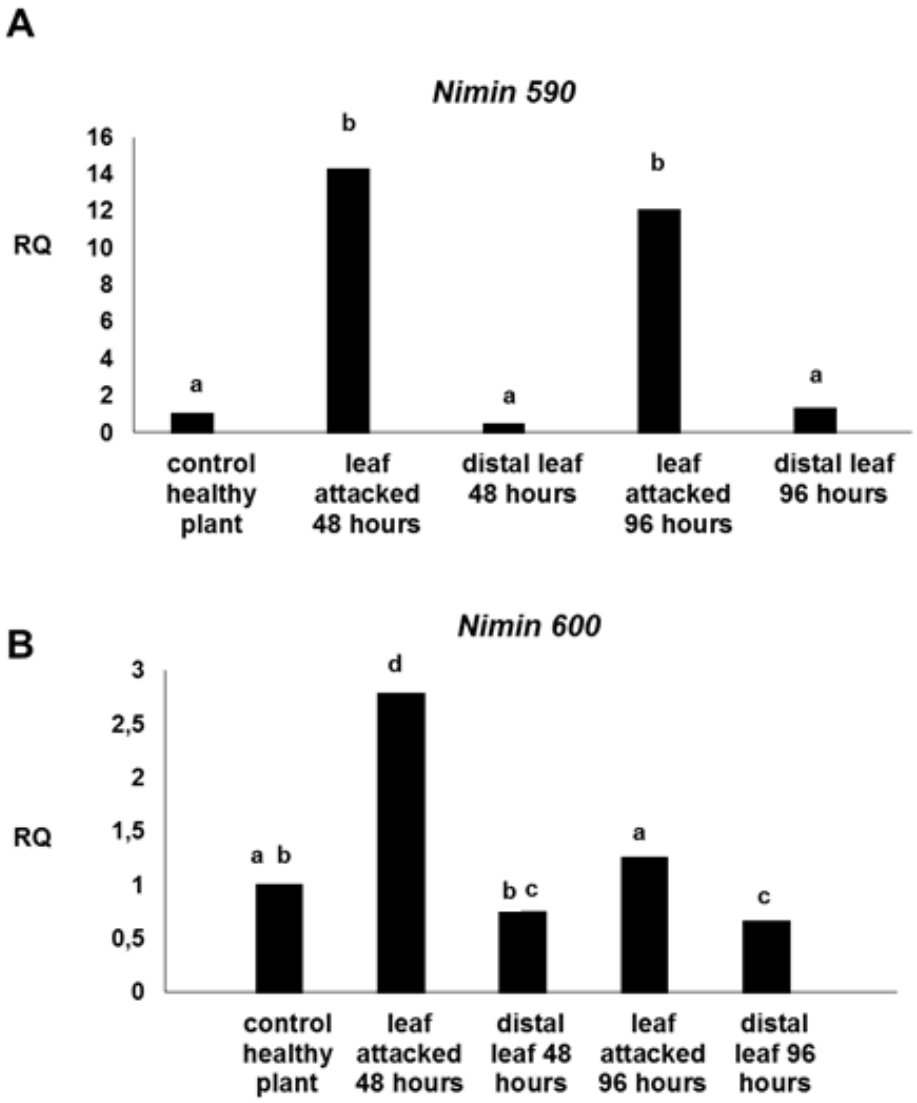

Figure 3.14: Analysis of *NIMIN 2C* (A: *NIMIN 590*, B: *NIMIN 600*) genes expression by real-time PCR performed on proximal and distal leaves after 48h and 96h from *Botrytis cinerea* attack. Expression levels are reported in relative terms. The calibrator is the healthy plant. Letters a-d indicate significant differences between samples according to Duncan test; $p < 0.05$.

The data confirm the different responses of the two *NIMIN 2C* genes to stress.

3.4 Assessment of the interaction between the tomato NIMIN 2C proteins and NPR1 by Bimolecular Fluorescence Complementation experiments (BiFC)

Bimolecular fluorescence complementation (BiFC) is very useful for the visualization of protein-protein interactions in living plant cells. The association of interacting proteins leads to the formation of a fluorescent complex by two non-fluorescent fragments of the yellow fluorescent protein (YFP) (Walter et al., 2004).

3.4.1 Cloning BiFC vectors

The first step of cloning was the amplification of two *NIMIN 2C* genes sequences with the primers pair NIMIN 590 P1P4 and NIMIN 600 P1P4. The amplification was performed using the Red Setter DNA as template. The expected size of PCR product for amplification are 397 bp for *NIMIN 590* and 325 bp for *NIMIN 600* as shown in figure 3.15.

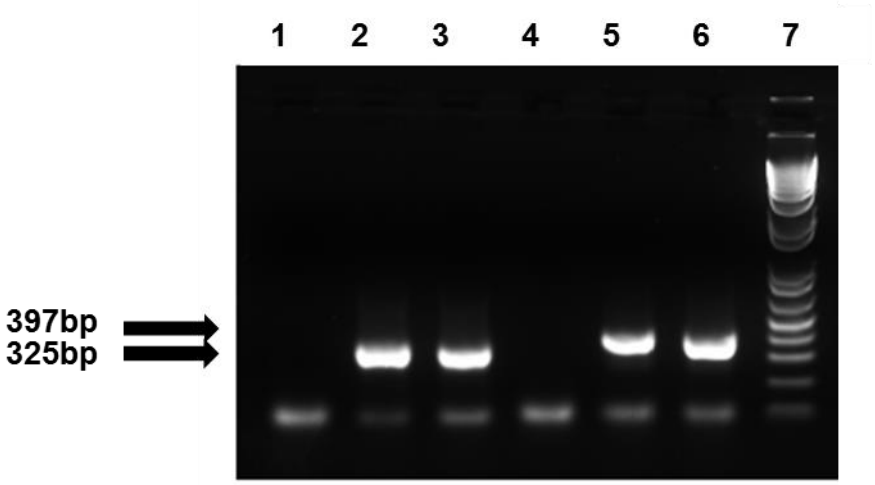

Figure 3.15: DNA fragment analysis on agarose gel electrophoresis after attB primers amplification of *NIMIN 590* and *NIMIN 600* genes from DNA sample. Lane 1: negative control *NIMIN 600* amplification, lanes 2-3: amplification of the Red Setter DNA with primers NIMIN 600 P1P4, lane 4: negative control *NIMIN 590* amplification, lanes 5-6: amplification of the Red Setter DNA with primers NIMIN 590 P1P4. 7: 1kb plus molecular weight ladder.

The purified amplification products were used for the recombination with the donor vector (pDonor221 P1P4). The recombination products, the entry clone pDonorNIMIN 590 P1P4 (pD590) and pDonorNIMIN 600 P1P4 (pD600), were used to transform *E.Coli* cells (InvαF') by thermal shock. The recombinant colonies were selected by colony PCR using the primers pair NIMIN 590 P1P4 and NIMIN 600 P1P4 (figure 3.16).

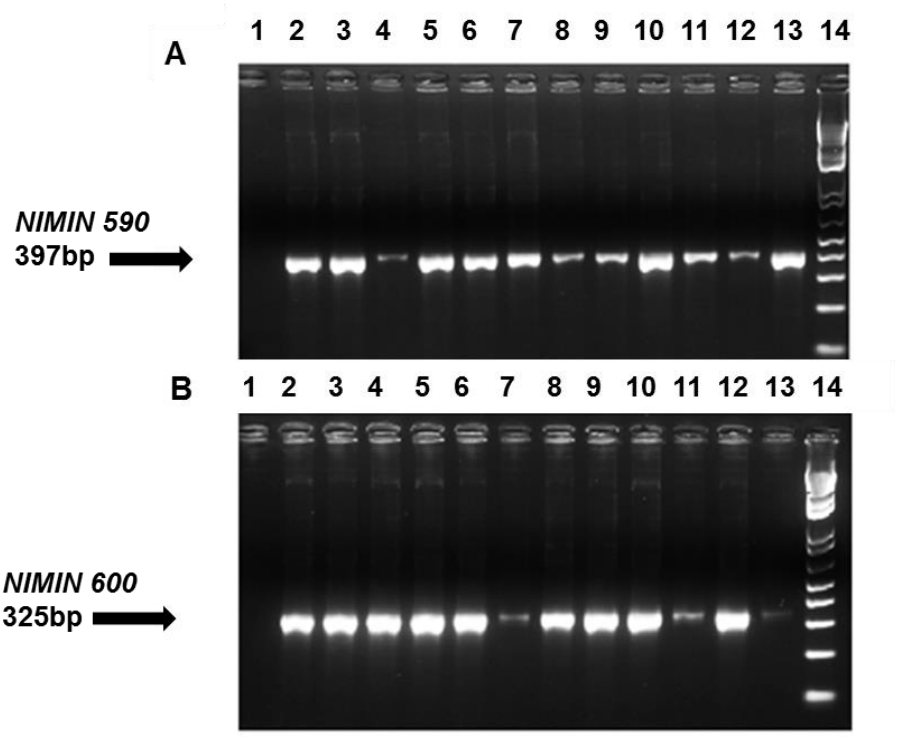

Figure 3.16: DNA fragment analysis on agarose gel electrophoresis of putatively recombinant plasmids using NIMIN 590 P1P4 (figure A) and NIMIN 600 P1P4 primers (figure B).
Lane 1 (A): negative control, lane 2 (A): DNA amplification, lanes 3-13 (A): colony PCR, putatively recombinant plasmid, 14 (A): 1kb plus molecular weight ladder.Lane 1 (B): negative control, lane 2 (B): DNA amplification, lanes 3-13 (B): PCR colony, putatively recombinant plasmid, 14 (B): 1kb plus molecular weight ladder.

The sequences of genes were confirmed by Sanger sequencing. Considering its length, the *NPR1* cDNA was synthesised by the GenScript Company with attL ends in a recombinant entry clone (pENPR1L2L3).
The production of the expression clone was achieved by the recombination reaction between the entry clones (pD590 or pD600), plus pENPR1 and destination vectors (BiFC vectors, pBiFCt-2in1-CC, pBiFCt-2in1-CN, pBiFCt-2in1-NC, pBiFCt-2in1-NN). The reaction product was used to transform *E.coli* by heat shock. A colony PCR was performed using the following primers pair; NIMIN 590 P1P4, NIMIN 600 P1P4 and NPR1 P2P3. Also in this case the sequences of genes were confirmed by Sanger sequencing. In the table 3.6 are listed all pBiFCt vectors, as well as the YFP fragments position and the name of expression clone obtained.

Table 3.6: List of the expression clones produced in this study.

Name of Expression Clone	YFP fragments position	pBifCt vectors
pBiFCt-590NPR1-CC	NPR1-YFP *carboxyl-terminus* NIMIN 590-YFP *carboxyl-terminus*	pBiFCt-2in1-CC
pBiFCt-590NPR1-CN	NPR1-YFP *carboxyl-terminus* NIMIN 590-YFP *amino-terminus*	pBiFCt-2in1-CN
pBiFCt-590NPR1-NC	NPR1-YFP *amino-terminus* NIMIN 590-YFP *carboxyl-terminus*	pBiFCt-2in1-NC
pBiFCt-590NPR1-NN	NPR1-YFP *amino-terminus* NIMIN 590-YFP *amino-terminus*	pBiFCt-2in1-NN
pBiFCt-600NPR1-CC	NPR1-YFP *carboxyl-terminus* NIMIN 600-YFP *carboxyl-terminus*	pBiFCt-2in1-CC
pBiFCt-600NPR1-CN	NPR1-YFP *carboxyl-terminus* NIMIN 600-YFP *amino-terminus*	pBiFCt-2in1-CN
pBiFCt-600NPR1-NC	NPR1-YFP *amino-terminus* NIMIN 600-YFP *carboxyl-terminus*	pBiFCt-2in1-NC
pBiFCt-600NPR1-NN	NPR1-YFP *amino-terminus* NIMIN 600-YFP *amino-terminus*	pBiFCt-2in1-NN

3.4.2 Bimolecular Fluorescence Complementation (BiFC) experiments

Agrobacterium tumefaciens (strain) cells were transformed by heat shock using the expression clones listed in the table 3.4 and screened by colony PCR. *Nicotiana benthamiana* plants were used for agro-infiltration.
For the infiltration with *Agrobacterium* a suspension of expression clones (table 3.4) and p19 vector was prepared in AS medium. This vector was used to suppress gene silencing in transformed tobacco leaves. The p19 protein is, in fact, an RNA silencing suppressor *by* Tomato Bushy Stunt Virus (*TBSV)* (Voinnet et al., 2003).
To monitor the effectiveness of the agro-infiltration transformation, as a positive control, two tobacco plants were agro-infiltrated with previously tested vectors that gave good results with the transformation protocol used.
In preliminary experiments, four weeks old plants and five weeks old plants were injected with Agrobacterium suspension in AS medium. Five weeks old plants were chosen for the following experiments since they seem to respond better to the treatments and agro-infiltrations carried out. The figure 3.17 displays the five weeks old plants before and 48 hours after agro-infiltration.

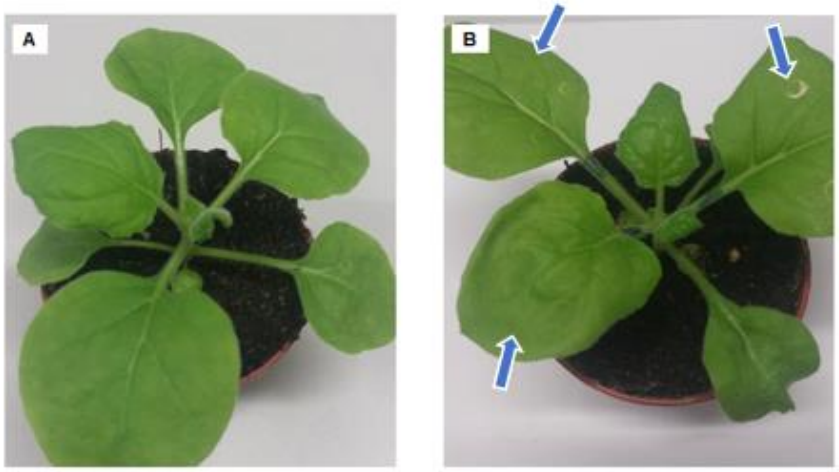

Figure 3.17: Five weeks old *Nicotiana benthamiana* plants. The figure shows an example of Nicotiana benthamiana plant before (A) and 48 hours after Agrobacterium infiltration (B). The infiltration was performed in three different leaves. The infiltrated leaves are indicated in figure by blue arrows.

