UNIVERSITY OF CALIFORNIA
RIVERSIDE

The Analysis of WRKY70/ LURP1 Dependent Defense Mechanism in Arabidopsis and Tomato

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Biology

by

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March 2013

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Acknowledgments

I would like to thank Dr. Thomas Eulgem for his support and encouragement throughout my PhD program. Dr. Eulgem's guidance and enthusiasm not only helped me enhance my scientific knowledge but his logic made me understand things at a whole new level. I am extremely thankful and proud of his mentorship that will resonate with me for all times to come. I would also like to thank all the members of the Eulgem lab particularly Dr. Tokuji Tsuchiya for his critical comments and guidance that helped me evolve as a researcher. I thank my dissertation committee members Dr. Isgouhi Kaloshian for her wise words and constant support and Dr. Hailing Jin for her warm greetings and critical evaluation of my PhD work. I also thank Dr. Julia Bailey-Serres for inducting me in to Chem-Gen IGERT program that helped me approach science from an integrated point of view. I am also very thankful to the many people in the UCR community and in the Department of Botany and Plant Sciences that supported me throughout these years. I would particularly like to thank my funding agency Fulbright/ HEC for supporting my PhD program. It was wonderful to be a part of Fulbright program. I am extremely thankful to my sisters Nadia and Sofia for having faith in me all these years. It was their constant push that has always helped me move forward. Last but not least I would like to thanks my mom for whatever I am today is because of her countless blessings and prayers. I miss my dad but it was his influence that got me this far.

Dedication

To my Parents

ABSTRACT OF THE DISSERTATION

The Analysis of WRKY70/ LURP1 Dependent Defense Mechanism in Arabidopsis and Tomato

by

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Doctor of Philosophy, Graduate Program in Plant Biology
University of California, Riverside, March 2013
Dr. Thomas Eulgem, Chairperson

The Arabidopsis *LURP1* gene of *LURP* gene cluster (Late Up-regulation in Response to *Hpa*) is a member of a 15-member gene family termed *LOR* (*LURP-one related*). Of all Arabidopsis *LOR* family members, only *LURP1* shows an unusually pronounced up-regulation in response to *Hyaloperonospora arabidopsidis* (*Hpa*) where as *LOR1* shows strong constitutive expression based on microarray data. Reverse genetic studies using transposon insertion mutants revealed an important role of *LURP1* in disease resistance mediated by the *R*-gene *RPP*5, while *LOR1* plays a significant role in basal defense against *Hpa*. The promoter swap expression lines $^{-1004}$*pLURR1::LOR1* and $^{-1093}$*pLOR1:: LURP1* were constructed to determine if the promoter or slight differences in their protein sequences are important for the differences in their defense-related roles. Confocal microscopy with stably expressed GFP fusion proteins showed GFP-LURP1 and GFP-LOR1 localized at the plasma membrane and in to the nucleus. Homozygous GFP-LURP1 and GFP-LOR1 restore wild type immunity in the respective mutant backgrounds during compatible and incompatible

interaction with *Hpa*. Yeast two hybrid (Y2H) screens with LURP1 and LOR1 as baits identified no significant interacting partners from a screen of 2.3 x 10^5 cDNA clones.

In Arabidopsis LURP expression is controlled by the WRKY70 transcription factor. Multiple transgenic tomato lines (cultivar VFNT Cherry tomato) containing a *Hpa*-responsive GUS reporter gene containing a promoter fragment of the *LURP* member *CaBP22 (pCaBP22^{-333}::GUS*) , responded to the defense inducing chemicals SA, BTH and DCA indicating that the mechanism of *LURP* regulation is likely conserved between Arabidopsis and tomato. Phylogenetic analysis revealed that *WRKY70*, which is a single copy gene in Arabidopsis, has two orthologs in tomato termed *SlWRKY70a* and *SlWRKY70b*. I made three silencing constructs to individually or co-silence *SlWRKY70a* and *SlWRKY70b*. TRV-VIGS silencing construct in homozygous tomato plants containing *pCaBP22^{-333}::GUS* reporter gene showed that at least one of the two tomato WRKY70 orthologs is required for mediating responses to BTH or DCA defense induction. Thus, the *WRKY70/LURP* regulatory module is likely conserved between Arabidopsis and tomato.

Table of Contents

List of Figures

List of Tables

General Introduction

The worldwide food security demand has highlighted the importance of disease resistance in high-yielding crops of agricultural importance. With modern molecular biological and genomics-related tools and research, it is now becoming increasingly possible to recognize and understand key molecular mechanisms underlying disease resistance in various food crops (Huynh, Dahlbeck et al. 1989; Hu, Xiong et al. 2005; Richardson, Vales et al. 2006). This has already helped scientists to develop some crops resistance against various plant pathogens (Edgerton, Fridgen et al. 2012). For many years, plant breeding has provided mankind the means to select plants that are high-yielding and disease resistant. Flor's gene-for-gene hypothesis (Flor 1971), according to which a plant to be resistant against a certain disease, it must have a resistance gene matching a corresponding pathogen avirulence gene, still holds true today. However, with technological and conceptual advancements in many plant biology-related disciplines (Katagiri and Glazebrook 2009; Kaufmann, Muino et al. 2010) it has become increasingly apparent that the actual molecular mechanisms underlying plant immunity are not simple. The numbers of genes involved in plant-pathogen recognition are large and often parts of large families of structurally related and functionally redundant members (Jones and Dangl 2006; Dodds and Rathjen 2010; Nishimura and Dangl 2010; Schwessinger and Ronald 2012). In addition, individual components of the plant immune system do

not act in simple linear pathways and appear rather to be functionally linked to each other forming a complex network (Qi, Tsuda et al. 2011). This has made the task to identify critical gene functions required for the regulation and execution of plant immune responses even more challenging.

Continuous efforts are needed to provide farmers with new disease resistant crop varieties, as virulent pathogen strains adapted to existing crop varieties can quickly evolve. For example, quick adaptation to resistant host varieties has recently been observed in the case of Potato Late blight caused by *Phytophthora infestans* and Southern corn leaf blight caused by the fungus *Helminthosporium maydis*. Over the years, farmers and scientists have exploited various strategies to develop durable resistant varieties by applying a variety procedures ranging from conventional breeding strategies, such as crosses with wild germplasm, to genetic modification of established crop varieties by introducing transgenes that enhance their immune functions (Xu, Wang et al. 2012).

For nearly three decades, the Brassicaceae species *Arabidopsis thaliana* (Arabidopsis) has been the main model system in plant molecular biology and genetics. Research using this model plant has resulted in the discovery of numerous components of the plant defense system, such as a variety of immune receptors, signal transducers, transcription factors as well as defense-executing enzymes and antimicrobial toxins (Jones and Dangl, 2006; Tsuda, Sato et al. 2009; Dodds and Rathjen 2010; Tsuda and Katagiri 2010; Qi, Tsuda et al. 2011).

As general concepts of many plant immune functions appear now well understood in Arabidopsis, and important new tools for molecular genetics and genomics are now in place for many crop systems, it has become feasible to translate basic knowledge on the plant immune system into crop protection strategies. Several crop systems have emerged to play a leading role in such translational efforts, serving a bridging function between sole model systems, such as Arabidopsis, and crop species of agricultural significance.

One example for a crop species that is highly suitable for such translational research is tomato (*Solanum lycopersicum; Sl*). Tomato is a crop system of worldwide importance, serving as a major cash crop in California (California Agri Resource Dir 2011). Tomato breeding is highly developed and numerous genomics tools have been generated. For example, high density genetic maps and the whole sequence of the tomato genome are available (Vision, Brown et al. 2000; Hobolth, Nielsen et al. 2006; Wang, Diehl et al. 2008; Consortium 2012). In addition, tomato can be stably transformed and a convenient transient gene silencing procedure has been developed that is based on virus-induced gene silencing (VIGS) (Liu, Schiff et al. 2002a; Ekengren, Liu et al. 2003).

In particular, the VIGS system has enabled researchers to translate findings from Arabidopsis to tomato (Bhattarai, Atamian et al. 2010; Atamian, Eulgem et al. 2012). Based on these technological improvements for tomato and the abundance of knowledge that has been built up for Arabidopsis, it seems of

paramount importance and of high potential to further the transition between basic and applied plant immune biology utilizing these two plant systems.

In this general introduction, I summarize key areas of plant immune biology, where successful transitions between both systems have been achieved. This general introduction is followed by two chapters reporting on my research, which was focused on the analysis of a pathogen defense mechanism that is dependent on WRKY70-type transcription factors and which appears conserved between Arabidopsis and tomato.

The plant immune system

Plants possess an innate immune system that is based on a complex transcriptional network that is inducible upon plant-pathogen recognition (Katagiri 2004; Eulgem 2005). This innate immune system consists of two interconnected branches termed PTI (Pattern-Triggered Immunity) and ETI (Effector-Triggered Immunity) (Dangl and McDowell 2006). PTI is activated by conserved microbe-associated molecular patterns (MAMPs) which are conserved molecular signature features associated with large phylogenetic classes of microbes and are recognized by plant cell surface pattern-recognition receptors (PRRs) (Gomez-Gomez and Boller 2002). PTI in plant cells is often attenuated by pathogen effectors proteins that intercept MAMP activated defense signals and enhance pathogen virulence and plant susceptibility (Nomura, Melotto et al.

2005; Abramovitch, Anderson et al. 2006). The remaining weak immune response is termed as basal defense (Glazebrook 2001).

ETI is a form of gene-for-gene mediated disease resistance (Flor 1971) where *R* genes involved in effector recognition are the key component of ETI. This type of non-self recognition results in incompatible plant-pathogen interaction making the plant resistant and the pathogen avirulent (Dangl and Jones 2001). One of the main characteristics of ETI is the hypersensitive response (HR), a programmed death of plant cells at the site of infection (Dangl, Dietrich et al. 2000; Dangl and Jones 2001). *R* genes involved in ETI typically encode proteins containing multiple leucine-rich repeats (LRRs), a central nucleotide binding site (NB) and a N-terminal coiled-coil (CC) or Toll/Interleukin 1 resistance (TIR) domains (Baker, Zambryski et al. 1997; Dangl and Jones 2001).

Microarray studies have revealed that ETI, PTI and basal defense are associated with extensive transcriptional reprogramming and that the differences between these type of immune responses are quantitative rather than qualitative (Maleck, Levine et al. 2000; Eulgem, Weigman et al. 2004; Navarro, Zipfel et al. 2004). It has been reported that these defense responses share numerous signaling mechanisms such as an oxidative burst and the accumulation of the defense hormone salicylic acid (SA) (Klessig, Durner et al. 2000; Zhang and Klessig 2001). SA accumulation also results in systemic acquired resistance (SAR) that provides long-term and broad-spectrum systemic protection against a wide variety of pathogens (Gaffney, Friedrich et al. 1993). In addition to SA, the

stress phytohormones ethylene (ET), and jasmonic acid (JA) are also implicated in defense regulation (Dong, 1998; Reymond and Farmer, 1998; Dempsey et al., 1999; Pieterse and van Loon, 1999). SA is involved in the activation of expression of pathogenicity-related (*PR*) genes such as *PR1*, *PR2*, and *PR5* whereas ET and JA activate the defense related genes *PR3*, *PR4*, and *PDF1.2* (Malamy et al., 1990; Thomma et al., 1998; Dempsey et al., 1999).