The infiltration was performed in three different leaves to evaluate if the leaf state of development could affect the expression of genes and fluorescence light intensity. Leaves were cut in small pieces and analysed by a Nikon Eclipse 90i microscope. The interaction of proteins gives positive results only for the vectors pBiFCt-590NPR1-NN (nYFP-NPR1 amino-terminal fusion; cYFP-NIMIN 590 amino-terminal fusion), pBiFCt-600NPR1-NN (nYFP-NPR1 amino-terminal fusion; cYFP-NIMIN 600 amino-terminal fusion). The figure 3.18 shows a schematic, out of scale, vector maps depicting the pBiFCt-590NPR1-NN and pBiFCt-600NPR1-NN.

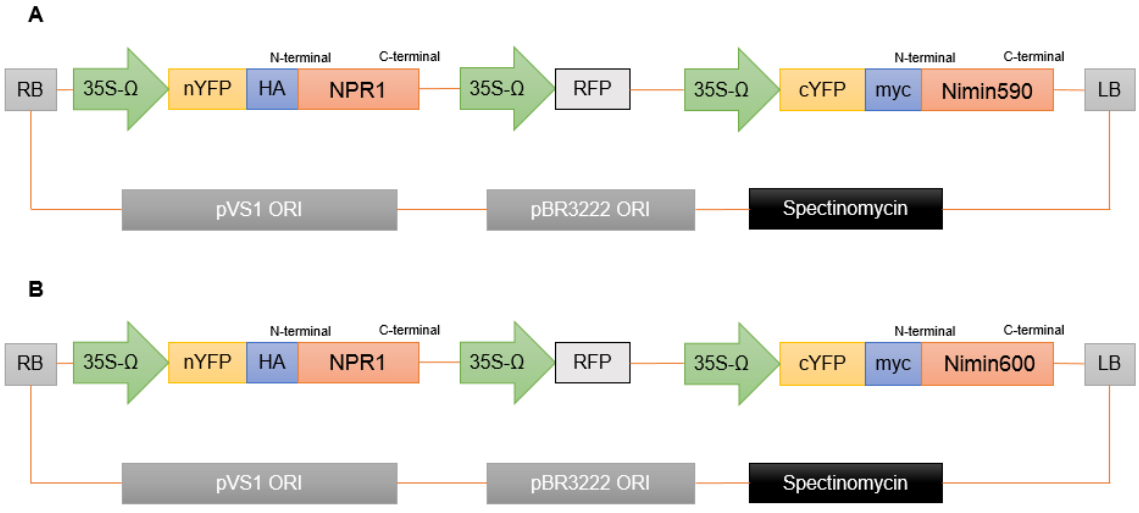

Figure 3.18: Schematic vector maps depicting the pBiFCt-590NPR1-NN (A) and pBiFCt-600NPR1-NN (B) (not to scale).
The map of vectors reports important sites like the two recombination sites in which NIMIN genes and NPR1 gene were inserted, the origin of replication (pBR322 ori), the 35s RNA CaMV promoter (35S), the pVS1 ori, the origin of replication in Agrobacterium tumefaciens. The maps also report the genes for selection (spectinomycin resistance) and the sites of fluorescence proteins such as *red fluorescent protein* (RFP) and the N- or C-terminal EYFP halves [nYFP (1–155 amino acids), cYFP (156–239 amino acids)]. The vectors contain also the sequence to produce Myc and HA-tagged peptides.

To see if the age of leaves could affect the interaction strength, the experiment was repeated transforming the youngest and older leaves of five weeks old plants. The figures 3.19 and 3.20 show epi-fluorescence microscope images of the interaction of NIMIN proteins and NPR1 by using pBiFCt-590NPR1-NN and pBiFCt-600NPR1-NN in younger (3.19) and older leaves (3.20). Due to bigger cell size and lower protein expression levels (enable to avoid artefacts caused by too high expression), the older leaves were used in further experiments.

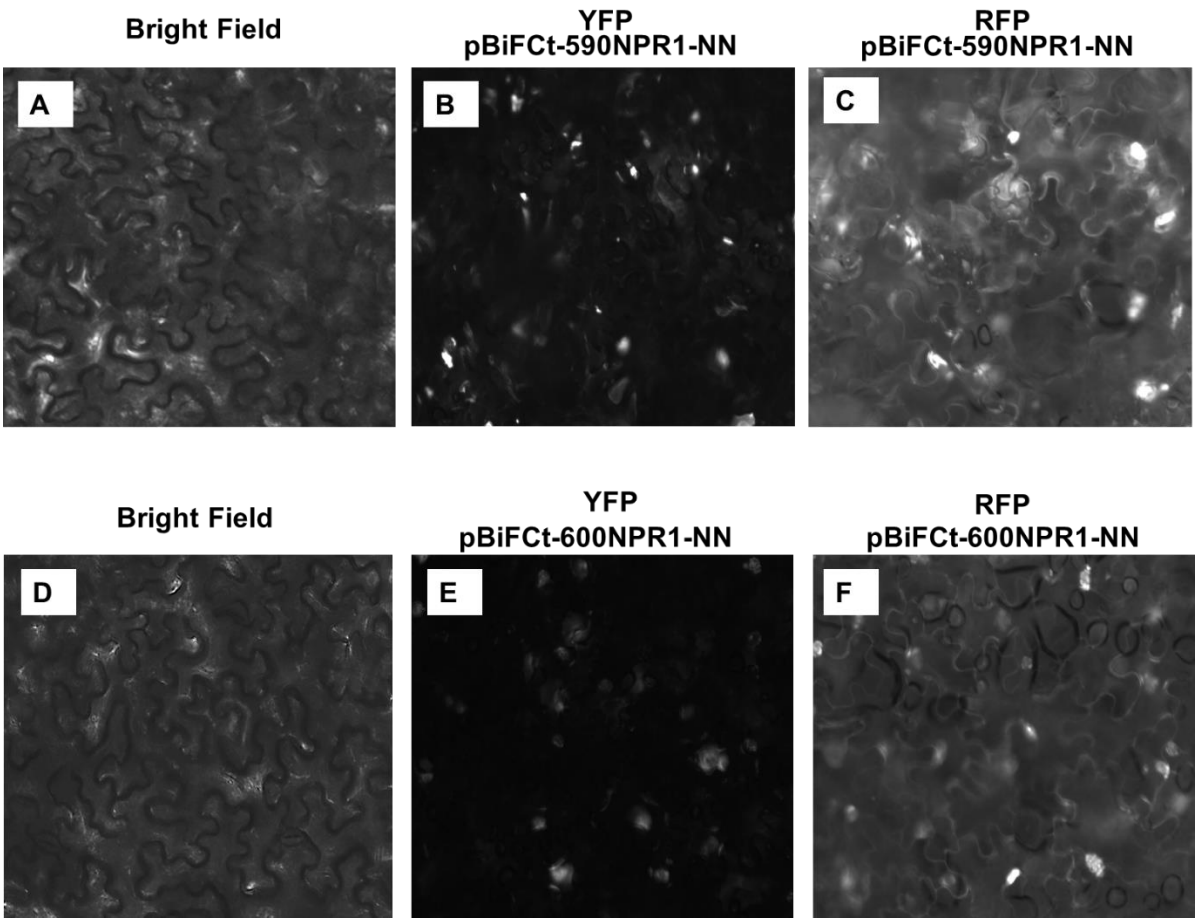

Figure 3.19: Epi-fluorescence microscope images (X20) in younger leaves. The figures displays the interactions between NIMIN proteins and NPR1.
Younger tobacco leaves were infiltrated with *Agrobacterium tumefaciens* transformed by the vector pBiFCt-590NPR1-NN (A-C) and by pBiFCt-600NPR1-NN (D-F). The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (B,E). The reconstitution of YFP from its' fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (C,F) was checked.

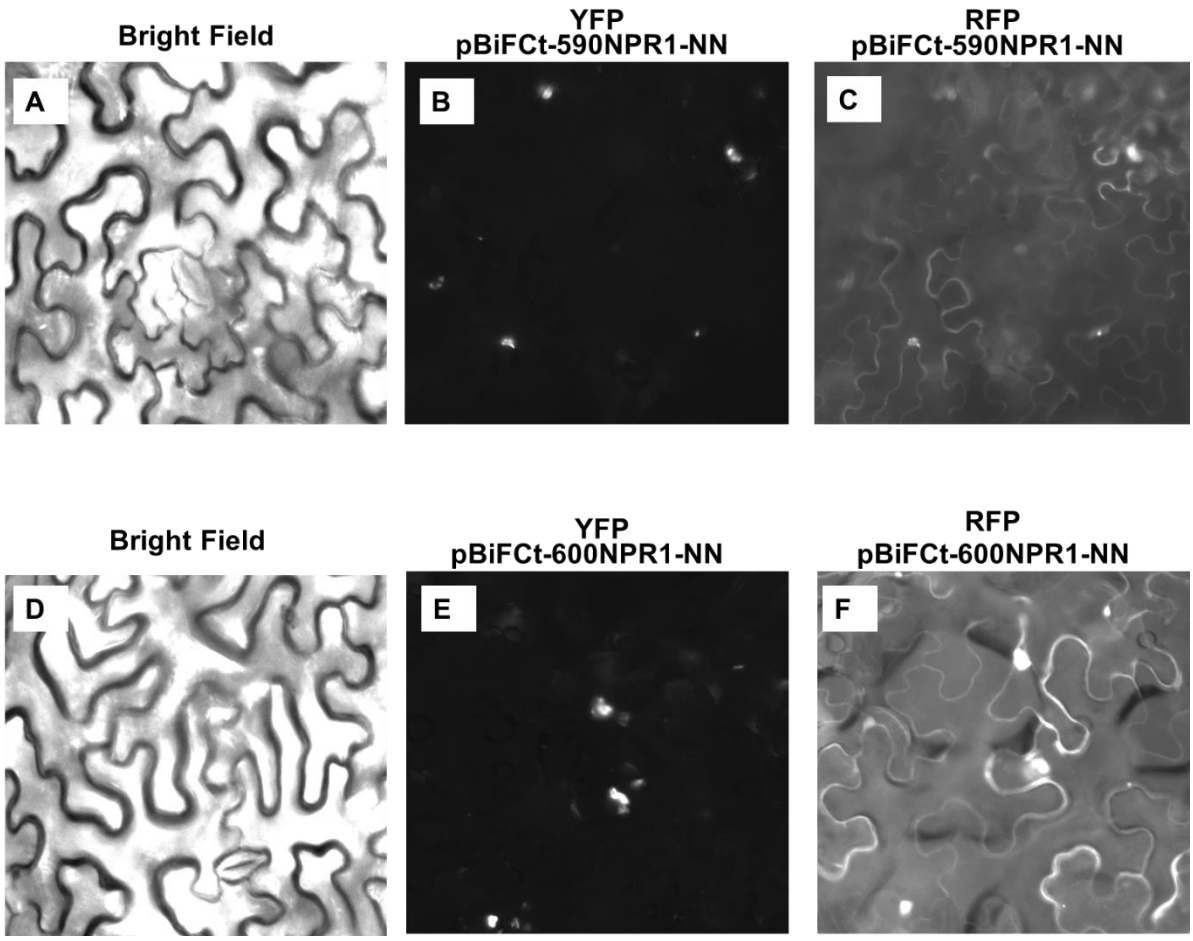

Figure 3.20: Epi-fluorescence microscope images (X20) in older leaves. The figures displays the interactions between NIMIN proteins and NPR1.
Older tobacco leaves were infiltrated with *Agrobacterium* tumefaciens transformed with the vector pBiFCt-590NPR1-NN (A-C) and with pBiFCt-600NPR1-NN (D-F). The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (B,E). The reconstitution of YFP from its' fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interaction among the proteins of interest. To control the infiltration performed the signal of *red fluorescent protein* (RFP) (C,F) was checked.

To verify the correct size of recombinant proteins in the cells following the agro-infiltration, a western blot analysis was performed on protein extracts of transformed plants with pBiFCt-590NPR1-NN and pBiFCt-600NPR1-NN vectors. The figure 3.21 shows the results of the analysis.

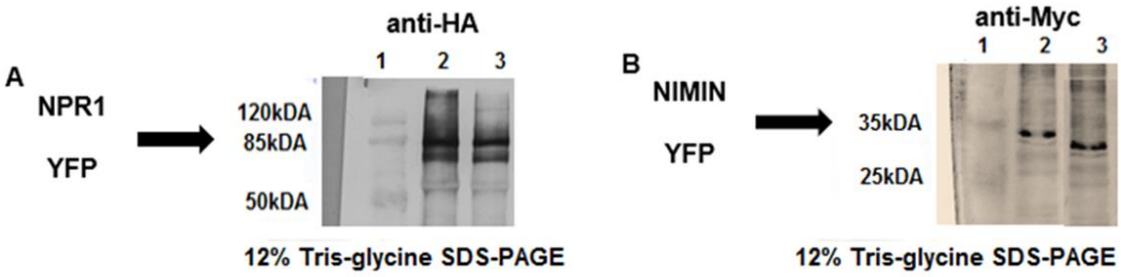

Figure 3.21: Western Blot analysis of two NIMIN proteins (B) and NPR1 protein (A). Samples were resolved on a 12% polyacrylamide gel, transferred to a membrane and detected with anti-Myc (NIMIN proteins) and anti-HA (NPR1 protein) antibody. Lane 1: molecular weight marker, lane 2: protein extract from the leaves transformed with pBiFCt-590NPR1-NN, lane 3: protein extract from the leaves transformed with pBiFCt-600NPR1-NN vector.

The recombinant proteins had the attended size ~ 85kDA for nYFP-NPR1, ~ 33kDA for cYFP-NIMIN590 and ~ 30kDA for cYFP-NIMIN590.
To verify if the interaction can be influenced by the time in which the leaves were injured by cutting into the small pieces for microscopy, an analysis at different time points from the first damage of plant was carried out by cutting off three small pieces from leaves. Leaves pieces were positioned on a glass slide and analysed at three different times t0 (immediately after the cut), t1 (30 min after the cut) and t2 (one hour after cut). The figures 3.22 and 3.23 show the results of analysis for the construct pBiFCt-590NPR1-NN and pBiFCt-600NPR1-NN at two different time points (t0 and t2).

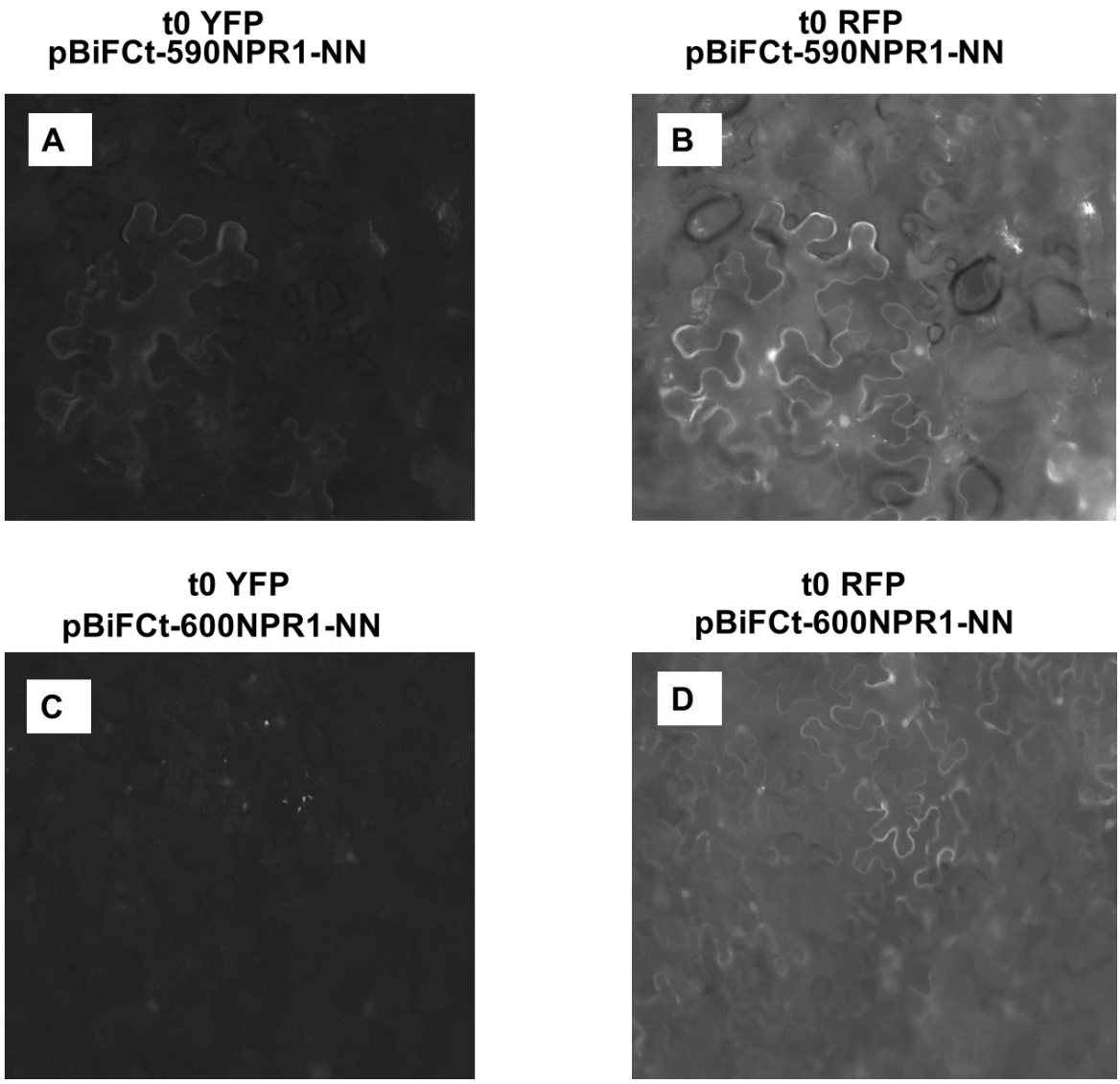

Figure 3.22: Epi-fluorescence microscope images (X20) in older leaves. The figure display the interaction between NIMIN proteins and NPR1 at time point t0 (immediately after the cut).
Older tobacco epidermal leaf cells were infiltrated with *Agrobacterium tumefaciens* transformed with the vector pBiFCt-590NPR1-NN (NPR1-YFP *amino-terminus* NIMIN 590-YFP *amino-terminus)* (A-B) and with pBiFCt-600NPR1-NN (NPR1-YFP *amino-terminus* NIMIN 600-YFP *amino-terminus)* (C-D). The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (A,C). The fusion of YFP fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (B,D) always present in the vector used was checked.

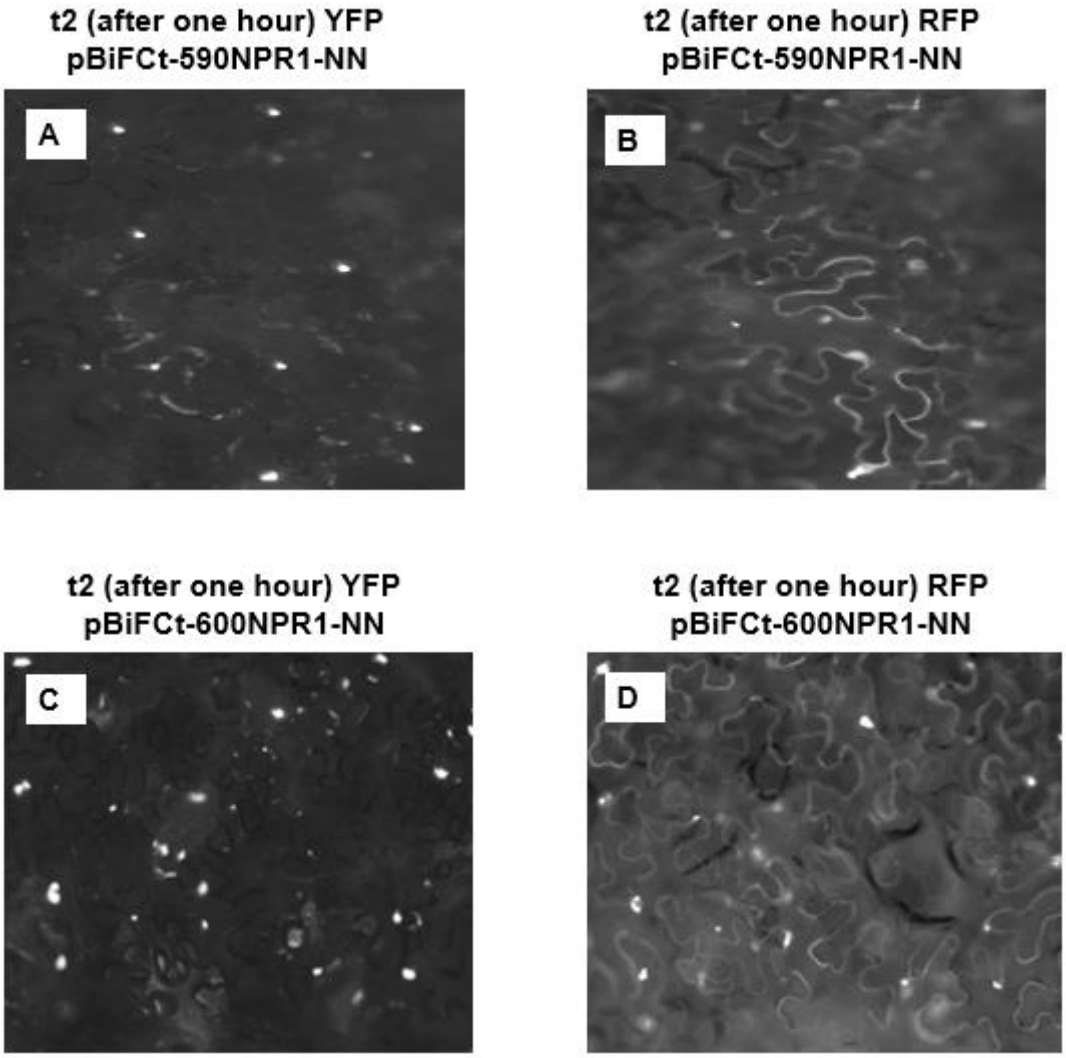

Figure 3.23: Epi-fluorescence microscope images (X20) in older leaves. The figure display the interaction between NIMIN proteins and NPR1 at time point t2 (one hour after the cut).
Older tobacco epidermal leaf cells were infiltrated with *Agrobacterium tumefaciens* transformed with the vector pBiFCt-590NPR1-NN (NPR1-YFP *amino-terminus* NIMIN 590-YFP *amino-terminus) (A-B) and with* pBiFCt-600NPR1-NN (NPR1-YFP *amino-terminus* NIMIN 600-YFP *amino-terminus) (C-D).* The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (A,C). The fusion of YFP fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (B,D) always present in the vector used was checked.

Through the experiments described, it was not possible to determine the site of the interaction between different proteins NIMIN and NPR1.
To achieve this goal leaves epidermis obtained by peeling of infiltrated leaf were analysed (figure 3.24). This data were collected one day after the first wound of the leaf.

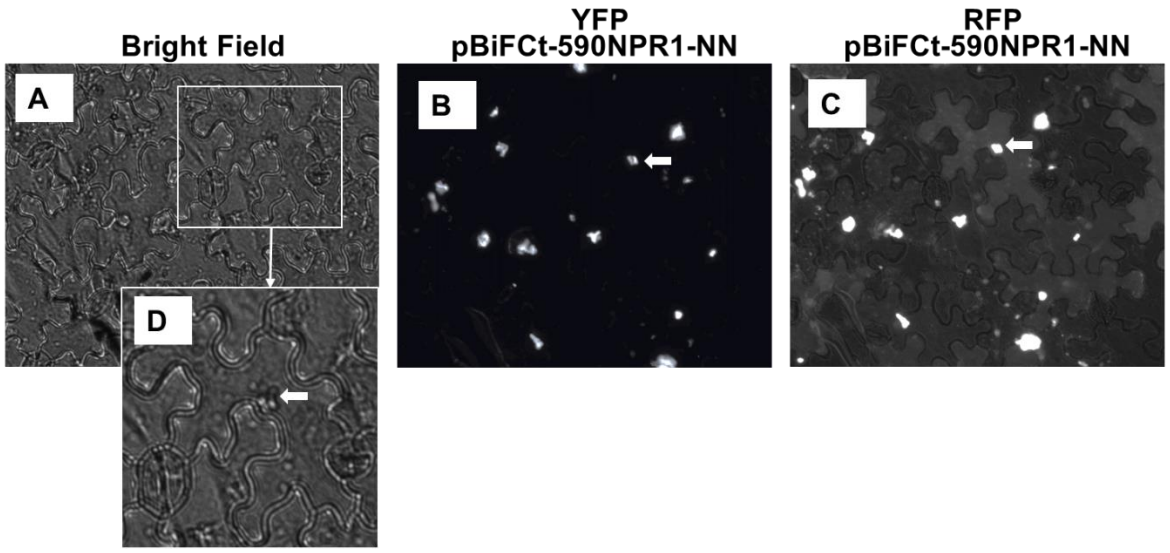

Figure 3.24: Epi-fluorescence microscope images (X20) in older leaves after peeling. The figure display the interaction between NIMIN 590 protein and NPR1.
Older tobacco leaves obtained by peeling were infiltrated with *Agrobacterium tumefaciens* transformed with the vector pBiFCt-590NPR1-NN. The figure displays also a magnification of a single cell, the nucleus is indicated by a white arrow.The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (B). The reconstitution of YFP from its fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (C) was checked.

The interaction was also confirmed by Leica SP2 confocal microscopy at different time points after wounding. The figure 3.25 shows a confocal image acquired about fifteen minutes after the first wound of the leaf agro-infiltrated with the plasmid pBiFCt-590NPR1-NN.

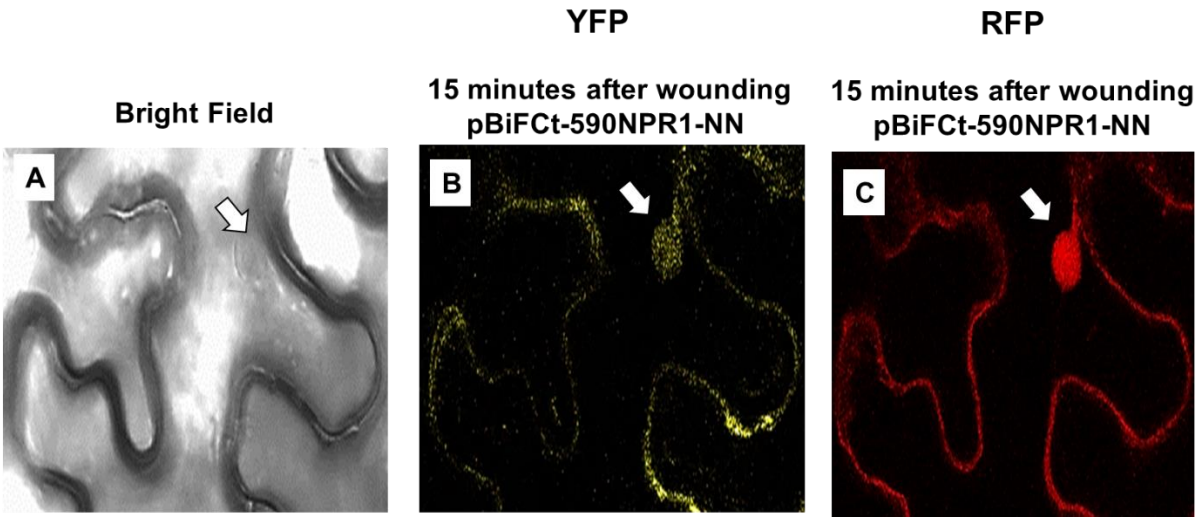

Figure 3.25: Confocal microscope images of older leaves. The figure display the interaction between NIMIN 590 protein and NPR1 15 minutes after wounding.
Older tobacco epidermal leaf (C) were infiltrated with *Agrobacterium tumefaciens* transformed with the vector pBiFCt-590NPR1-NN (NPR1-YFP *amino-terminus* NIMIN 590-YFP *amino-terminus).* The figure displays *a* magnification of a single cell, the nucleus is indicated by a white arrow. The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (A). The fusion of YFP fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (B) always present in the vector used was checked.