MAMP-recognition by plant PRR

The flagellin receptor of Arabidopsis FLAGELLIN SENSING2 (FLS2), involved in PTI, is a LRR receptor kinase (Gomez-Gomez and Boller 2000). FLS2, initially identified in Arabidopsis, has orthologs in tomato (Robatzek, Bittel et al. 2007). Both *At*FLS2 from Arabidopsis and *Sl*FLS2 from tomato respond to flg22, a 22 amino acid comprising peptide which is a highly conserved part of flagellin (Meindl, Boller et al. 2000; Bauer, Gómez-Gómez et al. 2001; Chinchilla, Bauer et al. 2006; Robatzek, Bittel et al. 2007). Arabidopsis protoplasts transformed with *Sl*FLS2 showed high responsiveness to the flg15 peptide with only the C-terminal 15 amino acids of flg22. This high sensitivity to flg15 is linked to the first 10 LRRs of *Sl*FLS2. *Sl*FLS2 as compared to *At*FLS2 shows higher affinity for flagellin. This shows that Arabidopsis and tomato respond differently towards the C-terminal part of the flg22. Swapping of the LRR domain of *Sl*FLS2 with *At*FLS2 resulted in a functional receptor that interacted with the flg22. *Sl*FLS2 gave full response with LRRs 1 to 24 indicating that distinct interaction

sites are present over all the entire 28 LRRs ectodomain of FLS2 (Mueller, Bittel et al. 2012).

Early PTI signaling by protein kinases

Many transmembrane receptor-like protein kinases (RLKs) play an important role in plant defense activation (Afzal, Wood et al. 2008) during both PTI and ETI. It has been found Arabidopsis that FLS2 hetero-dimerises with the regulatory LRR-RLK BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 (BAK1) upon MAMP recognition activating downstream signaling. Arabidopsis BAK1 is also called SERK3. It is a member of a family of five somatic embryogenesis receptor kinases (SERKs) (Hecht, Vielle-Calzada et al. 2001). SERKs consist of five extracytoplasmic LRRs, a hinge region, a transmembrane domain, a cytoplasmic Ser/Thr kinase domain and a C-terminal end (Chinchilla, Zipfel et al. 2007; Chinchilla, Shan et al. 2009). BAK1/SERK3 functions are also conserved in tobacco and tomato (Heese, Hann et al. 2007; Fradin, Zhang et al. 2009; Bar, Sharfman et al. 2010). In Arabidopsis, knocking out of *AtSERK3/BAK1* impairs PTI mediated by the PRR RLKs, LRR receptor kinases EF-TU RECEPTOR (EFR) and FLS2 (Chinchilla, Zipfel et al. 2007; Zipfel 2008). A direct link between SERK1 to innate immunity has been found in rice, where overexpression of *OsSERK1* leads to a decrease in host susceptibility to the blast fungus *Magnaporthe grisea* (Hu, Xiong et al. 2005).

Another important class of protein kinases involved in PTI are mitogen-activated protein (MAP)-kinases (Pedley and Martin 2005; Zhang, Yang et al. 2006). Typically three different types of protein kinases act sequentially in these signaling processes. MAP kinases (MAPKs) are phosphorylated by MAPK kinases (MAPKKs). In this phosphorylated form, MAPKs can phosphorylate their target proteins (Mishra, Tuteja et al. 2006). Often MAPKs, which are cytoplasmically localized move into the nucleus upon phosphorylation by MAPKKs. In the nucleus they can activate transcription factors. It has been found that WRKY transcription factors are phosphorylated by MAPKs (Menke, Kang et al. 2005). MAPKKs can get activated by phosphorylation mediated by MAPKK kinases (MAPKKKs), which often act closely associated with the respective stimulus perception process (Zhang and Klessig 2001). In plants such MAPK modules have been shown to act in SA signaling, PTI, but also other types of stress-related stimuli, such as ethylene perception (Zhang and Liu 2001; Nakagami, Pitzschke et al. 2005; Pedley and Martin 2005).

In tomato VIGS-induced silencing of *Le*MPK1, *Le*MPK2, and *Le*MPK3 identified different but overlapping roles of these MAPKs in HR and resistance of tomato to *Cladosporium fulvum* effector *Avr4*-mediated immunity to *Cf4 R*-gene (Stulemeijer, Stratmann et al. 2007). Two MAPKK, *Le*MKK2 and *Le*MKK4 in turn were found to phosphorylate these MAPKs. Where *Le*MPK2 and *Le*MPK3 were found activated by *Le*MAPKKKa (Pedley and Martin 2004).

Transfer of Arabidopsis LRR-RLKs EFR involved in bacterial Ef-Tu recognition, that is absent from the solanaceous species, into tomato resulted in increased resistance to *Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas perforans* (Lacombe, Rougon-Cardoso et al. 2010). This shows that MAMP signaling components in tomato are intact for EFR mediated disease resistance and shows how PTI could be exploited across species to confer wide range disease resistance against various pathogens.

Receptor-like proteins

In addition to RLKs, receptors like proteins (RLPs) appear to be required for PTI-related immune responses. *At*RLP52 and *At*RLP30 RLPs are involved in basal defense against the powdery mildew fungus *Erysiphe cichoracearum* and the bacterium *Pseudomonas syringae* pv. *phaseolicola* (Ramonell, Berrocal-Lobo et al. 2005; Wang G, Ellendorff U et al. 2008). *At*RLP51 was found to regulate defense against the downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) and *Pseudomonas syringae* pv. *tomato* (*Pst*; Zhang, Yang et al. 2010).

Some RLPs are critical for ETI, such as the tomato Cf proteins that provide gene-for-gene resistance against fungal pathogen *C. fulvum* (Jones, Thomas et al. 1994; Thomas, Jones et al. 1997). Cf-4 and Cf-9 confer resistance to *C. fulvum* through recognition of the Avr4 and Avr9 effectors, respectively (Cai, Takken et al. 2001).

The *Verticillium resistance 1* (*Ve1*) gene of tomato which encodes an LRR-RLP protein (Kawchuk LM, Hachey J et al. 2001; Wang G, Fiers M et al. 2010a), provides resistance in tomato against various species of fungal plant pathogen of the genus Verticillum (Fradin, Zhang et al. 2009). Arabidopsis expressing the tomato *Ve1 gene* was resistant to strains of *V. dahliae* and *V. albo-atrum* (Fradin, Haliem et al. 2011). In transgenic Arabidopsis plants expressing *Ve1,* the defense regulators EDS1 (enhanced disease susceptibility) and NDR1 (non-race specific disease resistance) were required for function of this *R*-gene, suggesting that EDS1 and NDR1 orthologs may be important for *Ve1* signaling in tomato also (Hu, Xiong et al. 2005; Fradin, Zhang et al. 2009). Both EDS1 and NDR1 are required for the activation of SA-dependent immune responses in Arabidopsis (Delaney, Uknes et al. 1994; Century, Shapiro et al. 1997; Aarts, Metz et al. 1998; Falk, Feys et al. 1999). This suggested some similarity between the Arabidopsis and tomato *Ve1*-dependent disease resistance.

The *At*SERK1, *At*SERK3/BAK1, and to lesser extent *At*SERK4 were required for full *Ve1*-mediated resistance in Arabidopsis. *Sl*SERK3 was also shown to be required for *Ve1*-mediated resistance in tomato (Fradin, Haliem et al. 2011). Similarly, *SlSERK1* was shown to be required for *Ve1*-mediated Verticillium resistance in tomato by VIGS-mediated silencing (Fradin, Haliem et al. 2011).

Salicylic acid signaling

In Arabidopsis two signaling branches converge upstream from SA. One that is dependent on EDS1 and PAD4 and the other one on NDR1 (Aarts, te Lintel Hekert et al. 1998; Wang, Ruan et al. 1998; Rustérucci, Aviv et al. 2001). Disease resistance mediated by SA is also partially dependent on NPR1 (nonexpresser of pathgenesis related gene; Cao, Glazebrook et al. 1997). NPR1 is a transcriptional co-factor that interacts with TGA-bZIP transcription factor during plant defense (Dong 1998; Dong, Li et al. 2001). Additional transcription factors like WRKYs play important role in the regulation of the SA-responsive plant defense transcriptome and SA-dependent disease resistance (Eulgem 2005). WRKY transcription factors generally bind to the W box (TTGAC/CT) in Arabidopsis defense gene promoters to assert their function (Eulgem and Somssich 2007). It has been shown that WRKY70 acts downstream of SA and downstream or independent of NPR1 in Arabidopsis disease resistance (Knoth, Ringler et al. 2007). It also acts as a point of conversion for SA and JA pathways (Li, Brader et al. 2004). .

Enhanced disease resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Kim, Kwon et al. 2009; Kwon, Kim et al. 2009) of the suppressor of *rps4-RLD1* (srfr1) mutant was found to be dependent on EDS1. Similar to the interaction of EDS1 with the TIR-NB-LRR proteins RPS4, SNC1, and RPS6, the EDS1-SRFR1 interaction was found in cytoplasmic microsomal fractions. *AvrRps4* and *HopA1* effectors also interacted with EDS1

(Bhattacharjee, Halane et al. 2011). As EDS1 interacts with three different R-proteins this regulatory protein could be manipulated as a regulatory node to explore different defense related underlying mechanisms in tomato to enhance disease resistance.

Tobacco mosaic virus (TMV) resistant transgenic tomato plants VF36 (*N*) containing the TIR-NBS-LRR *N* tobacco gene were mutagenized to identify TMV-susceptible plants (Hu, deHart et al. 2005). Mutant *sun1-1* seedlings failed to develop HR response and showed TMV infection. *sun1-1* seedlings were stunted and wilted in response to Verticillium and Fusarium infection (Kawchuk LM, Hachey J et al. 2001; Sela-Buurlage, Budai-Hadrian et al. 2001). The *sun1-1* mutation showed no effect on *Mi-1* mediated resistance to the root-knot nematode *Meloidogyne javanica*. SA application partially suppressed enhanced susceptibility in *sun1-1* (*N*) plants and is not sufficient to restore the cell death pathway in *sun1-1* mutant. Fine mapping identified *SUN1* and the phenotype associated with *sun1-1* (*N*) mutant were caused by disruption of the *EDS1* like gene in tomato thus named *Le_EDS1*. In addition to exhibiting impaired *R* gene-mediated resistance, *Le_eds1-1* mutant plants appear more susceptible to invading pathogens than plants that lack the corresponding *R* genes. This suggests a role for Le*_EDS1* in basal defense (Hu, deHart et al. 2005) and demonstrates that the function of *EDS1* is conserved between Arabidopsis and tomato.

Systemic acquired resistance (SAR) is a broad spectrum long-lasting immune response triggered throughout the entire plant organism by hypersensitive responses associated with localized avirulent pathogen infections or disease-related lesions developed during local compatible interactions (Ryals et al., 1996). SAR induction results in NPR1 localization to the nucleus, where it interacts with TGA factors (Kinkema et al., 2000; Subramaniam et al., 2001; Fan and Dong, 2002). As outlined above, NPR1 stimulates the DNA binding activity of TGA factors to SA-response elements in defense gene promoters (Lebel, Heifetz et al. 1998; Despres, DeLong et al. 2000; Niggeweg, Thurow et al. 2000; Fan and Dong 2002).

The TGA family of basic domain/ Leu zipper (bZIP) transcription factors (Zhang, Fan et al. 1999; Despres, DeLong et al. 2000; Niggeweg, Thurow et al. 2000; Zhou, Trifa et al. 2000; Chern, Fitzgerald et al. 2001) binds to DNA elements containing core TGACG region and were, therefore named TGA-bZIPs. Of the 10 TGA-bZIPs in Arabidopsis seven (TGA1–TGA7) were found to be able to interact with NPR1 (Jakoby, Weisshaar et al. 2002).

The redox status of certain cystine residues in TGA1 and TGA4 is controlled by SA and affect interaction with NPR1 (Despres, Chubak et al. 2003). NPR1 stimulated the DNA binding activity of the reduced form of TGA1. *In vivo* experiments confirmed that SA reduces the cystine residues in TGA resulting in the stimulation of NPR1 and TGA1 interaction (Despres, Chubak et al. 2003).

The tomato Pto pathway

The tomato *R*-gene *Pto* encodes a ser/thr protein kinase (Martin, Brommonschenkel et al. 1993; Pedley and Martin 2003) which confers resistance to *Pst* expressing the effector proteins AvrPto and AvrPtoB (Ronald, Salmeron et al. 1992; Kim, Lin et al. 2002). The Prf protein, which is similar to CC-NBS-LRR proteins is required for *Pto*-mediated disease resistance (Salmeron, Oldroyd et al. 1996; Pedley and Martin 2004). VIGS studies in tomato expressing *Pto* gene showed that silencing of *Nicotiana benthamiana* derived cDNAs of two MAPKs, *NTF6* and wound induced protein kinase (*WIPK*; ortholog of tomato *MPK2*) and two *Nicotiana tabacum* derived MAPKKs (*MEK1* and *MEK2*) were involved in symptom development against this bacterial pathogen. It has been found that *MEK1* with *NTF6* or *MEK2* with *WIPK* mediate *Pto*-induced cell death in *N. benthamiana* (Pedley and Martin 2003; Liu, Schiff et al. 2004; Nakagami, Pitzschke et al. 2005; Oh and Martin 2010). In addition *NPR1, TGA1a* and *TGA2.2* also resulted in the enhanced development of bacterial speck disease symptoms after *Pst* strain T1 (*avrPto*) infection. Thus silencing of these genes result in bacterial speck disease on Pto expressing tomato leaves when inoculated with avirulent *P.syringae* pv. *tomato* T1(*avrPto*). This shows that genes identified as tomato ortholog in *N. benthamiana* and *N*. *tabacum* are involved in *Pto*-mediated disease resistance in tomato (Ekengren, Liu et al. 2003).

Responses caused by the phytotoxin cornonatine and the phytohormone jasmonic acid

Bacterial speck disease caused by *Pst* DC3000 on tomato results in the formation of necrotic lesions surrounded by chlorotic halos (Bender, Stone et al. 1987; Bender, Alarcon-Chaidez et al. 1999; Preston 2001). Chlorosis is mainly due to the phytotoxin coronatine (COR) produced by *Pst* DC3000 (Mittal and Davis 1995; Bender, Alarcon-Chaidez et al. 1999; Zhao, Thilmony et al. 2003; Uppalapati, Ayoubi et al. 2005; Uppalapati, Ishiga et al. 2007) and results in virulence of *Pst* DC3000 in Arabidopsis, tomato, collards and turnip (Zhao, Thilmony et al. 2003; Brooks, Hernandez-Guzman et al. 2004; Elizabeth and Bender 2007; Uppalapati, Ishiga et al. 2007). As COR is structurally related to the JA derivative methyl-JA, it simulates responses triggered by JA. It is well known that in Arabidopsis JA- and SA-dependent signaling processes are mutually antagonistic (Xie, Feys et al. 1998; Glazebrook, Chen et al. 2003; Spoel, Koornneef et al. 2003; Li, Brader et al. 2004; Li, Brader et al. 2006). Consistent with this, COR was found to suppress SA signaling and stimulate the JA pathway in Arabidopsis and tomato (Kloek, Verbsky et al. 2001; Schmelz, Engelberth et al. 2003; Zhao, Thilmony et al. 2003; Block, Schmelz et al. 2005; Melotto, Underwood et al. 2006; Uppalapati, Ishiga et al. 2007).

In Arabidopsis, co-chaperone Sgt1b (suppressor of G2 allele of *skp1*) is required for the proper function of several R proteins as well as regulation of HR (Tör, Gordon et al. 2002; Liu, Schiff et al. 2002b; Holt, Hurbet et al. 2003). The

SlSGT1-VIGS silenced plants compared with control plants after *Pst* DC3000 inoculated resulted in a complete loss of COR-induced chlorosis in tomato (Uppalapati, Ishiga et al. 2011). Chlorosis was strongly reduced in the Arabidopsis *sgt1b* mutants (Austin, Muskett et al. 2002; Tör, Gordon et al. 2002; Holt, Belkhadir et al. 2005; Azevedo, Betsuyaku et al. 2006; Noe¨l, Cagna et al. 2007). This shows that SGT1 is needed for full disease symptom development during a compatible interaction in tomato and Arabidopsis (Muskett and Parker 2003; Shirasu 2009). Thus this regulatory protein could be used as a common cue for various defense signaling processes.

Tomato leaf tissues treated with purified COR show chlorosis (Gnanamanickam, Starratt et al. 1982; Uppalapati, Ayoubi et al. 2005; Uppalapati, Ishiga et al. 2007 Kloek, Verbsky et al. 2001). A VIGS-based (Liu, Brutlag et al. 2001; Liu, Schiff et al. 2002a; Anand, Vaghchhipawala et al. 2007) screening of a *N. benthamiana* cDNA library indicated that silencing Altered COR Response 1 (*ALC1*) gene resulted in a hypersensitive/necrosis-like phenotype rather than a typical chlorotic phenotype observed in response to COR application. *ALC1* has homology to the Arabidopsis gene *THF1* Thylakoid Formation1 (Wang, Sullivan et al. 2004). The loss of *ALC1/THF1* was found to result in accelerated cell death in response to *Pst* DC3000 infection in tomato and Arabidopsis (Wangdi, Uppalapati et al. 2010).

Signaling processes triggered by the tomato R protein Mi-1

The tomato *R* gene *Mi-1* has been found to mediate strong gene-for-gene resistance to pests from various phylogenetic clades such as potato aphids (*Macrosiphum euphorbiae*), sweet potato whitefly (*Bemisia tabaci*) and root-knot nematodes (RKNs; *Meloidogyne* spp.) (Kaloshian, Lange et al. 1995; Milligan, Bodeau et al. 1998; Rossi, Goggin et al. 1998; Nombela, Williamson et al. 2003; Kaloshian 2004; Kaloshian and Walling 2005). *Mi-1* encodes a typical NB-LRR type R protein with an N-terminal coiled-coil domain (Milligan, Bodeau et al. 1998).

To identify components involved in *Mi-1* signaling, a high throughput suppressor screen using tobacco rattle virus (TRV)-based VIGS was performed in *N. benthamiana* (Mantelin, Peng et al. 2011). Attenuation of HR was used as a visible maker for VIGS-mediated disruption of *Mi-1*-dependent defense signaling processes. Screening of a cDNA library prepared from tobacco mosaic virus (TMV)-infected *N. benthamiana* (Liu, Schiff et al. 2002a; Liu, Schiff et al. 2002b) identified *NbSERK1* as a suppressor of the HR phenotype. Its tomato ortholog, *SlSERK1,* was found in VIGS studies to be required for *Mi-1*-mediated resistance against aphids but not against RKNs. *SlSERK1* was also not found to be involved in basal defense against potato aphids (Mantelin, Peng et al. 2011).

Various defense signaling and regulatory proteins such as Sgt1 (Austin, Muskett et al. 2002; Azevedo, Sadanandom et al. 2002; Muskett and Parker 2003; Azevedo, Betsuyaku et al. 2006), Rar1 (required for *Mla*12 resistance)

(Muskett, Kahn et al. 2002) and Hsp90 (heat shock protein 90) a chaperon molecule (Hubert, Tornero et al. 2003; Liu, Burch-Smith et al. 2004; Shirasu 2009) form a stable signaling complex involving many R proteins (Azevedo, Sadanandom et al. 2002; Takahashi, Casais et al. 2003; Schulze-Lefert 2004; Noe¨l, Cagna et al. 2007). It was found that TRV-*NbSgt1* VIGS silenced tomato plants resulted in attenuation of *Mi-1*-mediated resistance to potato aphid but not to RKNs. *Rar1* silenced plants did not indicate a role for this gene in *Mi-1*-mediated resistance to aphids and nematodes. However Hsp90 silenced plants were compromised in both aphid and RKN *Mi-1*-mediated disease resistance indicating to an extent a common role for these components in *R*-gene-mediated disease resistance against various pathogens and pests (Bhattarai, Li et al. 2007).

WRKY transcription factors

WRKY transcription factors constitute large families in plant species (Eulgem, Rushton et al. 2000). In Arabidopsis, 72 members of this family have been described (Eulgem and Somssich 2007; Rushton, Somssich et al. 2010). Characteristic for these factors is their conserved WRKY DNA-binding domain and their ability to bind to W-box promoter elements (Eulgem, Rushton et al. 2000). Many members of this family have so far been shown to be required for proper immune responses in Arabidopsis (Chen and Chen 2002; Eulgem and Somssich 2007).

Using VIGS, the two tomato orthologs of Arabidopsis *WRKY72*, *SlWRKY72a* and *SlWRKY72b*, were found to be involved in basal defense and *Mi-1*-mediated disease resistance against potato aphids and RKNs (Bhattarai, Atamian et al. 2010). The sole Arabidopsis ortholog of these two closely related genes *AtWRKY72* was also found to be important for basal defense. Reverse genetics experiments using Arabidopsis T-DNA mutants demonstrated *AtWRKY72* to contribute to basal defense against RKNs, aphids and *Hpa*Noco2. However *wrky72* mutants tested for gene-for-gene resistance in Arabidopsis showed that this gene is not required for *RPM1, RPS2 and RPP4* resistance during incompatible interactions with *Pst* and *Hpa*. Comparative profiling of transcriptome changes triggered during a compatible *Hpa* interaction in *wrky72* mutants and wild type plants suggested that this transcription factor control defense processes independent of SA. This suggested that the role of WRKY72-type transcription factors in basal defense is conserved between Arabidopsis and tomato, while these transcription factors appear to have been specifically recruited to the *Mi-1*-pathway, but not to those triggered by other tested *R*-genes.