The same analysis were conducted for the NIMIN 600 gene using pBiFCt-600NPR1-NN (NPR1-YFP amino-terminus NIMIN 600-YFP amino-terminus*)* construct, a comparable result was obtained. As time goes on the signal of interaction between NIMIN590/600-NPR1 proteins become more intense in the nucleus. The figure 3.26 shows Leica SP2 confocal microscope after 30 minutes from the wounding of the leaf agro-infiltrate with the plasmid pBiFCt-590NPR1-NN.

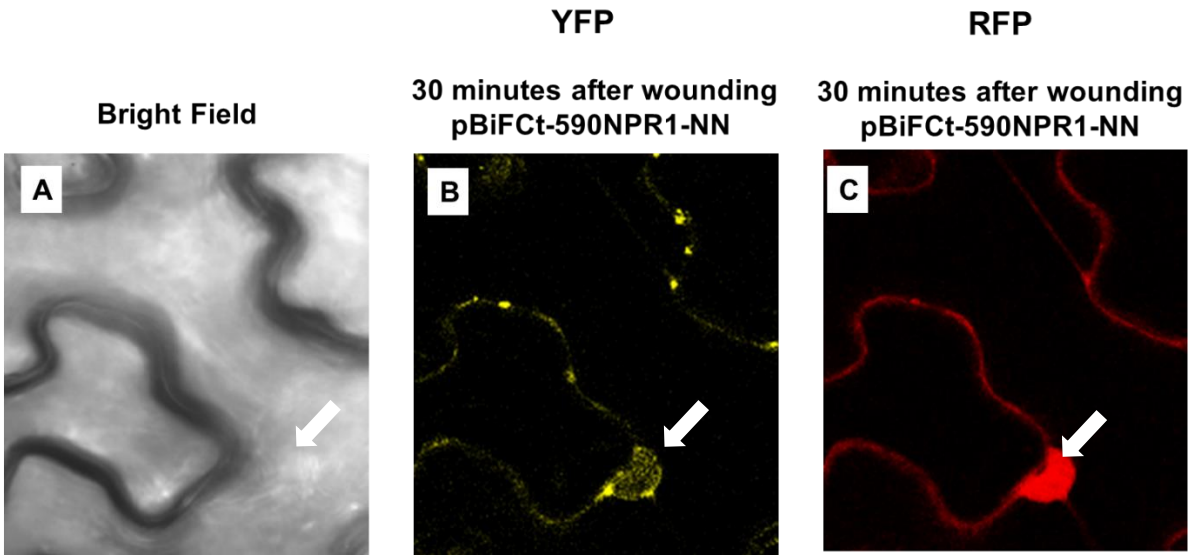

Figure 3.26: Confocal microscope images of older leaves. The figure display the interaction between NIMIN 590 protein and NPR1 30 minutes after wounding.
Older tobacco leaves were infiltrated with *Agrobacterium tumefaciens* transformed with the vector pBiFCt-590NPR1-NN (NPR1-YFP *amino-terminus* NIMIN 590-YFP *amino-terminus).* The figure displays a magnification of a single cell, the nucleus is indicated by a white arrow. The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (A). The reconstitution of YFP from its fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (B) always present in the vector used was checked.

Finally, in the figure 3.27 the results for NIMIN590/600-NPR1 proteins interactions after one hour after wounding are shown.

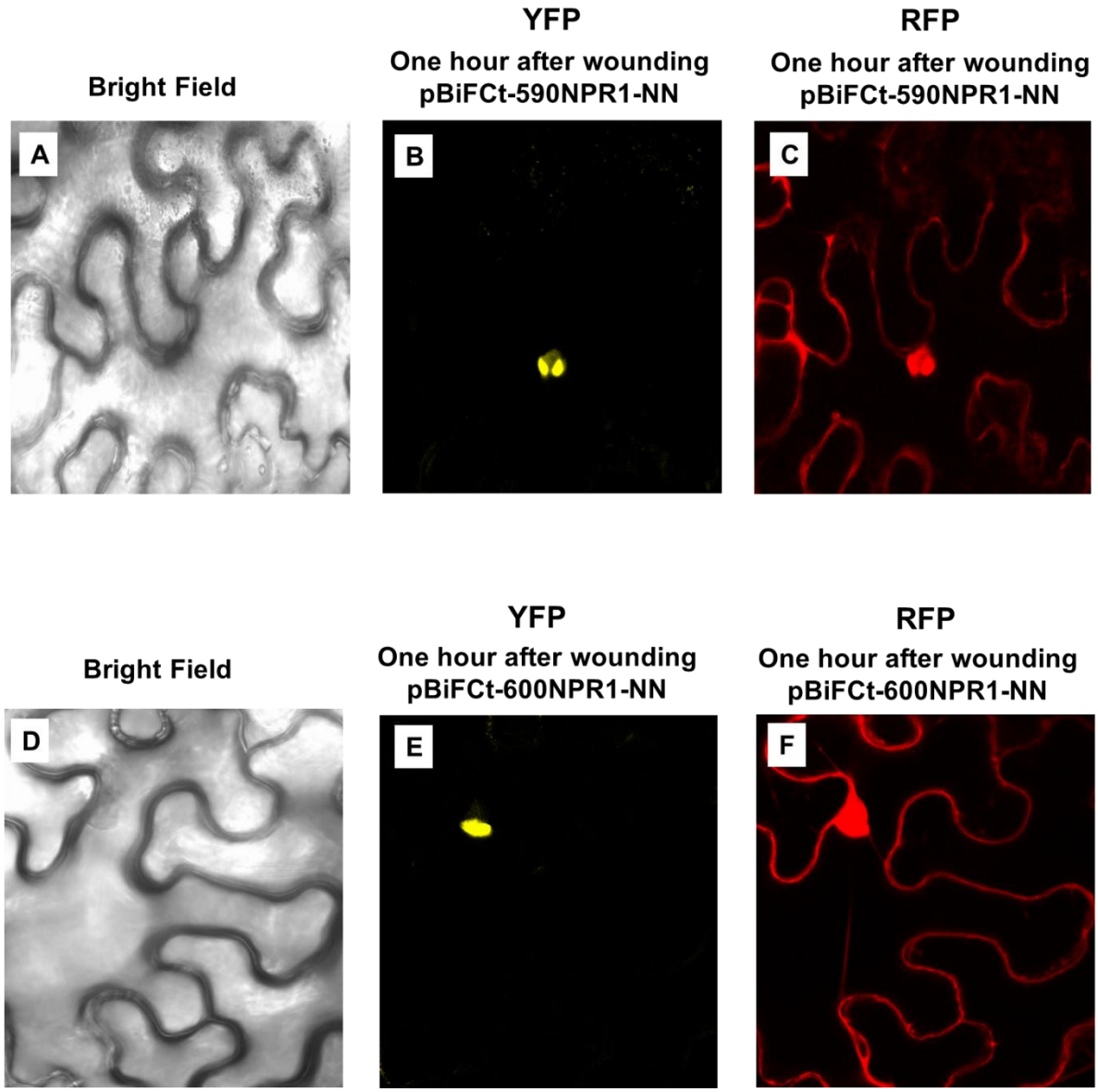

Figure 3.27: Confocal microscope images of older leaves. The figure display the interaction between NIMIN590/600 protein and NPR1 one hour after wound.
Tobacco leaves were infiltrated with *Agrobacterium tumefaciens* transformed with the vector pBiFCt-590NPR1-NN *(A-C)* and with pBiFCt-600NPR1-NN *(D-F).* The interaction among proteins give a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (B,E). The reconstitution of YFP from its fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (C,F) was checked.

Therefore, to summarize the confocal microscopy results, NIMIN 2C and NPR1 proteins seem to interact in the nucleus. The intensity of fluorescence signals increase with time after wounding (one hour). This is consistent to bioinformatics analysis highlighted. *NIMIN 2C* genes also appear to respond positively to stress such as the wounding, as in experiments conducted. The data obtained validate an interaction between tomato NIMIN proteins and NPR1, whose role in the activation of genes involved in plant defence mechanisms is known and widely studied in the literature.

Finally the effect of salicylic acid on NIMIN590/600-NPR1 interaction was monitored. To achieve this goal five weeks old tobacco plants were agro-infiltrated, and after 24 hours the leaves were treated with 1 mM SA. The salicylic acid, dissolved in ethanol at 2M concentration and diluted to 1mM concentration in 0.1% Triton X-100 solution, was applied to the leaf by using a brush. As negative control were used leaves brushed with 0.1% Triton X-100 solution. After 48hours from the agro-infiltration, treated leaves were analysed through an epi-fluorescence microscope (figure 3.28). The interaction was visualized at t0 time point.

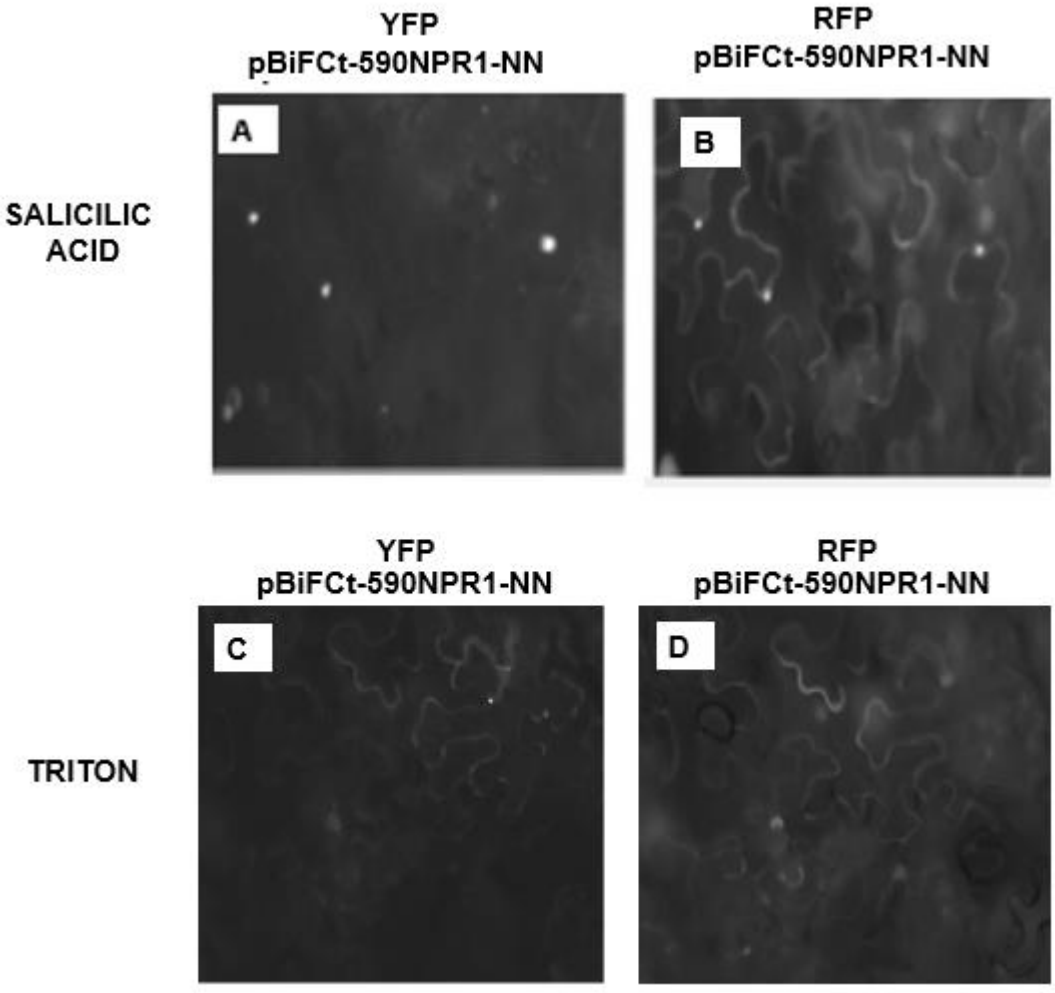

Figure 3.28: Epi-fluorescence microscope images (X20) in older leaves. The figure display the interaction between NIMIN 590 protein and NPR1 in freshly cut leaves after the application of salicylic acid. Older tobacco leaves were infiltrated with *Agrobacterium tumefaciens* transformed with the vector pBiFCt-590NPR1-NN. The interaction among proteins gives a yellow fluorescence signal due to the fusion of two YFP non-fluorescent fragments (A,C). The reconstitution of YFP from its fragments (YFP^N, N-terminal fragment [amino acids 1–155]; YFP^C, C-terminal fragment [amino acids 156–239]) is mediated by the interactions among the proteins of interest. To control the infiltration performed the signal of red fluorescent protein (RFP) (B,D) was checked.

Preliminary results have shown that the application of salicylic acid is able to activate more rapidly the interaction between NIMIN 2C and NPR1 proteins; good levels of interaction also occur at time zero after wounding.

3.5 Preparation of recombinant vector pHellsgateNiminAll (pHNA)

The bioinformatics analysis, the gene expression profile and the BIFC results, indicated that both tomato NIMIN genes are expressed in different tissues and that their proteins interact with NPR1. In order to study the role of *NIMIN 2C* genes in tomato-aphid interaction a vector of dsRNA mediated gene silencing of the two genes was prepared. The sequence of NIMIN 2C (Solyc03g119590.1.1) was inserted into the pGemT Easy Vector using the T/A cloning strategy (not shown). After Sanger sequencing, the coding sequence of NIMIN 590 was amplified by primers NIMINAll fw(AttB1) and NIMINAll rv (AttB2) for Gateway cloning into pHellsgate 12. The figure 3.29 shows an alignment of *Solanum licopersicum NIMIN 2C* genes sequences.

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)

Solyc03g119590.1.1      ATGCTACTTATGGACGGAGAAAAGAAGAGGAAGAGAACATCAATCGTCGCCGGAGATCGG
Solyc03g119600.1.1      ---------ATGGACCGGCACAAGAAGCGGAAGAGAA---CTGACAACGGCGGTGATCGA
                                ****** *  * ****** *********   *   *  ** *** *****

Solyc03g119590.1.1      AGTAACGATGACGTAAAACCTACTGTGAAGGAGGA------------GGAGCCGCCG---
Solyc03g119600.1.1      AGCAGACATGAGAGAGAAAGTTCTGTGAAGGCGAACACCGTAGTATCGAAGCCGCCGCCG
                        ** *   ****   * **  * ********* * *           * ********

Solyc03g119590.1.1      ---TCAGAGGCGGAGGTTAACGAGTTCTTCGCAATTTTACGGAGGATGCACATGGCCGTA
Solyc03g119600.1.1      CCATCGGAGGCGGAAGTTAACGAATTCTTCGCGATCTTACGGAGGATGAACGTGGCAGTG
                           ** ******** ******** ******** ** ************ ** **** **

Solyc03g119590.1.1      AAATATCTTCAGAGAAACGCTCAGATTCAGCCGGAAAACGTTAACGCTCACGGCAGCAAG
Solyc03g119600.1.1      AAATATCTTCAGAAAAATGCTCAGAT---------------------------------
                        ************** *** ********

Solyc03g119590.1.1      TTAACCGCATCGCCGGCCGGTGTTAACGGAGATGCAACTGGACAGAAGAGAGAACGGGGA
Solyc03g119600.1.1      ----------------CGGTG------------AAGTAGA------------GGATA
                                       *****            ** * **            **  *