A second member of the WRKY family has been implicated in *Mi-1*-mediated defense induction. Levels of transcripts related to those of the Arabidopsis transcription factor WRKY70 were induced in tomato during both basal defense to aphids and RKNs, as well as *Mi-1*-mediated resistance to these pests (Atamian, Eulgem et al. 2012). Suppression of these transcripts by VIGS resulted in loss of *Mi-1*-mediated immunity against RKNs and aphids.

Furthermore, *SlWRKY70* transcripts were found to be up-regulated by exogenous application of SA and down-regulated by methyl-jasmonate (MeJA) in tomato. In Arabidopsis, *AtWRKY70* shows the same response pattern and seems to act as a node of convergence integrating SA- and JA-dependent signals. This suggests that mechanisms regulating *WRKY70* expression are largely conserved between Arabidopsis and tomato.

ERF transcription factors

Besides TGA bZIPs and WRKYs, ethylene response factors (ERFs) have been implicated in the regulation of plant immune responses. They typically bind to GCC boxes or related stress-response promoter elements (Ohme-Takagi and Shinshi 1995; Suzuki, Suzuki et al. 1998). The Arabidopsis genome harbors genes encoding these transcription factors (Riechmann, Heard et al. 2000; Pedley and Martin 2003) The tomato ERF transcription factors, Pti4, Pti5, and Pti6 were identified by their specific interaction with Pto (Zhou, Tang et al. 1997; Gu and Martin 1998; Gu, Yang et al. 2000). Transient expression of fusions of the GUS reporter protein to Pti4, Pti5 or Pti6 showed these chimeric proteins were localized to the nucleus of tobacco cells. The expression of *Pti5* and *Pti6* increased the GCC box–mediated transcription of luciferase (*LUC)* gene (Gu, Wildermuth et al. 2002).

Expression of *PR1* in Pti4/5/6 transgenic Arabidopsis plants increased whereas the *PDF1.2* transcripts decreased by SA application indicated SA

suppressed induction of GCC box–containing *PDF1.2* gene and repressed the induction of SA responsive *PR1* gene (Gu, Wildermuth et al. 2002). This shows that these genes from tomato can be engineered in Arabidopsis and vice versa for disease resistance, a strategy that can be extended to other crops as well.

Papain-like cystein proteases

Papain-like Cystine proteases (PLCPs) such as RCR3 are required to trigger HR in tomato plants carrying the *Cf-2* resistance gene infected by *C. fulvum* expressing the Avr2 effector (Kru¨ger, Thomas et al. 2002). RCR3 and PIP1, which are closely related PLCPs from tomato (Shabab, Shindo et al. 2008; Van Esse, Van't Klooster et al. 2008), are inhibited by EPIC1 and EPIC2B, two closely related apoplastic cystatin-like effector proteins *of P. infestans (Pinf)* (Tian, Win et al. 2007; Song, Win et al. 2009). EPICs have a higher affinity to the C14 which is a secreted papain-like cysteine protease of tomato and potato (*Solanum tuberosum*) that is a target of EPIC1 and EPIC2B (Kaschani, Shabab et al. 2012). The Arabidopsis RD21 was found as the closest ortholog of tomato C14 protein and *Hpa*EPIC-B and -C as the most likely orthologs of *Pinf*EPIC1 and -2B from *Hpa* isolate Emoy2 (Baxter, Tripathy et al. 2010. However, the *rd21* mutant (Wang, Gu et al. 2008) showed no difference during compatible and incompatible interactions with *Hpa* and *Pst* DC3000, representing an example for clear differences in Arabidopsis-tomato pathogen interaction mechanisms.

With Arabidopsis as a model plant and tomato as a major crop, the various defense related signaling components and their convergent and divergent roles were discussed above. This provides an opportunity where the disease related signaling pathways and defense transcriptome in both Arabidopsis and tomato could be used for not only understanding the defense mechanisms operating in these two diverse species but also to engineer different strategies for disease resistance in other crops against various pathogens. The recently annotated sequenced tomato genome and the wealth of information available from eudicots now provide ample opportunity for scientists and crop growers to integrate such information for practical applications of such research based plant science.

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Chapter 1: Biological and Molecular characterization of Arabidopsis *LURP1* gene and its paralog *LOR1* (*LURP-one related1)* and their role in disease resistance against *Hyaloperonospora arabidopsidis* (*Hpa*).

Summary

LURP1 is a member of the *LURP* gene cluster that shows an unusually pronounced transcriptional up-regulation in response to *Hyaloperonospora arabidopsidis* (*Hpa*). Mutations in *LURP1* resulted reduced immunity mediated by the *R*-gene *RPP5* against *Hpa*. *LURP1* is a member of a fifteen member gene family in Arabidopsis termed *LOR* (LURP-one related). Of the *LOR* family members, *LOR1* shows strong constitutive expression based on microarray data whereas *LURP1* is the only family member showing a transcriptional induction in response to *Hpa*. Mutation in *LOR1* revealed a significant role of this gene in basal defense against *Hpa*. I constructed transgenic promoter swap expression Arabidopsis lines with $^{-1004}$*pLURR1::LOR1* and $^{-1083}$*pLOR1:: LURP1* to determine if the promoter or minor differences in the protein sequences are important for the differences in *LURP1* and *LOR1* defense induction. Based on the resemblance of the LOR1 protein to human Phospholipid scramblase1 (PLSCR1) stable GFP-LURP1 and GFP-LOR1 expression lines were created to analyze the subcellular localization of these proteins. Like PLSCR, both GFP-LURP1 and GFP-LOR1 appeared to be localized at the plasma membrane and in the nucleus. GFP-LURP1 and GFP-LOR1 also complemented loss of resistance in

their respective mutant backgrounds. LURP1 and LOR1 used as bait protein in yeast two hybrid did not result in the identification of any interacting proteins from a screen of $2.3x10^5$ cDNA from control and disease sampled plants treated with *Hpa* isolates.

Introduction

The innate immune system of plants consists of two inter-connected branches termed PTI (Pattern-Triggered Immunity) and ETI (Effector-Triggered Immunity). PTI is activated by receptor mediated recognition of microbe-associated molecular patterns (MAMPs), which are highly conserved molecular signatures widely present in certain types of microbes. Plants have the capacity to perceive pathogenic microorganisms by pattern recognition receptors (PRRs) which interact with such conserved molecular signatures or elicitors (Boller 1995; Ebel and Mithöfer 1998) including flagellin of bacterial plant pathogen *Pseudomonas syringae* (Felix, Duran et al. 1999). Flagellin is recognized in Arabidopsis by the cell surface receptor-like kinase (RLK) FLS2 (Gomez-Gomez, Bauer et al. 2001; Shiu and Bleecker 2001). FLS2 is composed of an extracellular leucine-rich-repeat (LRR) domain, a single membrane-spanning domain and an intracellular serine/ threonine protein kinase domain (Gomez-Gomez and Boller 2000). The responses to elicitors like flagellin include the production of reactive oxygen species, ethylene, and the induction of pathogenesis-related (PR) proteins involved in disease resistance.

In mammals, Toll-like receptors (TLR) which are structurally similar to plant PRRs have been implicated in innate immunity (Kopp and Medzhitov 1999; Imler and Hoffmann 2001; Sieling and Modlin 2002). Like many PRRs, TLRs have an extracellular LRR domain and an intracellular domain involved in protein-protein interaction. TLRs are involved in the recognition of MAMPS such

as lipopolysaccharides, lipoproteins glycolipids and fungal cell-wall components (Hoffmann and Reichhart 2002). In mammals, the recognition of such MAMPs results in inflammatory responses, including the production of reactive oxygen and antimicrobial proteins (Silverman and Maniatis 2001). Toll-like receptors are known to homodimerize or to heterodimerize with other TLRs to form functional receptors. Signalling via TLR in mammals (Silverman and Maniatis 2001) involving receptor dimeriztion include adaptor MyD88 that activate protein kinases such as IRAK (Gomez-Gomez and Boller 2002; Ausubel 2005). This results in the transport of the transcription factor NF-κB into the nucleus (Akira, Takeda et al. 2001; Silverman and Maniatis 2001; Gomez-Gomez and Boller 2002; Hoffmann and Reichhart 2002; Ausubel 2005) where it mediates massive transcriptional reprogramming. It has been shown in Arabidopsis that flagellin signalling also activates the *At*MEKK1 component of a mitogen activated protein (MAP) kinase cascade. This phosphorylates the MAP kinase kinases *At*MKK4 and *At*MKK5 which activate the MAP kinases *At*MPK3 and *At*MPK6 (Asai, Tena et al. 2002; Gomez-Gomez and Boller 2002). The downstream targets of such signaling cascade are the WRKY transcription factors (Eulgem, Rushton et al. 2000), which are responsible for the activation of genes involved in defense responses (Asai, Tena et al. 2002).

It has been shown that many pathogens secrete effectors into plant host cells that intercept MAMP triggered defense signals thereby attenuating PTI (Nomura, Melotto et al. 2005; Abramovitch, Anderson et al. 2006; Jones and

Dangl 2006). The remaining weak immune response called basal defense can limit the growth of pathogen in the host tissue but is typically insufficient to prevent disease resulting in compatible interactions (Glazebrook 2001). Co-evolution of virulent pathogens with their hosts frequently resulted in the establishment of ETI, a typically manifestation of gene-for-gene resistance that results in strong race-specific immunity (Flor 1971). Of key importance for ETI are plant disease resistance *R*-genes encoding nucleotide-binding (NB) and LRR containing receptors (NLRs) that mediate specific recognition of pathogen effectors and trigger highly efficient defense reactions (Gómez-Gómez and Boller 2000). ETI results in incompatible plant-pathogen interactions in which the plant is resistant and the pathogen is avirulent. In plants, after NLR activation, generation of reactive oxygen species, a sustained increase in cytosolic Ca^{2+} and transcriptional reprogramming occur followed by a rapid host-cell death at the site of infection. The latter phenomenon is termed hypersensitive response (HR) and is based on programmed death of plant cells in direct contact with invading pathogens (Dangl and Jones 2001). HR appears to be an efficient immune response against biotrophic pathogens, which depend on living plant tissue to complete their life cycles (Dangl, Dietrich et al. 2000; Staskawicz, Mudgett et al. 2001).