Solyc03g119590.1.1      ATCGTGAGAAAAGGTGATTTGGACCTCAACACTTTGCCGGACTGCGGAGACTAA
Solyc03g119600.1.1      GCCAT-AAAAGAGTTGATTTGGATCTGAATACACTACCGGAAGGCGGAGATTAA
                          * * * ** ** ********** ** ** **  * *****  ******* ***
```

Figure 3.29: *Solanum licopersicum NIMIN 2C* genes sequences alignment. Underlaned the sequence chosen to design appropriate primers sequences. The choice of these sequences made in order to allow the silencing of both genes of the NIMIN gene family in *Solanum licopersicum*. Asterisk (*) indicates positions which have a conserved residue.

The amplification product was purified with the PCR Purification Kit (Invitrogen) and subjected to quantification on 1% agarose gel (figure 3.30).

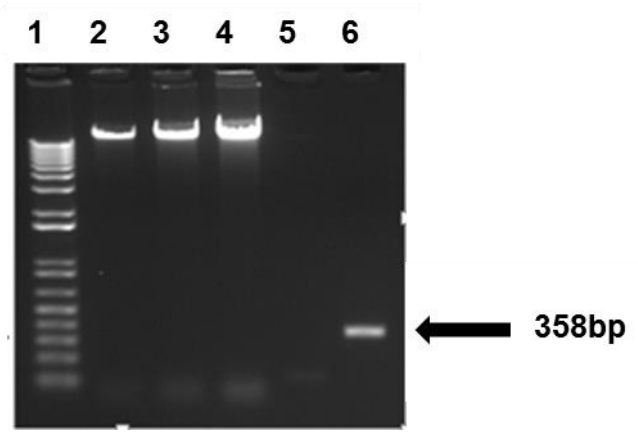

Figure 3.30: DNA fragment analysis on agarose gel electrophoresis after attB primers amplification of *NIMIN 590*-pGemT Easy vector. Lane 1: 1 kb plus molecular weight ladder, lane 2: 50ngλ phage DNA, lane 3: 100ng λ phage DNA, lane 4: 200ng λ phage DNA, lane 5: negative control, lane 6: amplification of the plasmid pGEM-T containing the gene *NIMIN 590* (358bp).

The purified amplification product was used for the recombination with the donor vector (pDonor/Zeo, Invitrogen). To check the insertion of the PCR product into pDonor vector was performed an amplification reaction (figure 3.31). The PCR product has an expected size of 588bp. Sanger sequencing was used to control the absence of variations in the cloned sequence.

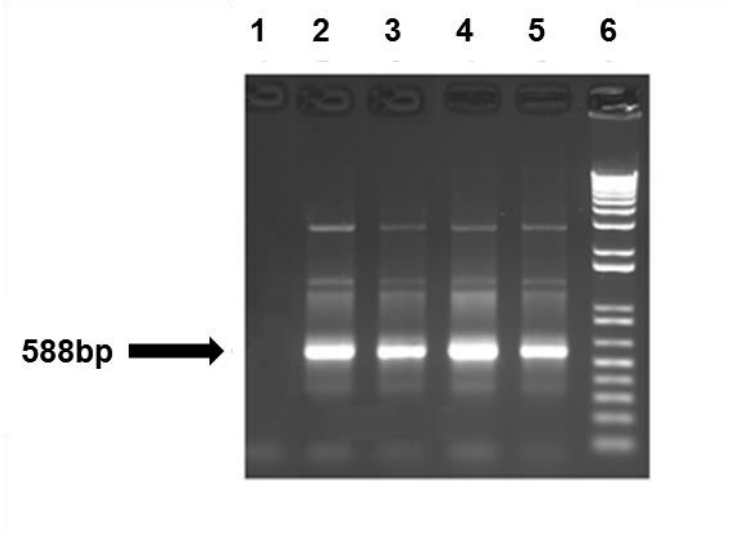

Figure 3.31: DNA fragment analysis on agarose gel electrophoresis of putatively recombinant plasmids using M13 primers (table 2.1). Lane 1: negative control, lanes 2-5: putatively recombinant plasmid DNA, PCR product has expected size of 588bp, lane 6: 1 kb plus molecular weight ladder.

The following step was the recombination reaction between the entry clone (pDNA) and the destination vector (pHellsgate12). The reaction product was used to transform *E.coli* by heat shock.

To verify the insertion of the fragment into the vector in both recombination sites, an amplification reactions was performed using the primer AGRI 51-56 and AGRI 64-69. The figure 3.32 shows an example of a run on agarose gel of such amplification products.

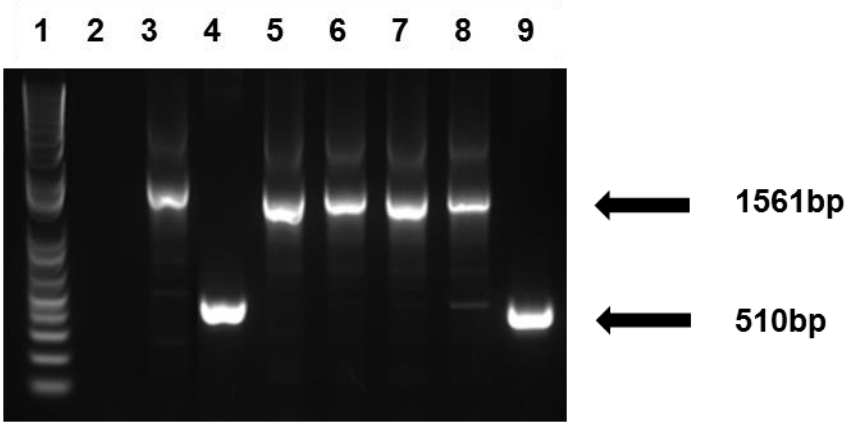

Figure 3.32: DNA fragment analysis on agarose gel electrophoresis of putatively recombinant plasmids using AGRI 51-56 primers (table 2.1). Lane 1: kb plus molecular weight ladder, lane 2: negative control, lanes 3-9: plasmid DNA amplification, PCR product has the size of 510bp for recombinant plasmid (putatively recombinant, lane 4 and lane 9) and 1561bp for the empty vector.

The samples were, also analysed by PCR using the primer pair AGRI 64-69 (not shown). Figure 3.33 shows schematically the expression cassette of the binary vector pHNA and the location of the primers used to check the correct insertion of the sequences of interest.

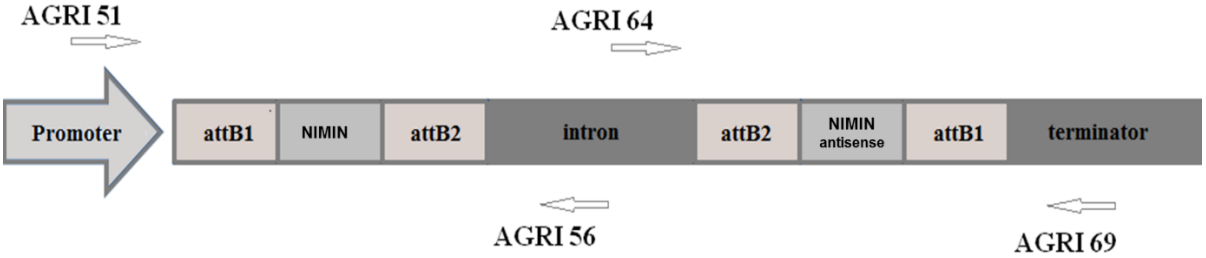

Figure 3.33: pHellsgate12 vector after recombination reaction whit primers AGRI regions. AGRI primers were designed close to the two-recombination regions to allow the verification insertion of the fragment of interest in the two sites.

To check the correct insertion in both directions of the fragment, a new amplification reaction (for the samples tested positive in PCR with primers AGRI 51-56 and AGRI 64-69) was set up using primers that were specific for both fragments inserted: AGRI attB2 51-and 64 AGRI-attB1, shown in table 2.1 (figure 3.34).

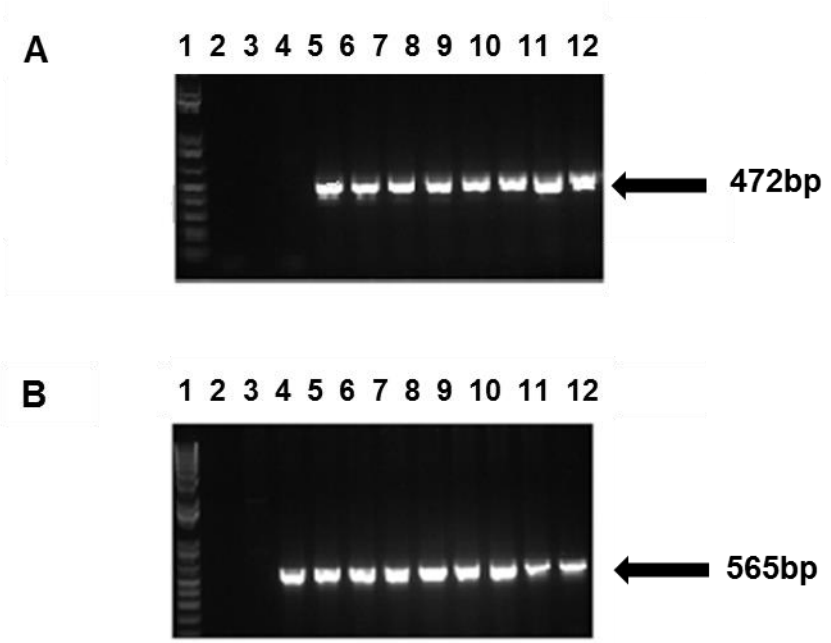

Figure 3.34: DNA fragment analysis on agarose gel electrophoresis of putatively recombinant plasmids using AGRI 51-attB2 (A) or AGRI 64-attB1 primers (B).
Lane 1: kb plus molecular weight ladder, lane 2: negative control, lane 3: not recombinant pHellsgate12 vector, lanes 4-12: plasmid DNA amplification, PCR product has the size of 632bp for recombinant plasmid.

The recombinant plasmids (pHNA) were purified and the inserts Sanger-sequenced. Plasmids that contained the two fragments of the gene with the right sequence and orientations were used for the stable transformation of tomato plants (genotype Red Setter) in collaboration with Dr Pasquale Chiaiese (University of Federico II, Naples).

3.6 Tomato genetic transformation

Agrobacterium tumefaciens cells were transformed by heat shock using the pHNA vector. Cells were screened by PCR colony.
A total of 800 explants from 600 cotyledons were co-cultivated with the *Agrobacterium* and selected on medium with 50mg l^{-1} kanamycin. The cotyledon explant were cut before the development of true leaves Generally, the mortality of cotyledons determined four weeks after inoculation and after two cycles of antibiotic selection was about 16%, even if the majority of them not already survived the first antibiotic selection.
From the explants, approximately 500 green *calli* were obtained. After culture in selection and shoot elongation medium for six weeks, 53 regenerated shoots were selected.

Figure 3.35 illustrates different stages in the plant transformation and regeneration process of cotyledon explants.

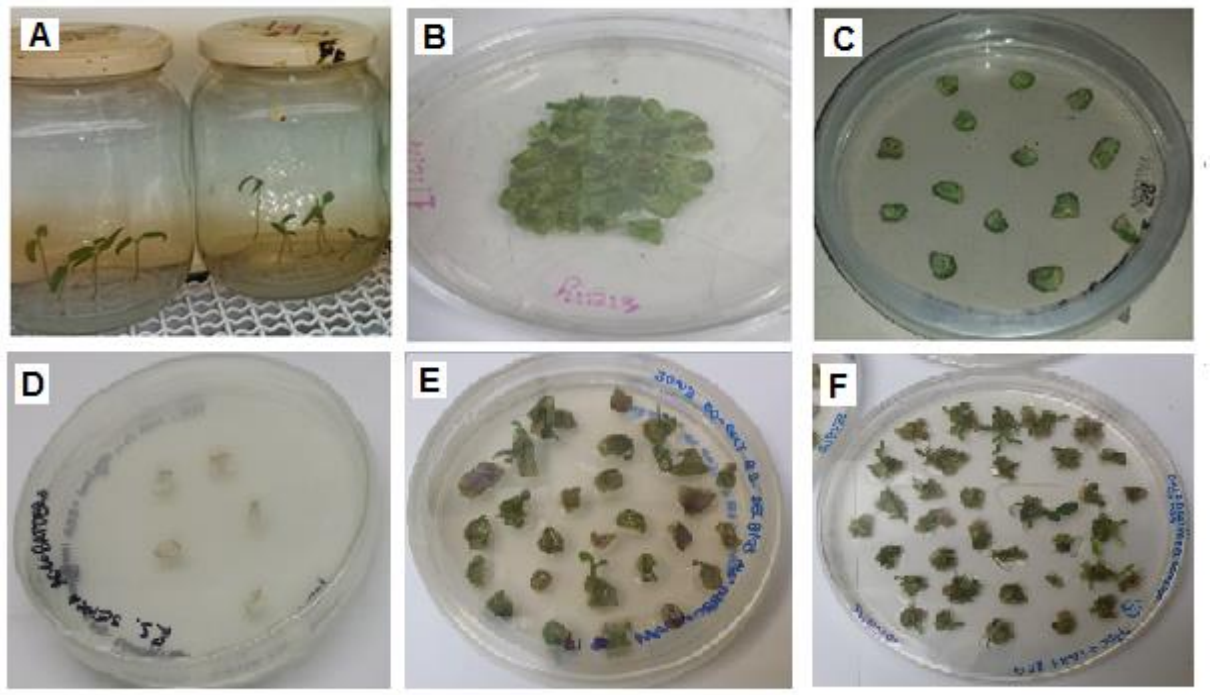

Figure 3.35: Different stages in the tomato transformation and regeneration process of cotyledon explants. A: Red Setter plants from witch were cut cotyledons explants. B: *Agrobacterium* explants co-cultivation on MS30 plate. C: Co-coultivated explants at beginning of differentiation process on MS30 medium containing 1 mg l^{-1} zeatin, 0.4mg l^{-1} of IAA, 100mg l^{-1} of claforan and 50 mg l^{-1} of kanamycin. D: Not co-cultivated explants after four weeks on MS30 medium containing 1mg l zeatin, 0.4 mg l^{-1} of IAA, 100mg l^{-1} of claforan and 50 mg l^{-1} of kanamycin. E: Co-cultivated explants and callus after four weeks on MS30 medium containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} of IAA, 100 mg l^{-1} of claforan and 50 mg l^{-1} of kanamycin. F: Control explants after four weeks on MS30 medium containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} of IAA, 100 mg l^{-1} of claforan.

Once elongated, shoots were separated by callus and placed in a rooting substrate containing the antibiotic kanamycin. After two weeks, the shoots were transferred in a rooting substrate without selective agent. Figure 3.36 represents the state of the growth of regenerated shoots from about three months since the cut of cotyledon explants.

Figure 3.36: Regenerated shoots from about three month to the cut of cotyledon explants.

After rooting and propagation, the DNA of putative transformed plants was isolated and analysed by PCR reaction with specific primers. To perform the PCR reactions the primers pair AGRI 51-56 (yielding a 510 bp fragment) and AGRI 64-69 (545 bp) were employed. To date two transgenic lanes have been obtained. Three plants for each of the two transgenic lanes were analysed by PCR to verify the efficiency of the plant's transformation (figure 3.37).

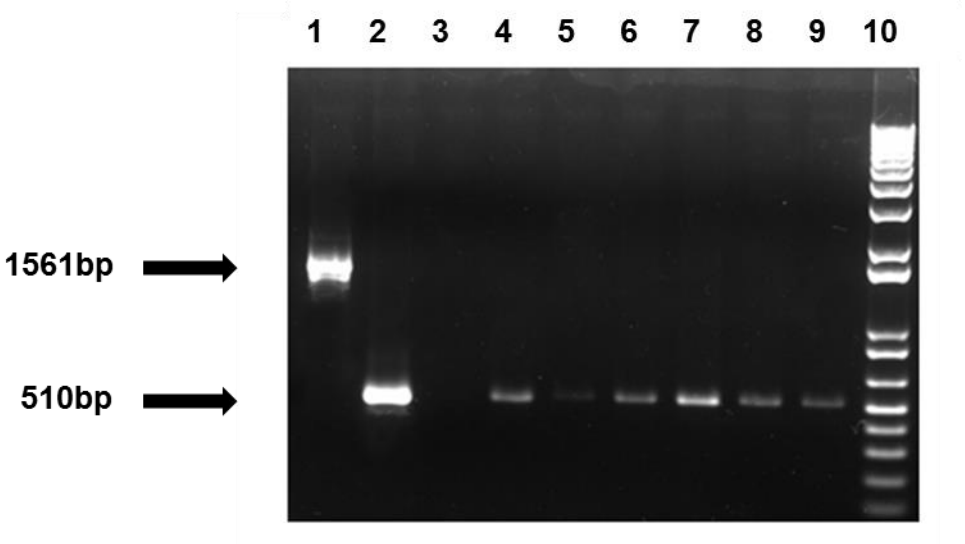

Figure 3.37: DNA fragment analysis on agarose gel electrophoresis using primers AGRI 51-56 (table 2.1). Lane 1: pHellsgate12 vector, lane 2: pHNA vector, lane 3: negative control, lane 4-6: transformed plants TGA RA, lanes 7-9: transformed plants TGA RB, lane 10: kb plus molecular weight ladder. The fragment in recombinant plants have to be 510bp like a control, whereas 1561bp for not recombinant vector.

PCR- positive plants were transferred *in vivo*.

3.7 Characterization of transformed tomato plants

Both the growth and molecular analysis were followed using, as control of transformation, the shoots regenerated by the explants not treated with *Agrobacterium*. The growth pattern of plants was monitored from following days the shift in vivo, to seed production (figure 3.38). There were not obvious differences between the growth of the control plants and those transformed in terms of plant size and the production of flowers and seeds.

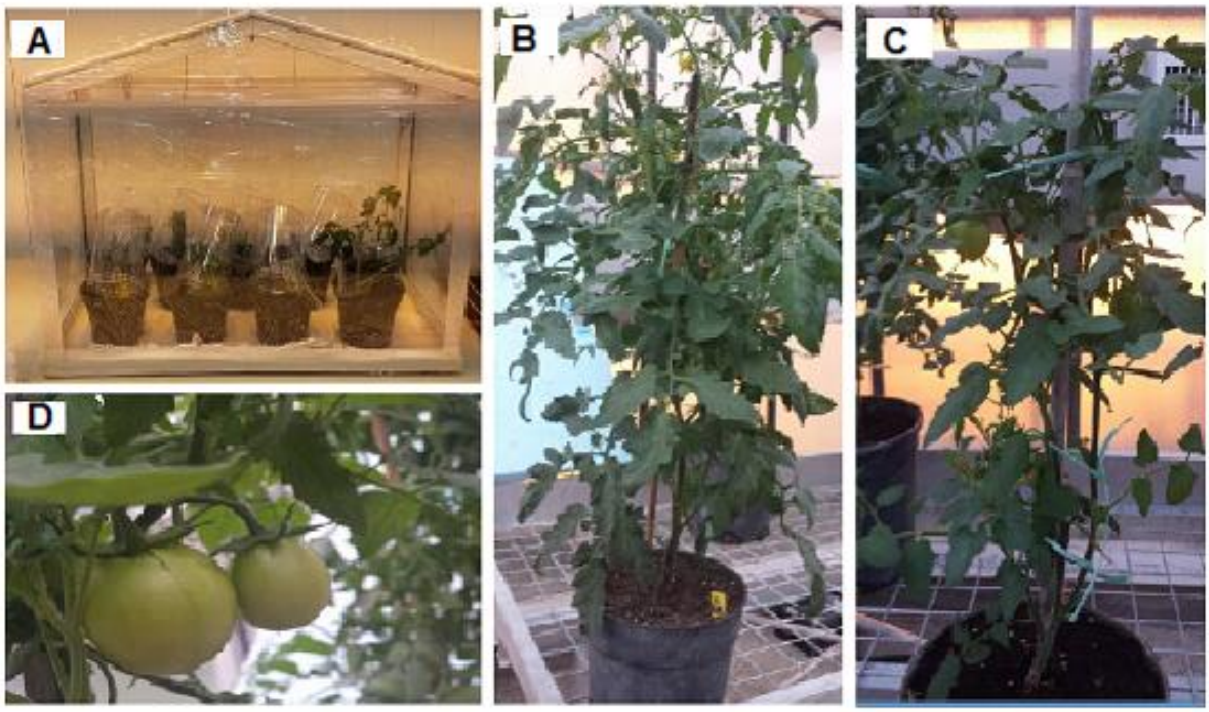

Figure 3.38: *In vivo* transformed tomato plants and control. A: Plants transferred from “in vitro” experiments. B: Red Setter plant from in vitro tissue culture. C: Transgenic plants lane TGA-RB. D: Tomato fruits of transgenic plants.

Leaves form three weeks old plants were taken to perform the extraction in order to control the expression values of the *NIMIN 2C* genes compared to the plant control. The control plant derives from the differentiation of no co-cultivated explants.
On leaf material a RNA extraction was performed to carry out an expression gene analysis to verify the silencing of genes *NIMIN 2C* genes.
The figure 3.39 show the result obtained for expression analysis of NIMIN 600 gene in transgenic plants. The statistical analysis performed on real-time results obtained was carried out by using the Duncan test to check for significant differences between means ($p < 0.05$).

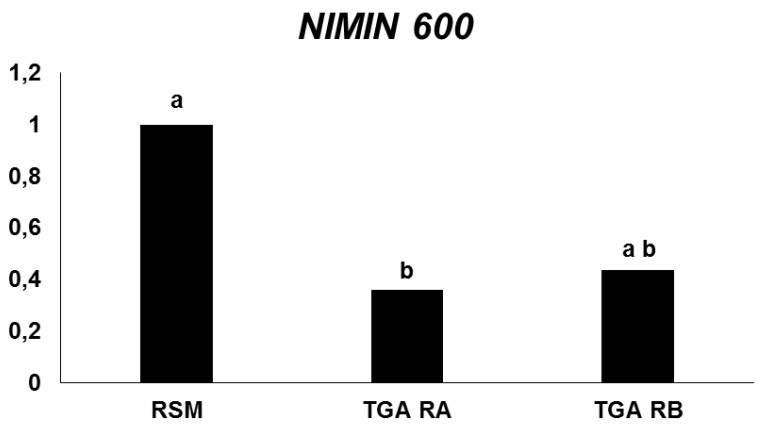

Figure 3.39: Expression analysis of *NIMIN 600* genes in transgenic plants. The analysis was carried out using a comparative Ct method, the endogenous control was *EF1-α* gene. The Red Setter plant (RSM) not transformed was used as a calibrator. The analysis was performed for two transgenic lanes. The statistical analysis performed was the Duncan test ($p < 0.05$) letters a-b indicate significant differences between data.

To test the silencing of NIMIM genes I used primers specifically to NIMIN 600 table 2.1, primers NIMIN600 Rt forward and reverse) because these primers did not amplified the cloned sequences and their reverse products.

Figure 3.40 shows an alignment of two *NIMIN 2C* genes; in orange the sequences on which the primers were constructed for *NIMIN 2C* genes silencing is highlighted, in red the sequences on which the primers were constructed for real time analysis of putatively transformed plants is highlighted (table 2.1).

Figure 3.40: *Solanum licopersicum NIMIN 2C* genes sequence alignment. Underlaned the sequence choosen to design appropriate primers sequences. In orange is highlighted the sequence on which the primers were constructed for NIMIN cloning, in red is highlighted on which the primers were constructed for real time analysis of putatively transformed plants. Asterisk (*) indicates positions which have a conserved residue.

These results show that for the line TGA RA there was a significant reduction of the basal gene expression level (around 60%). A similar reduction of *NIMIN 2C* genes expression was also observed for the TGA RB line although the experimental variability did not allow a statistical separation from the control untransformed plant.

It is likely that differences in gene expression will be higher when *NIMIN 2C* genes will be induced by a biotic stress and further work will be carried out toward this direction.

3.8 Gateway cloning, preparation of recombinant vector pHellsgate 12 for silencing of *102* gene in *Spodoptera littoralis*