Mammals have class of immune receptors related to plant NLRs, which are also termed NLR (NOD-like receptor) (Holt, Hurbet et al. 2003; Nimchuk, Eulgem et al. 2003). Plant and mammalian NLRs are of similar structure, as both

contain central NB domain and C-terminus LRRs domain (Hoffmann and Reichhart 2002). However, while plant NLRs seem entirely to be involved in effector recognition, mammalian NLRs, like TLRs, are activated by MAMPs or endogenous substances released after pathogen attack and are similar in that term to animal PRRs. The activation of NLRs in animals results in the secretion of antimicrobial peptides and the induction of cell death responses (Georgel, Naitza et al. 2001; Rämet, Manfruelli et al. 2002).

NLRs in animals and plants show diversity in their N-terminal domains. In plants, coiled-coil (CC) or Toll–interleukin 1 (IL-1) receptor (TIR) domains are present whereas caspase activation and recruitment domains (CARDs), pyrin and NACHT domains are found only in animal NLRs (Belvin and Anderson 1996; Ausubel 2005; Ting and Davis 2005). LRR motif is involved in effector-pathogen recognition specificity in plants (Georgel, Naitza et al. 2001; Gottar, Gobert et al. 2002; Chamaillard, Girardin et al. 2003; Chamaillard, Hashimoto et al. 2003; Hoffmann 2003; Athman and Philpott 2004; Girardin and Philpott 2004; Philpott and Girardin 2004; Vialaa, Sansonettib et al. 2004; Ting and Davis 2005). In animals direct interaction of microbial structures with animal NLRs has not been reported so far except for NLR-related protein where Apaf-1 directly interacts with its elicitor cytochrome-c (Tschopp, Martinon et al. 2003; Martinon, Agostini et al. 2004; Maekawa, Kufer et al. 2011). Indirect recognition in plants was reported for the tomato NLR Prf and its associated Pto serine-threonine protein kinase. The *Pseudomonas syringae* pv. *tomato* effector protein AvrPto binds to the Pto, that

inhibits its kinase activity thus activating Prf (Martin, Brommonschenkel et al. 1993; Shan 2008; Xiang, Zong et al. 2008). It has been shown that intramolecular conformational changes are critical for NLR activation (Couillault, Pujol et al. 2004) and in turn downstream signaling (Kim, Liberati et al. 2004). The N-terminal CC and TIR domains of the plant NLRs MLA and L6 were found to form homodimers that is critical for the disease resistance (Dangl and Jones 2001; Axtell and Staskawicz 2003; Maekawa, Kufer et al. 2011). Homotypic TIR domain associations are also important for animal TLRs intracellular signal transduction pathways such as MyD88 (Lemaitre, Nicolas et al. 1996; Hoffmann and Reichhart 2002; Ausubel 2005).

Transfer of NLRs between nuclei and the cytoplasm is important for the function of these receptors in animals such as Rx (Slootweg, Roosien et al. 2010; Tameling, Nooijena et al. 2010; Maekawa, Kufer et al. 2011; Slootweg, Roosien et al. 2010). In contrast in Arabidopsis, TIR-type NLR, RPS4 nuclear localization is critical for pathogen growth restriction (Wirthmueller, Zhang et al. 2007; García, Baufumé-Blanvillain et al. 2012). Coordinated movement of RPS4 or Rx between cytoplasmic and nuclear compartments is critical for these immune receptors (Wirthmueller, Zhang et al. 2007; Cheng 2009). In humans, the NLR such as NLRC5 and the transcriptional coactivator CIITA move into the nucleus, where as in Arabidopsis, NLRC5 and CIITA are involved in transcriptional reprogramming (Hake, Masternak et al. 2000; Meissner, Li et al. 2010; Maekawa, Kufer et al. 2011).

Both PTI and ETI were found associated with massive transcriptional reprogramming in plants (Katagiri 2004; Glazebrook 2007). Microarray studies further suggested that differences between PTI, ETI, and basal defense in Arabidopsis are quantitative rather than qualitative (Maleck, Levine et al. 2000; Tao, Xie et al. 2003; Eulgem 2005). Abundant genetic evidence supports the existence of signaling mechanisms shared by some R proteins and PRRs (Tao, Xie et al. 2003; Navarro, Zipfel et al. 2004; Eulgem 2005) . Plants also use phytohormones and secreted peptides for the regulation of immune responses, similar to mammalian cytokines and interferons (Shen, Saijo et al. 2007; Pieterse, Leon-Reyes et al. 2009). In plants phytohormones, such as salicylic acid (SA), appear to be central components of defense signaling processes.

In Arabidopsis, *LURP* (Late up-regulated in response to *Hpa* recognition) genes operate in an SA-dependent pathway that mediates resistance to *Hyaloperonospora arabidopsidis* (*Hpa*). *LURP* genes exhibit a particularly pronounced coordinated increase of transcript levels after pathogen recognition by the R-proteins RPP4 or RPP7 (Figure 1.1).

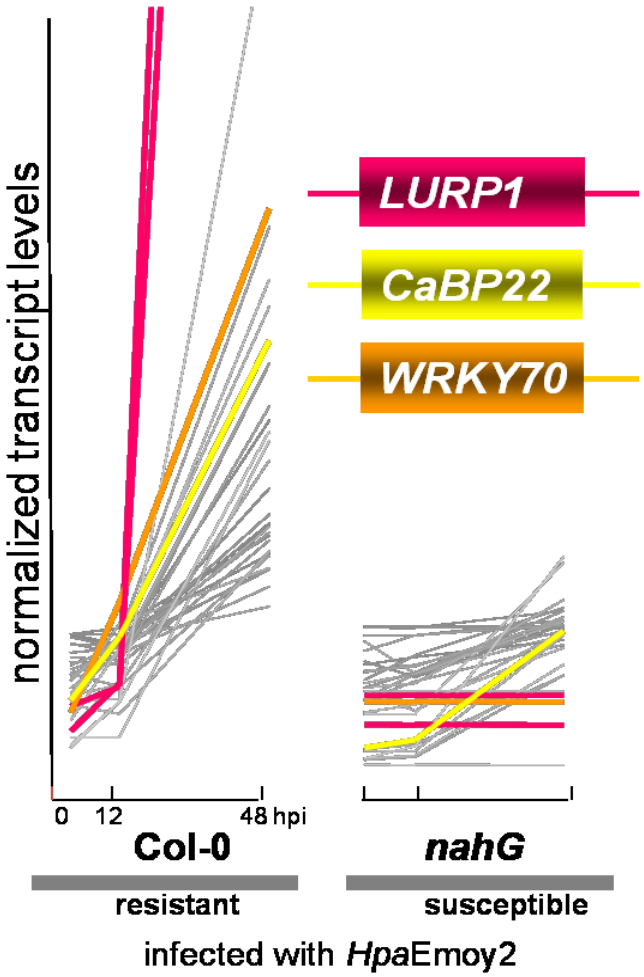

Figure 1.1: Transcript profiles of *LURP1, CaBP22, and WRKY70* (highlighted) in response to Peronospora. Normalized transcript levels of *LURP* (Late upregulation in response to *Hpa*) gene cluster in Col-0 and *nahG* 0, 12 and 48hrs after *Hpa* infection (Eulgem, et al. 2004).

Accumulation of *LURP* transcripts is delayed or attenuated in susceptible lines lacking *RPP4* or *RPP7* function, which suggested a role of *LURP* genes in resistance to *Hpa*. Of all *LURP* genes, *LURP1* showed the most pronounced up-regulation in response to *Hpa*. *LURP1* is a member of a fifteen-member gene family termed *LOR* (LURP-one related). Of the *LOR* family members, only *LOR1*

exhibits strong constitutive expression based on microarray data (Eulgem and Somssich 2007) whereas other family members do not show any detectable expression or only weak constitutive expression (Table 1.1). *LURP1* is the only family member showing a transcriptional induction in response to *Hpa*.

Genes	Col-5 0hpi	Col-5 12hpi	Col-5 24hpi	Col-5 48hpi
At1g33840_at	52.435	41.12	54.58	64.225
At1g80120_at	15.54	10.48	15.755	11.46
At2g05910_at	4.6	10.17	5.06	5.66
LURP1	30.74	160.38	172.315	682.45
At2g30270_at	36.425	41.905	40.335	41.235
At2g38640_at	43.66	29.39	64.475	49.79
At3g11740_at	11.455	12.53	11.855	13.44
At3g14260_at	0.5	2.985	2.5	0.49
At3g15810_at	42.18	36.035	44.81	32.96
At3g16900_at	4.35	2.58	2.875	1.22
At3g56180_at	1.93	0.91	1.555	1.285
LOR1	825.83	556.34	442.66	608.23
At5g20640_at	2.41	0.49	0.1	1.815

Table 1.1: Microarray data showing *LURP1* and *LOR1* expression profiles after infection with avirulent *Hpa*Hiks1. Shown are absolute signal intensities reflecting mRNA steady state levels at the indicated time points. Signal intensities below 25 are considered experimental noise (Eulgem, Weigman et al. 2004). *LURP1* (At2g14560, red) shows a massive transcriptional induction between 24 and 48 hours post infection (hpi) with, whereas *LOR1* (At5g01750, blue) exhibits strong constitutive expression which remains unchanged at 12, 24 and 48hpi (Eulgem, Tsuchiya et al. 2007).

LOR family was found to be structurally related to the mammalian PLSCR protein family. The three-dimensional structure of the *Arabidopsis* protein LOR1 (At5g01750) solved by X-ray crystallography provided the first structural model for this family (Bateman, Finn et al. 2009).

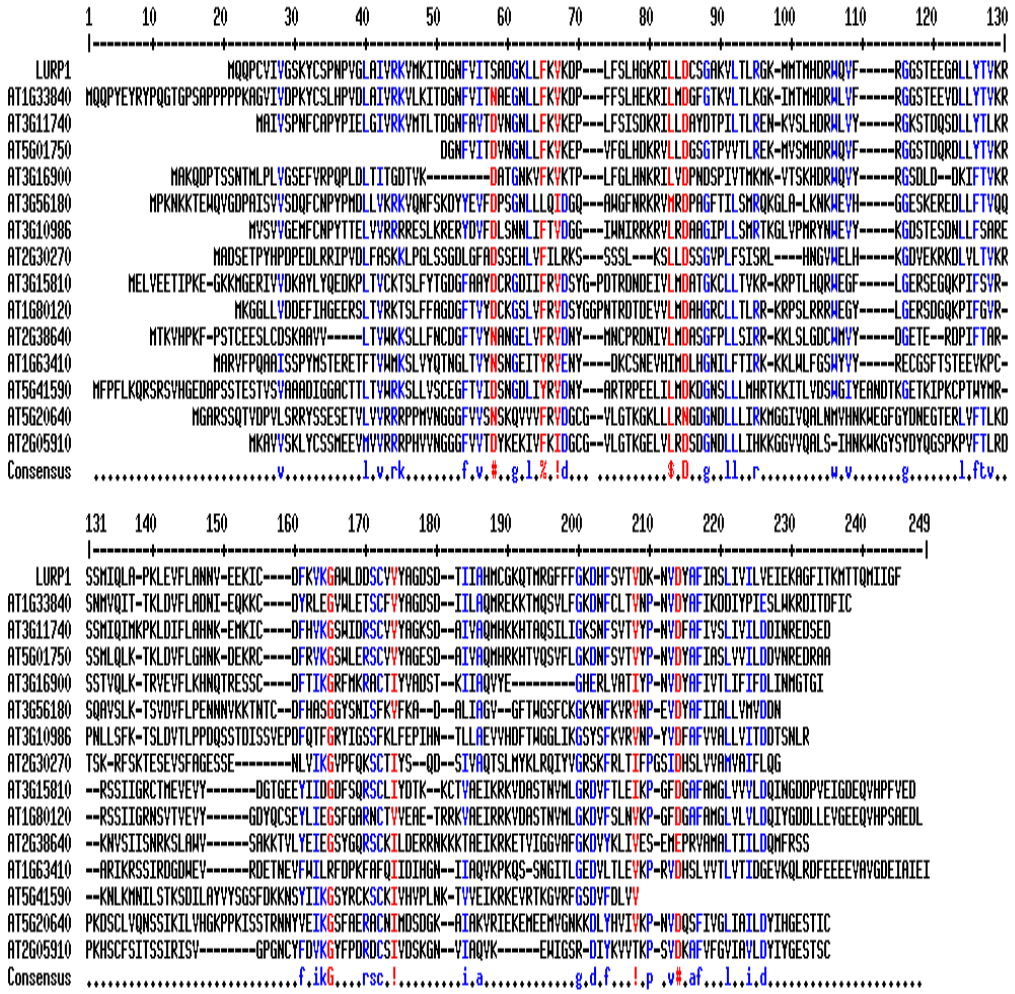

Figure 1.2: The Arabidopsis LOR (LURP one-related) protein family featuring domain of unknown function-DUF567. Amino acid sequence alignment of Arabidopsis LOR family members. High consensus color: Red (at least 90% identity), Low consensus color: Blue (<50% identity) (multalin.toulouse.inra.fr/multalin/) (Corpet 1988).

Human PLSCR1 a known member of the PLSCR family is a multiply palmitoylated lipid bound protein that is normally localized to the plasma membrane where it had originally been suspected to be involved in the transfer of phospholipids across plasma membrane (Zhou, Zhao et al. 1997).PLSCR1 was also found to move in to the nucleus where it acts as a transcription factor by directly transcriptionally regulating inositol 1,4,5-triphosphate receptor type 1 (*IP3R1*) gene expression (Zhou, Ben-Efraim et al. 2005). PLSCR1 was also recently found to interact directly with the CD4 receptor at the cell surface of T lymphocytes and to serve as a receptor of the Secretory Leukocyte Protease Inhibitor (SLPI) involved in the inhibition of HIV-1 transfer and replication (Py, Basmaciogullari et al. 2009). In this study molecular and defense-related roles of LURP1 and LOR1 were explored. I also focused on possible functional similarities of LURP1 and LOR1 to PLSCR1. Results from this study will further help in dissecting the molecular mechanisms underlying the immune systems in both plants and animals.

Results

The *lurp1-2* and *lor1-1* transposon mutants are compromised in different aspects of innate immunity

Arabidopsis lines with insertions in *LURP1* and *LOR1* were obtained from sequence indexed transposon mutant collections (Parinov, Sevugan et al. 1999; Sessions, Burke et al. 2002; Alonso, Stepanova et al. 2003). SGT4080 (*lurp1-2*) in the Landsberg erecta (L*er*) background has a transposon insertion in the second exon whereas GT11546 (*lor1-1*) has a transposon insertion in the first exon downstream from the translation start site (Figure 1.3). Homozygous T3 individuals for the respective insertions were selected by PCR-based genotyping (Alonso, Stepanova et al. 2003) and selfed. Their progeny were used for all the experiments described here. The genomic location for insertion in *lurp1-2* and *lor1-1* was confirmed by sequencing. *LURP1* transcript levels have been shown by microarray analysis to be extremely low in uninfected plants, but show a massive relative up-regulation 48hpi with avirulent *Hpa* (Eulgem, Weigman et al. 2004). It has also been shown by RNA blotting that *LURP1* transcripts are visibly up-regulated 48hpi after avirulent *Hpa* but was not detectable in untreated samples (Knoth and Eulgem 2008). *LOR1* on the other hand, exhibited in microarray experiments (Eulgem, Tsuchiya et al. 2007) strong constitutive expression which remained unchanged at 12, 24 and 48hpi.

The *lurp1-2* mutant was infected with *Hpa*Noco2, which is exclusively recognized by the *R* gene *RPP5* in the Ler background (Slusarenko and Schlaich 2003). *RPP4* in the Col-0 background and *RPP5* in Ler are encoded by orthologous genes and trigger SA-dependent defense mechanisms (van der Biezen, Freddie et al. 2002).

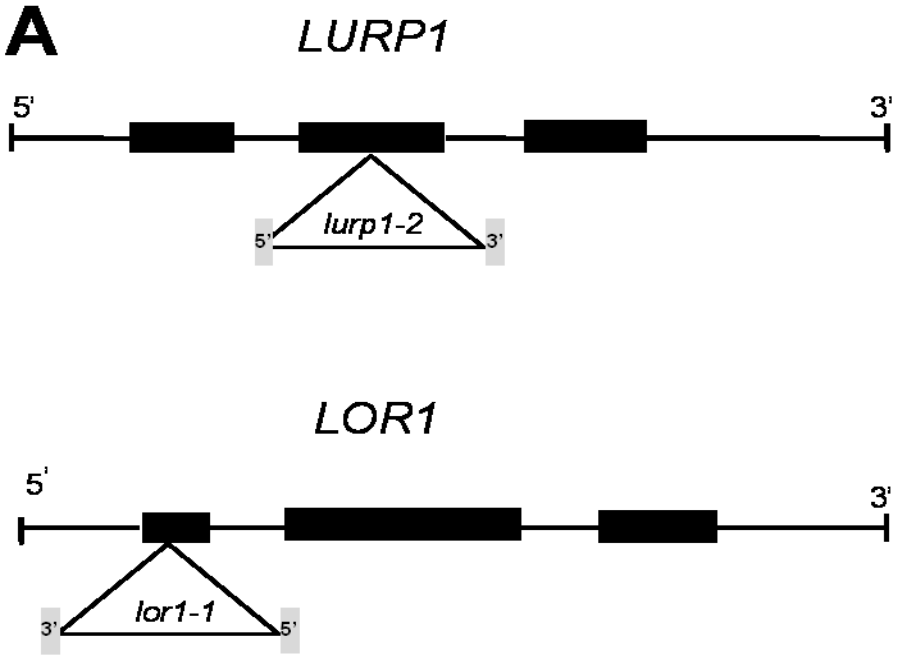

Figure 1.4: Characterization of *lurp1-2* and *lor1-1* transposon insertion mutants. Diagrammatic representation of transposon insertions. *lurp1-2* (SGT4080) and *lor1-1* (GT 11546) homozygous mutant are in Arabidopsis ecotype Landsberg erecta (Ler) background.

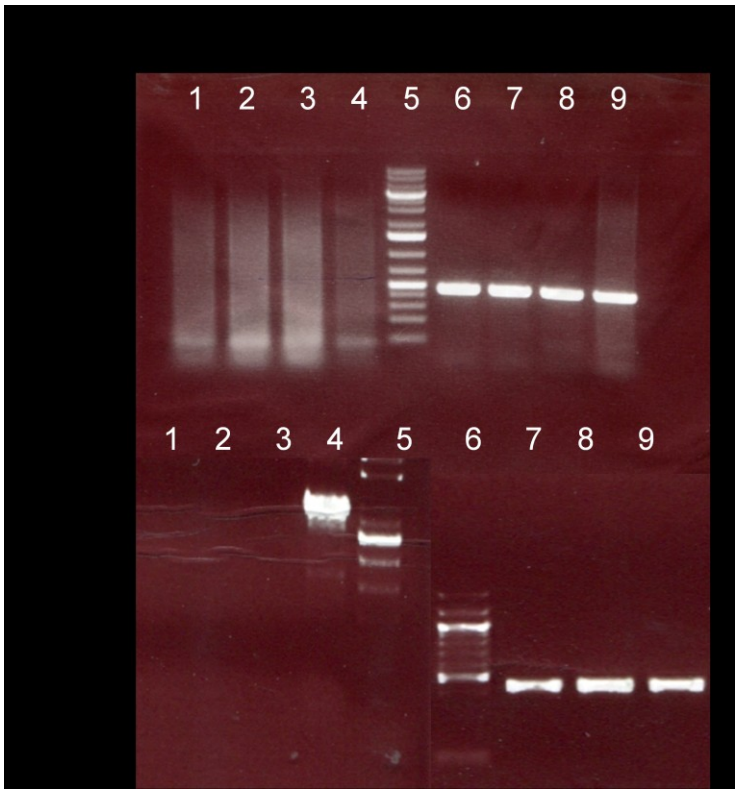

Figure 1.3: Characterization of *lurp1-2* and *lor1-1* transposon insertion mutants.
A: Diagrammatic representation of homozygous transposon insertion mutants *lurp1-2* (SGT4080) and *lor1-1* (GT11546) in the Arabidopsis ecotype Landsberg erecta (Ler) background. Black boxes represent exonic coding sequences. Grey boxes represent the 5' and 3' ends of the transposon insertions.
B: Upper gel representing the transposon mutant *lurp1-2* with the absence of PCR product of 820bp from two gene specific primers from 4 technical replicates (Lane 1-4) .Positive PCR with *lurp1-2* forward and transposon specific primer Ds5'-1 from 4 technical replicates (Lane 6-9) with 563bp band size.1kb plus ladder was used (Lane 5). Lower gel representing no PCR band of 789bp with *lor1-1* forward and reverse primers (Lane 1-3) with 3 technical replicate. Ler was used as positive control with PCR band from *lor1-1* forward and reverse primers (Lane 4) with 789bp band size. Presence of PCR product in *lor1-1* mutant with *lor1-1* forward and Ds3'-3 reverse primer with 4 technical replicate (Lane 7-10) 550bp band size. 1kb ladder was used (Lane 5 and 6).

Basal defense is compromised by mutations in *LURP1* and *LOR1*

Defense-related functions of LURP1 and LOR1 were further examined by determining their roles in basal defense against the virulent *Hpa* isolate *Cala2*. *HpaCala2* is not recognized by any *R*-gene in the Ler background and is, therefore, virulent in this ecotype. One-week old Ler, *lurp1-2* or *lor1-1* seedlings were spray-inoculated with 3 X 10^4 *Hp*Cala2 spores and analyzed 7 dpi. Mutant plants of *lurp1-2* and *lor1-1* exhibited increased numbers of spores per gram fresh tissue as compared to Ler (Figure 1.4). This increase, however, was only clear and significant in the case of *lor1-1*.