A previous studies, carried out in the laboratory of Prof. Pennacchio (University of Naples Federico II) indicated that *102* gene was one of the most important gene in immune response of the larva of the tobacco budworm (Heliothis virescens), a moth of the Noctuidae family. This gene is involved in the localization of the process of melanisation of the hemocyte capsule around the foreign objects and is essential for the formation of the capsule itself (Di Lelio et al., 2014). The study has demonstrated that oral injection of dsRNA*102* gene into mature Lepidoptera larvae (*Spodoptera littoralis*) inhibited encapsulation and melanisation of injected chromatography beads. Therefore, the aim of this part of the project is the production of transgenic tobacco plants, which carries the construct for the production of dsRNA*102* that once introduced in larvae, can bring a down-regulation of the *102* gene. The amplified sequence was inserted into the vector pDonor constitutes the external amplified gene *102* (figure 3.41).

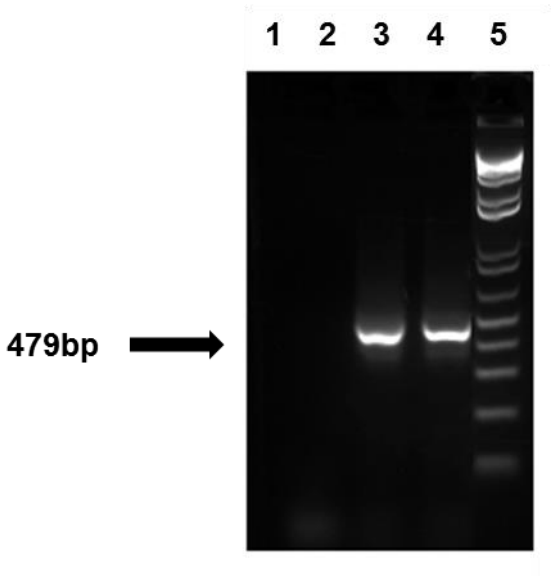

Figure 3.41: DNA fragment analysis on agarose gel of *102* gene amplification with attB primers: Lane 1: negative control, lanes 2-3: amplification products of *102* gene using different melting temperatures, lane 4: kb plus molecular weight ladder

The purified amplification product was used for the recombination with the donor vector (pDonorZeo, Invitrogen). The entry clone pDonor102 (pD102) was used to the transformation of *E.coli* cells (InvαF') by chemical shock. An electrophoretic analysis of a PCR-colony experiment is showed in figure 3.42. The expected amplification products is 709bp in size.

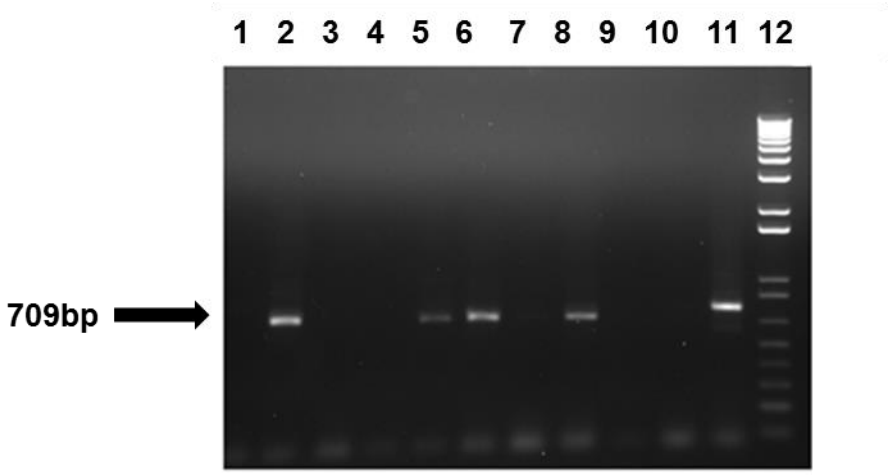

Figure 3.42: **DNA fragment analysis on agarose gel electrophoresis of putatively recombinant plasmids using M13 primers (table 2.1).** Lane 1: negative control, lanes 2-11: putatively recombinant plasmid, lane 12: 1 kb plus molecular weight ladder

As for the production of expression clone a recombination reaction between the entry clone (pH102) and the destination vector (pHellsgate12) was performed. The reaction product was used to transform *E.coli* by heat shock. The colony transformed was selected by PCR colony using the primers pair AGRI-attB. The colony selected was inoculate, the analysis of sequence to extracted plasmid was performed a Sanger sequencing. Figure 3.43 shows a DNA fragment analysis of two sequenced, plasmid PCR products using AGRI primers (table 2.1) run on an agarose gel.

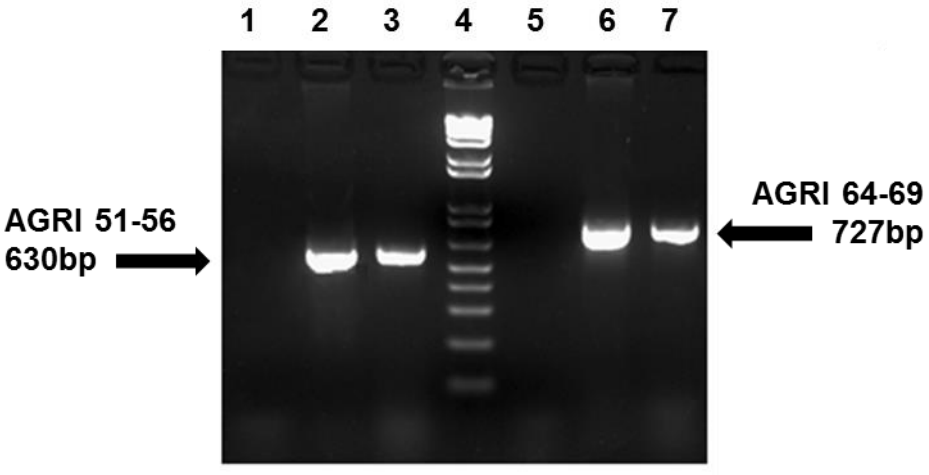

Figure 3.43: DNA fragment analysis on agarose gel electrophoresis of recombinant plasmids using AGRI primers (table 2.1). Lane 1: negative control, lanes 2-3: PCR products of putatively recombinant plasmid amplified with AGRI 51-56 primers, lane 4: 1 kb plus molecular weight ladder, lane 5: negative control, lanes 6-7: PCR products of putatively recombinant plasmid amplified with AGRI 64-69 primers

Plasmids that contained the two fragments of the gene with the right sequence and orientations were used for the stable transformation of tobacco plants (genotype

Nicotiana tabacum) in collaboration with Dr Pasquale Chiaiese (University of Federico II, Naples).

3.9 Tobacco genetic transformation

To perform the stable transformation of *Nicotiana tabacum* plants *Agrobacterium tumefaciens* cells were transformed by electric shock with the plasmid pH102. The tobacco cultivar used to a genetic stable transformation was *Nicotiana tabacum*. Explant of *Nicotiana tabacum* healthy fully expanded leaves (four-five week old tissue) were co-cultivated with a suspension of bacterial. A total of 100 explants from 25 leaves were co-cultivated with the *Agrobacterium* and selected on medium with 50 mg l^{-1} kanamycin. The mortality of explants, determined after one cycle of antibiotic selection, was about 10%. From putatively transformed explants, a total of 90 green calli were obtained. They produced 70 regenerated shoots. The emerging shoots showed root formation in one month.
Plants where than transferred in vivo in an isolated system. Figure 3.44 shows pictures of the different stages in the plant regeneration process from leaves explants and to transformed plants.

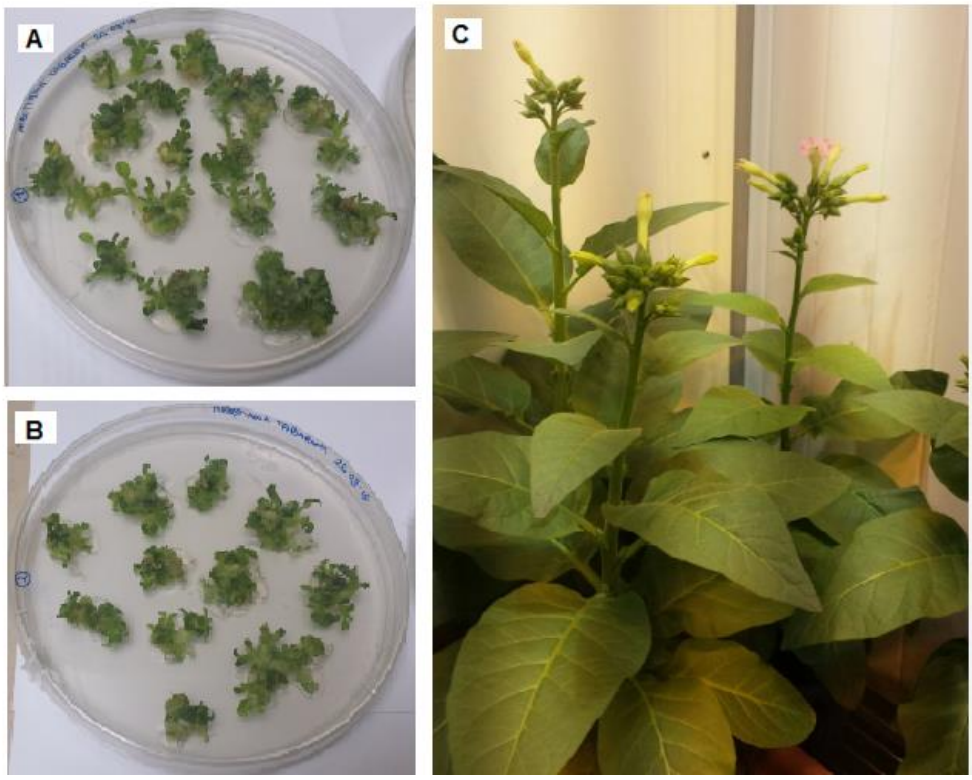

Figure 3.44: Different stages in the Nicotiana tabacum transformation, regeneration process of explants and adult transformed plants. A: *Nicotiana tabacum* not co-cultivated explants after four weeks on MS30 plate containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} of IAA, 100 mg l^{-1} of claforan and 50 mg l^{-1} of kanamycin. B: Co-cultivated explants at beginning of differentiation process on MS30 medium containing 1 mg l^{-1} zeatin, 0.4 mg l^{-1} of IAA, 100 mg l of claforan and 50 mg l^{-1} of kanamycin. C: Transgenic plants different lanes.

The DNA of putative transformed plants was isolated and analysed by PCR reaction with specific primers. Plants transformation was checked by PCR reaction using 102AGRI 51-102attb2, 102AGRI 64-102attb1 primers. Figure 3.45 shows the results of PCR reaction.

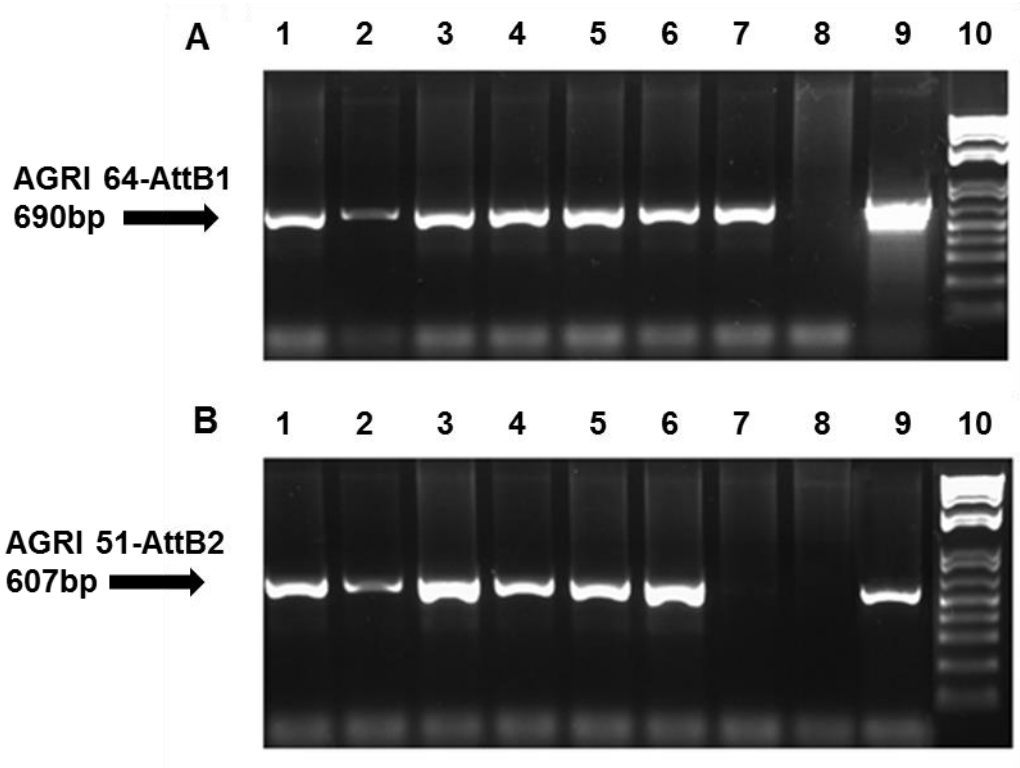

Figure 3.45: DNA fragment analysis on agarose gel electrophoresis of putatively transformed plants DNA using AGRI 51-attB2 (A) or AGRI 64-attB1 primers (B). Lane 1-7 (A): putatively transformed plants, PCR products have the size of 607 bp for transformed plants, lane 8 (A): negative control, lane 10 (A): 1 kb plus molecular weight ladder. Lane 1-7 (B): putatively transformed plants, PCR products have the size of 690 bp for transformed plants, lane 8 (B): negative control, lane 10 (B): 1 kb plus molecular weight ladder.

The plants which gave a positive result from the PCR analysis were transferred *in vivo*. On these plants an expression analysis will be performed in order to verify the expression of dsRNA in order to induce *102* gene silencing in lepidoptera larvae.

4. DISCUSSION AND CONCLUSION

Plants are able to decipher and interpret the cues created by harmful biotic agents and consequently, develop a defence response. The response to insect damage includes the activation of different metabolic pathways. Plants are able to distinguish and recognize pathogens with different life styles and type of nutrition and activate efficient responses according to the damage (Walling, 2000).
Different studies have investigated variations in gene expression following aphid attack through "omics" approaches (Kessler and Baldwin, 2002; Kuhn and Schaller 2004; Giri et al. 2006; Lippert et al. 2007). Recent transcriptomic studies have highlighted a large change in genetic expression profile of plants following the aphid attack (Thompson and Goggin, 2006; Smith and Boyko, 2006). Recent study had demonstrated that tomato plants have a dynamic transcriptional response following *Macrosiphum euphorbiae* attack (Coppola et al., 2013). In this study, a probe annotated as *NIMIN 2C* was among the genes with the higher level of expression. The aphids infection leads to an induction of genes related to salicylic acid pathway and a lower induction of genes related to wound and jasmonic acid (Coppola et al., 2013).
The involvement of *NIMIN 2C* (Solyc03g119590.1.1) in aphid response was confirmed by a study of the levels of expression at different time-points of *Macrosiphum euphorbiae* aphids infestation. The real-time results indicated that this gene is highly overexpressed during all phases of the infestation analysed.
In *Arabidopsis* , it has been found an increase in the levels of expression of defence genes (like PR1 and GluB) induced by salicylic acid following the attack of *M. euphorbiae. NIMIN* genes encode a protein able to interact with NPR1 and to regulate the expression of pathogenesis-related proteins, PR-1 (Weigel et al., 2001). In *Arabidopsis* and tobacco, it was observed a strong induction of this gene in response to salicylic acid (Weigel et al., 2001-2005; Zwicker et al., 2007).
In particular, it was observed that overexpression of the genes of tobacco *NIMIN 2* favours a delay in accumulation of PR-1 proteins while its subexpression increased the levels of PR-1 proteins (Zwicher et al., 2007). The same result were obtained in *Arabidopsis* plants, plants in which there was a *NIMIN 1* gene overexpress showed a suppression of induction of *PR-1* genes mediated by SA and a reduction of the SAR (Weigel et al., 2005).

Unlike the *Arabidopsis* genome, which contains four *NIMIN* genes (*NIMIN 1*, *NIMIN 1b*, *NIMIN 2*, *NIMIN 3*), a recent search in Sol Genomics database indicated the presence of two *NIMIN* genes in tomato: Solyc03g119590.1.1 and Solyc03g119600.1.1. They are both present on chromosome III (62207834..62208169 for Solyc03g11959; 62212939..62213202 for Solyc03g119600). The encoded proteins share a conserved regions, which hint for NIMIN proteins involvement and function in very same pathway. Considering the organization in *Arabidopsis*, their location in the tomato genome and the presence of several conserved regions suggest that a duplication event may have given rise to these two genes. This research was then followed by a bioinformatics analysis both in *Arabidopsis* and in *Solanum lycopersicum* plants in order to understand the possible functional specificity or the possible redundancy in the function of members of *NIMIN* gene family. In *Arabidopsis*, members of the *NIMIN* gene family (in particular *NIMIN 1* and *NIMIN 3*) are both expressed in the range of tissues and conditions that have been analysed. However they respond to stress in a different fashion. This implied a possible differential

regulation. The conditions where *NIMIN 1* expression was highly affected were essentially related to the presence of salicylic acid, biotic stress (mainly fungal or bacterial pathogens) or the presence of elicitors. In the same conditions, the level of expression of *NIMIN 3* is not greatly affected. Moreover, in *Arabidopsis*, members of the *NIMIN* gene family differ in the way they respond to stress. *NIMIN* genes are likely to be associated and probably regulated by different molecular network furthermore the co-expression analysis strengthen the possible link between the members of the *NIMIN* gene family, with important genes related to the response against stress.
In tomato, the two *NIMIN* genes are closely related, both are expressed in the range of tissues and conditions that have been analysed except during the growth phase and the maturation of the fruits in which show expression profiles different. As for the response to stress the two gene are differently regulated following the application of stress investigated such as the presence of the protein flagellin 22 (flg22) or flagellin II-28 (flgII-28) but also the presence of lipopolysaccarides (LPS), peptidoglycan (PGN), cold shock protein 22 (csp22) and finally some lanes of *Pseudomonas syringae*.