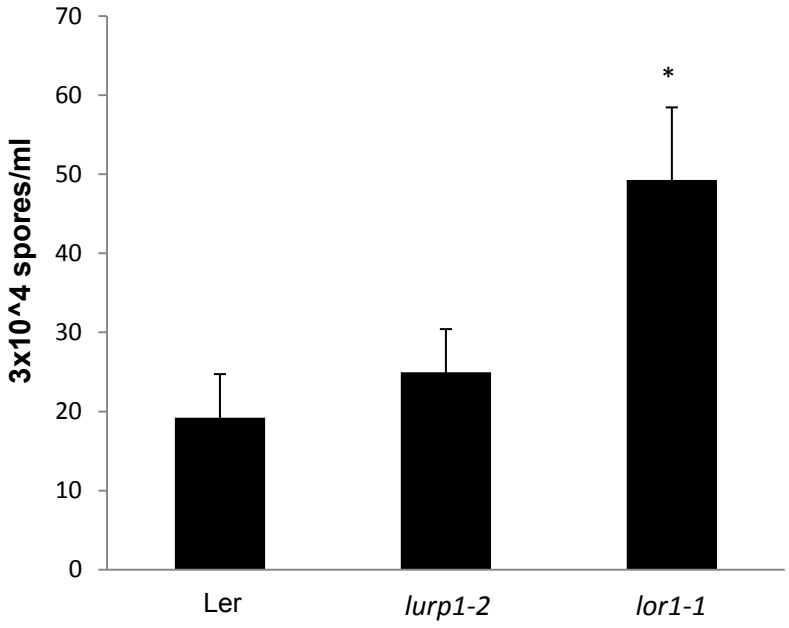

Figure 1.4: Analysis of *lurp1-2* and *lor1-1* during compatible interaction. One-week old Arabidopsis *lurp1-2* and *lor1-1* transposon mutants were sprayed with virulent *Hpa*Cala2 (3x10^4 spores/ ml). Spores were counted 7 days post infection. Significantly different spore numbers from Ler wild type plants (t-test, $p<0.05$) are marked by an asterisk. Error bars represent mean standard error based on at least three independent biological replicates.

Seedlings of *lurp1-2 and lor1-1* infected with *Hpa*Cala2 ($3x10^4$ spores/ml) were also evaluated for hyphal growth (Figure 1.5). Cotyledons were stained with trypan blue 7 days after infection to visualize hyphal growth (Hy) and cell death (HR) responses. Wild-type, Col-0 infected with *Hpa*Cala2, frequently show sites of discrete HR due to recognition of this *Hpa* isolate by the *R*-gene *RPP2*. Ler as well as *lurp1-2, lor1-1* plants which lacks this *R*-gene behaved fully susceptible to *Hpa*Cala2 and exhibited extensive growth of free hyphae (Hy) and sporangiophores (Sp). However, the extent of *Hpa* growth was clearly stronger in *lor1-1* plants compared to Ler and *lurp1-2* plants. Thus *LOR1*, but not *LURP1* appears to be required for basal defense of Arabidopsis to *Hpa*Cala2.

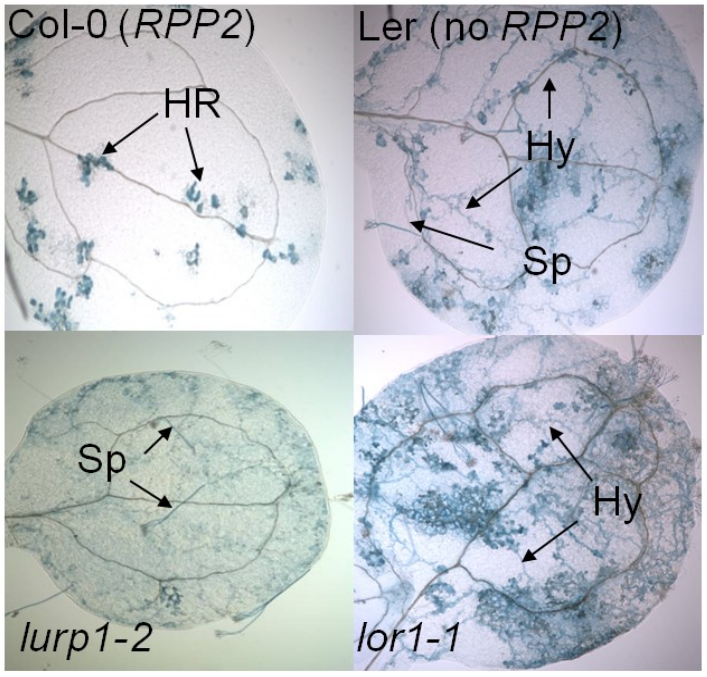

Figure 1.5: Arabidopsis Col-0, Ler, *lurp1-2* and *lor1-1* infected with *Hpa*Cala2. Two weeks old Arabidopsis cotyledons were stained with trypan blue 7 days post infection (dpi) with $3x10^4$ spores of *Hpa*Cala2. Col-0 showed sites of discrete sites of HR cell death (HR). Ler, *lurp1-2* and *lor1* infected cotyledons exhibit sporangiophores (Sp), dense networks of hyphae (Hy) and no signs of HR cell death.

Mutations in *LURP1-2,* but not *LOR1* compromise function of *RPP5*

As previously shown, mutation of *LURP1-2* affects *RPP5*-mediated resistance to *Hpa*Noco2 (Knoth and Eulgem 2008). I repeated these experiments for additional *Hpa* defense assays. Resistant wild type plants of the Ler ecotype predominantly showed discrete HR sites in response to *Hpa*Noco2 infection and did not allow for the development of sporangiophores however *lurp1-2* showed reduction in the development of discrete HR sites. Mutant *lurp1-2* also showed typical hyphae growth surrounded by trail of necrotic plant cells (Knoth and Eulgem 2008). Such necrosis is due to partially reduced disease resistance (Torres, Dangl et al. 2002). Mutant *lurp1-2* plants were clearly compromised in *RPP5*-mediated resistance by showing significantly enhanced numbers of sporangiophore per seedling compared to the Ler control. Col-0 plants, which are strongly susceptible to *Hpa*Noco2, due to the absence of an *Hpa*Noco2 recognition *R*-gene, exhibited extensive formation of sporangiophore (Figure 1.6).

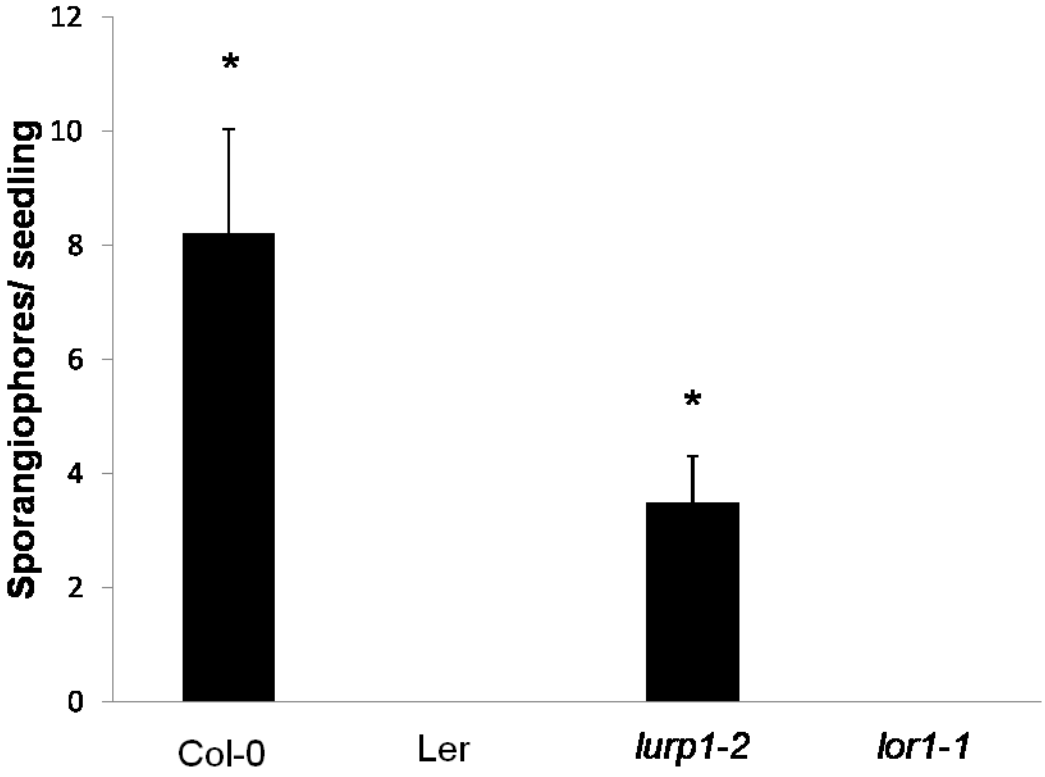

Figure 1.6: Analysis of *lurp1-2* and *lor1-1* during the incompatible *Hpa*Noco*2* interaction. Sporangiophores were counted on two-week-old Arabidopsis seedlings 7 days post infection (dpi) after spray-inoculation with $3x10^4$ spores/ml of the avirulent *Hpa*Noco2 isolate. Error bars represent standard errors calculated from three individual experiments. Significantly different sporangiopore numbers from Ler wild type plants (t-test, $p<0.05$) are marked by an asterisk.

Contrary to the *lurp1-2* mutant, the *lor1-1* mutant exhibited wild type HR development in response to *Hp*Noco2 and absence of sprongiophore formation (Figure 1.7). These data confirmed a role for *LURP1* in *RPP5*-mediated gene-for-gene resistance to avirulant *Hpa*Noco2, while *LOR1* appears not to play an important role in this defense pathway.

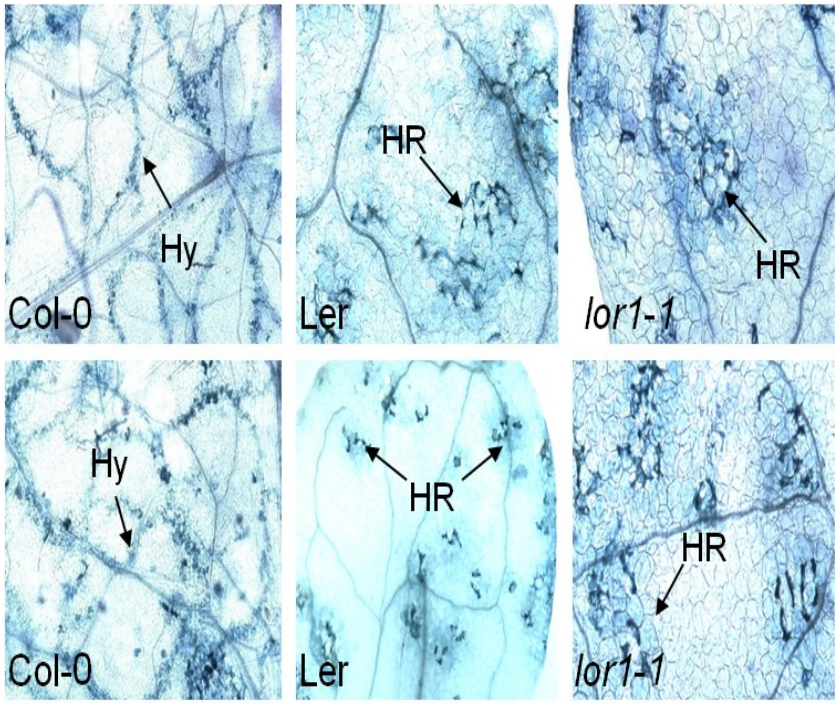

Figure 1.7: Arabidopsis Col-0, Ler and *lor1-1* infected with *Hpa*Noco2.
Two-weeks-old Arabidopsis cotyledons were stained with trypan blue 7 days post infection (dpi) with $3x10^4$ spores of *Hpa*Noco2. Col-0 infected cotyledons exhibit dense networks of hyphae (Hy) whereas Ler and *lor1-1* show sites of discrete sites of HR cell death (HR).