Moreover analysis of expression of the *NIMIN 2C* genes, Solyc03g119590.1.1 Solyc03g119600.1.1 in leaves attacked and distal 48h and 96h after inoculation of the fungus *Botrytis cinerea* was performed. In particular, it was possible to observe a rise of *NIMIN 2C* genes expression levels after 96 hours from inoculation in both distal and attacked leaves. It has been proposed that the plant is able to perceive in a similar way aphids and phytopathogenic fungi (Walling, 2000). This characteristic is justified in the literature by the similarity in plant cell penetration mode between the fungal hyphae and the aphid stylet. Moreover, the elicitors issued by hyphae during growth and released by aphid during feeding are similar (Bos et al., 2011).
The systemic acquired response, mediated by SA, is activated against a broad spectrum of oomycetes, fungi, bacteria and pathogenic viruses. The salicylic acid is able to regulate the defence mechanisms through the induction of PR proteins which show antifungal activity, antibacterial and against oomycetes (Vidhyasekaran, 2007).
Also a mechanical damage, which is also produced by chewing insects, can influence NIMIN 2C genes levels of expression.
Phytophagous insects leads to the production of signals that promote the accumulation of molecules involved in direct and indirect resistance (Mcgurl et al., 1994, Ryan, 2000). The jasmonic acid (JA) pathway plays a central role in response to chewing-insects (Stotz et al., 2000; Reymond et al., 2000). The SA and JA signalling pathways may be mutually antagonistic, because of their ability to inhibit each other (Kunkel and Brooks, 2002). In this way, plant can prioritize one defence response according to the type of pathogen attack. In the presence of SA, NPR1 was found to be involved in the suppression of defence genes mediated by JA (Spoel et al., 2003). In the absence of NIMIN proteins and with a subsequent overexpression of NPR1, in *Arabidopsis* plants was observed an increased susceptibility too many necrotrophic pathogen (Heil and Baldwin, 2002). NIMIN proteins may be indirectly involved in the resource adjustment of defence responses mediated by SA and JA or processes relating to growth and reproduction. It is also possible that aphids have developed mechanisms capable to induce in the host plant the activation of defence pathways mediated by SA and antagonized those mediated by JA (Walling, 2008).
An increase in *NIMIN* gene expression following a mechanical damage could bring to a reduction in the expression of defence genes linked to the pathway of salicylic acid.

Recent studies have, however, to assume that any biotrophy insects, such as aphids, induce pathways mediated defence from salicylic acid so as to antagonize those

regulated by the acid jasmonic (Walling, 2008). Ellis et al., 2002 have observed that *Arabidopsis* plants capable of expressing high levels of JA found to be more resistant to attack aphid compared to wild-type plants. It was also demonstrated that exogenous applications of methyl jasmonate (MeJA) are able to promote the resistance against aphids in *Arabidopsis* plants (Ellis et al., 2002). It is not still clear how the downstream of salicylic acid pathway are able to regulate the defence of plant.

Yeast two-hybrid studies have revealed that *Arabidopsis* AtNIMIN proteins interact at different sites with AtNPR1. NIMIN 3 interacts at the N-terminus, however, NIMIN 1, NIMIN 1b and NIMIN 2 interact at the C-terminus with AtNPR1 (Weigel, 2001). To check if the two NIMIN genes from tomato can interact with NPR1 a bimolecular fluorescence complementation (BiFC) analysis was performed. The use of BiFC assay to verify protein interaction can rise main advantages, which include the possibility to assay protein interaction directly *in planta* with comparative simplicity.
Only the vectors pBiFCt-590NPR1-NN (nYFP-NPR1 amino-terminal fusion; cYFP-NIMIN 590 amino-terminal fusion) *and* pBiFCt-600NPR1-NN (nYFP-NPR1 amino-terminal fusion; cYFP-NIMIN 600 amino-terminal fusion) show a positive result in the western blot and epi-fluorescence microscope analysis. The rise of a fluorescence signal when the two proteins of interest are N-terminally fused with YFP fragments may indicate a possible interaction of both proteins through the *N-terminus*. Tomato NIMIN 2C proteins do not seem to interact with NPR1 in different ways and this represent a difference with *Arabidopsis* NIMIN proteins and their interaction with NPR1 gene.

The interaction was verified at three different time points: t0 (immediately after the cut), t1 (30 minute after the cut) and t2 (one hour after cut). Interestingly, there is an increase of the interaction between the two proteins after one hour from the damage of the leaf. This result confirms a role in the defence mechanisms of the *NIMIN 2C* genes.

The final aim of the project was to developed biological tools to defence study and new strategies to increase resistance against insect pests.
For these reasons have been developed transgenic tomato and tobacco lanes, which were able to silence both the genes of *NIMIN 2C* family (tomato plants), and *102* gene in phytophagous larvae (tobacco plants). *NIMIN 2C* silenced tomato plants, since *NIMIN 2C* genes important roles in defence mechanism activated by salicylic acid pathways, will be used for a future biological assay with aphids in order to understand how the defence mechanisms are adjusted against these pathogens and agents such elicitors are able to control such response. Furthermore, this assay will give us some clarification about the role of this gene in the defence mechanisms. Particularly if the transgenic plants are more susceptible to aphids attack compared to wild-type plants it will prove that this gene is a key regulator in plant defence mechanisms against such pathogens as if the plant will be more resistant it will confirm the role of this gene as a negative regulator of the defence mechanisms of the tomato plant. A silencing of the *NIMIN 2C* genes may also be important for a better characterization of genes and defence mechanisms activated after different stress applied such as fungus attack, wound and all conditions analysed for wild type plants.

Whereas the RNAi-mediated silencing of the *102* gene, essential for parassitoids development and survival, by producing *in planta* appropriate dsRNA is currently providing the possibility to study and develop new control methods.

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6. SCIENTIFIC COURSES, MEETINGS AND SEMINARS

6.1 Courses and meetings

- Attestato di partecipazione alla Tavola Rotonda. Prof. Rosa Rao: "Le nuove tecnologie del DNA per la lotta alle frodi agroalimentari" - date 28th June 2013.
- Cambridge English: Preliminary (PET-B1). Centro Linguistico Ateneo (CLA) – date October 2013 / March 2014.
- Genetica Agraria Prof. Domenico Carputo. Dipartimento di Agraria, Università degli Studi di Napoli Federico II. – date October 2013 / May 2014.
- "Insect Science" 5 th Annual Meeting, Orosei (Nuoro,Italia)- date 7th and 8th June 2014.
- Prof. Domenico Carputo: "How to write a scientific paper" - Dipartimento di Agraria, Portici. - date 20th November 2014.
- Prof.ssa Marielena Furano: "Probabilità e Statistica" - Dipartimento Agraria, Portici. - date January and February 2015.
- Prof Rosa Rao: PGB Network "Plant genetics and Biothecnology"- Padova- date 15-17 June 2015.
- Dr. Sandra Richter (University Tübingen): "General rules for confocal operation Leica SP2 and SP8 at the ZMBP" - date 10th February 2016

6.2 Seminars

- Dr. Massimo Iorizzo: "Genomics and computing advances for precision breeding in plants"- date 5th May 2013.
- Prof. Peter Götz: "Dynamic processes in microbial metabolism"- date 27th May 2013.
- Prof. Richard R. Bélanger: "A biocontrol agent among plant pathogens: can comparative genomics explain lifestyle?"- date 4th June 2013.
- Dr. Jack A. Gilbert: "A focus on metagenomics: the earth microbiome project and the importance of data standards in modelling the planet's microbiome" - date 5th June 2013.
- Dr. Paolo Bagnaresi: "Experiences with RNAseq"- date 7th June 2013.
- Dr. Nunzio D'Agostino: "*Solanum dulcamara*: making your own model with NGS" - date 18th July 2013.
- Dr. Nunzio D'Agostino: "From ESTs to RNAseq"- date 20th June 2013.

Prof. Daniele Rossellini: "Genetics, genetic engineering, polyploids: diversify to survive"- date 14th November 2013

- Dr. Jalila Simann: "Lytic polysaccharide monooxygenases: recentlyidentified copper-containing enzymes involved in oxidative cleavage of cellulose" - date 24th June 2014.
- Dr Thierry Tron, Dr. Jalila Simann, Prof Danilo Porro: "Impact factor, citations, Journals reputation, editorial policy: how to choose the right journal" - date 24th June 2014.
- Dr. Anil Grover: "Molecular response to heat stress in rice"- date 23th March 2015
- Prof Angharad M.R. Gatehouse: "Biopesticides which target voltage-gated ion channels: efficacy and Biosafety" - date 14th January 2016.
- Dr. Thierry Tron: "Functionalized and artificial enzymes: new Bio-derived catalysts" - date 14th January 2016.
- Dr. Thierry Tron: "Bio and bioinspired oxidative catalysts: Odds and ends in recombinant proteins expression" - date 24th January 2016.

7. PAPERS AND COMMUNICATIONS

Poster presentation and oral comunication

- S.Passato, G.Corrado, R.Rao (Department of Agriculture, University of Naples Federico II, Itay): “Functional characterization of NIMIN genes in relation to aphid response” - “Insect Science” 5 th Annual Meeting, Orosei (Nuoro,Italia)-date 7th and 8th June 2014.

Abstract

Members of the NIMIN gene family are able to negatively regulate distinct functions of Non-Expressor of Pathogenesis Related Gene 1 (NPR1). The latter is a key gene involved in systemic acquired resistance in plants, thought to be involved in cross talk between jasmonic (JA) and salicyclic acid (SA).
In tomato, it has recently been demonstrated that NIMIN 2C is upregulated in plants at different time points of a compatible aphid infestation. This evidence suggests that NIMIN2c may be a crucial player of the active deception of plant defense carried out by aphids. As an introductory study to functionally characterized NIMIN 2C gene in tomato, we performed a bioinformatics analysis of NIMIN genes using published data. We studied the expression level of the members of NIMIN family across different stages of development and after specific pertubations, such biotic stress or elicitor treatment. NIMIN genes are differently expressed, both at different development stages and after specific pertubations, indicating a limited redundancy of their functions in relation to biotic stress. In tomato, the data indicated that only Solyc03g119600.1.1 is most influenced by biotic stress. This finding was confirmed by Real Time PCR of this gene in tomato after aphids infestation.

8. STUDY AND RESEARCH PERIODS ABROAD

- University of Tübingen with Dr Christina Chaban at ZMBP, Pflanzenphysiologie, Auf der Morgenstelle 32, D-72076 Tübingen / Germany (1-26/02/2016).

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