Generation of chimeric *LOR* expression constructs

Hpa infection assays clearly confirmed a defense-related role for *LURP1* and established *LOR1* as a new component of the plant immune system. Furthermore it was found that *LURP1* has a pronounced role in *RPP5*-mediated disease resistance and seems to be less important for basal defense. Conversely, *LOR1* plays a strong role in basal defense, but appears not to contribute to *RPP5*-mediated immunity. An amino acid sequence alignment of LOR family members showed only subtle differences between LURP1 and LOR1 (Figure 1.2). Therefore, difference in the defense-related roles of LURP1 and LOR1 may be attributable to the difference in their transcriptional regulation. While *LURP1* transcript levels are extremely low in the absence of pathogen infection, they dramatically increase in response to *R*-mediated *Hpa* recognition. Transcript levels of this gene also increase during basal defense. However, this up-regulation is delayed (Eulgem, Weigman et al. 2004; Eulgem and Somssich 2007; Knoth, Ringler et al. 2007). *LOR1* transcript levels, in contrast, are unaffected by *Hpa* recognition and are constitutively at high levels. Thus, a possible explanation for the functional differences between *LURP1* and *LOR1* may be that a general *LOR*-dependent defense activity is important for providing weak but constitutive protection during compatible interactions, due to the constant presence of LOR1. During incompatible interactions, this general *LOR*-dependent defense function is enhanced, due to the dramatic up-regulation of *LURP1*. Thus, the constitutive activity of *LOR1* may be insufficient to provide

strong immunity during incompatible interactions. To test if indeed differences in their transcriptional regulation are mainly responsible for the functional differences between *LURP1* and *LOR1*, I constructed chimeric expression constructs for both genes. A stretch of the *LURP1* upstream sequence that was previously found to be sufficient for the strong *R*-mediated up-regulation of this gene (Knoth and Eulgem 2008), was translationally fused to the LOR coding sequence and a *LOR1* promoter stretch was translationally fused to the LURP1 coding sequence (Figure 1.8 & 1.9).

AAATTTTGTTTCTATTTGAGTATGCAGAAAAATACAAGTACTATATATATATAAAACACTCGTTTTTTAAGTCATGTAAAT
TATCATTAATGAATGAAGGAATTTGTAAGTTACCAAAAAATTGCTTAACCAGCGAAGGGGATAAACCTTGCCAAAAAAG
AATAAAGAACAAGGATATATTTTGCATGTCAGAGATTAGGTATTACCGTATTAGCAAGCCAAAGCTGCATTAGGGAAAT
CCCAATAAGGTTTTTACGTTCATGATTTCTTGCACGACTATGATCTCGTCAATATGGAATTATACAACGTCTGCTATTT
GAATATGGCTGCCTTAGGCTCTCTCAAATCTTATCTATACTGCATATTCGATTGGACGAAATGGAACTATGATTATTTG
CCAATTTTGTCCATACATCAATATTTTCAAAGTATTCCCTGGAATTGTTTCTTATTGGTGTTAAGATTTTGTCATTGTT
GAAGTACTTTTGATGTTATTTTTATACGTAATTAATTTAAATGAAGTGAAAAACAAGAAATGAAACTTTTTTGTTTTTA
TTCAATTGTTTCTTTTGTTAGTTTTTCTATTTCTAAAATCTTATTTTTCATACTTTGGAATATTTTTTAGTTTTTTTGT
TCATCAAATATCCTATAATTTATTTAAACTTTTTAACCTAATGTAATAATACAAAATAAATTAGACGGTATGCTGTTGT
AAATGTTACAGAATTCAGATCAAATTTAATCAACTATTTTTAAAAAATTAACAAGTAAGTCATGAAGAACATATAGTCT
TTTTATGTTTAATGCATGTATCTACTATCTCTCCCACCTGCTTTATATATGACGCCACGTCTATGACGTAGGTCAAAAC
AGTAACATTAGATACTATACTATCAGACACACATTCATATGGCCAGAGATGACCAACCACTGGAGATGCTCTAAGAAAT
TTATCCTCGTAGGCTTTTCTCTTTTAAAAGTCAACATATTGGAGCCAGATGTTTATTTTATAAATACTAAGAACCACAC
CAAAGAAAAAACATTTCAAAGCAAAACAAAAGAGAGAGAGTGTATCCATTTTTGGAGGGGAAAACAAAG

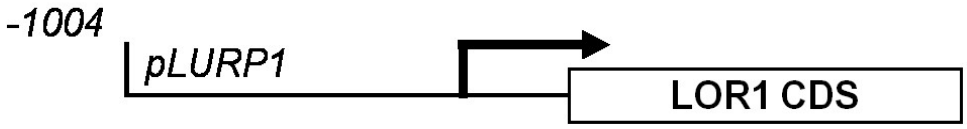

Figure 1.8: *LURP1-2* Promoter sequence for *p*$^{-1004}$*LURP1::LOR1.* Schematic representation of T-DNA construct with the *LURP1* promoter up to -1004 bp fused to the *LOR1* coding sequence. Blue: TATA box, Red: Skn-1 motif, Light green: Box-4 involved in light responsiveness, Purple: HES, Orange: GCN4 motif, Green: CAAT box, Pink: G-box, Dark Red: CGTCA motif involved in MeJA response, Grey: ATCT motif, Light grey: TC rich repeat, Violet: MRE Box, Yellow: Unnamed box, Black: TGACG-motif, Olive green: GAG-motif, Light blue: ERE, Aqua: ABRE, Tan: TATCCAT motif, W-Box: Underline (PlantCARE, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Two constructs with promoter ranging from -1004 up to base pair (bp) upstream of the translation start site (*LURP1*$^{-1004}$) and from -1093 to base pair (bp) upstream of the translation start site(*LOR1*$^{-1093}$) fused to LOR1 and LURP1 coding sequence respectively were created. The *p*$^{-1004}$*LURP1::LOR1* and *p*$^{-1083}$*LOR1::LURP1* constructs were each transformed into both *lurp1-2* and *lor1-1* mutant backgrounds to examine their role in plant disease resistance.

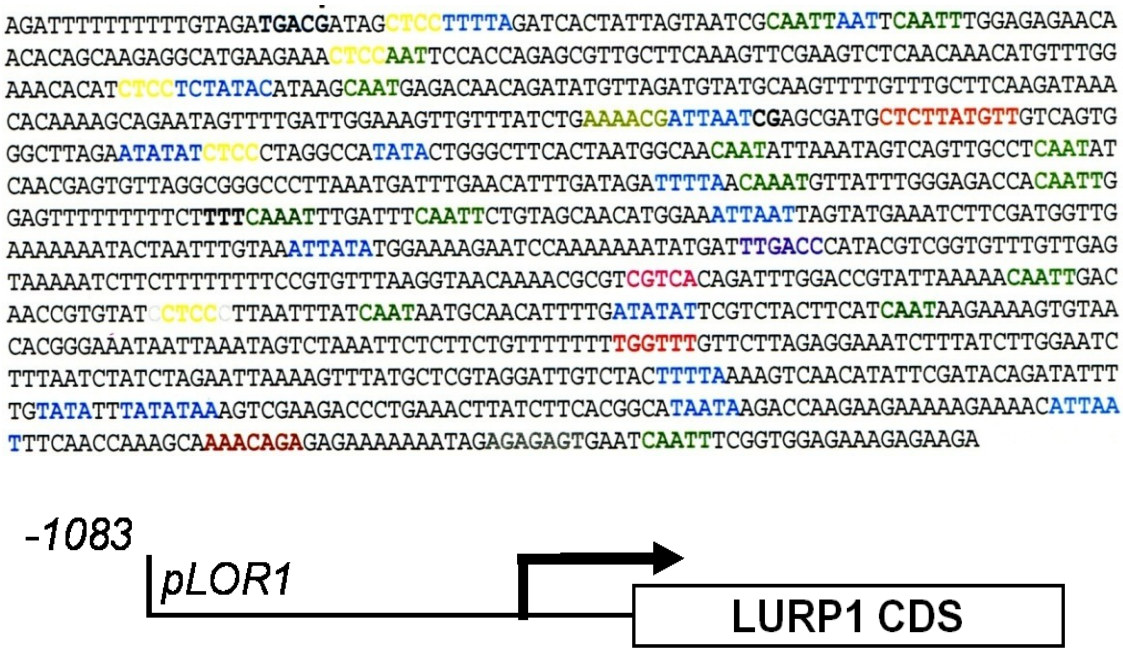

Figure 1.9: *LOR1-1* promoter sequence for *p*$^{-1083}$*LOR1::LURP1*. Schematic representation of T-DNA construct with *LOR1* promoter sequence of -1083 bp fused to the *LURP1* coding sequence. Dark blue: cis-element involved in MeJA response, Yellow: Unnamed box, Blue: TATA box, Green: CAAT-Box, Olive green: ACE, Black: Box-I, Light gray: Sp1, Pink: CGTCA Box, Red: ARE Box, Dark Red: GARE-motif. (PlantCARE, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)

Arabidopsis lines stably transformed with *p*$^{-1004}$*LURP1::LOR1* or *p*$^{-1083}$*LOR1::LURP1* and their status are listed in table 1.2. The T_0 plants were grown and seeds were collected after floral dip-mediated transformation (Clough and Bent 1998). The T_1 plants were grown on ½ strength MS medium with 50mg/ml hygromycin.

No.	Promoter constructs	Background	Status
1	*p*$^{-1004}$*LURP1::LOR1*	*lurp1-2*	T_1
2	*p*$^{-1004}$*LURP1::LOR1*	*lor1-1*	T_0
3	*p*$^{-1083}$*LOR1::LURP1*	*lurp1-2*	T_1
4	*p*$^{-1083}$*LOR1::LURP1*	*lor1-1*	T_1

Table 1.2: The *p*$^{-1004}$*LURP1::LOR1* and *p*$^{-1083}$*LOR1::LURP1* constructs transformed in to *lurp1-2* and *lor1-1* mutant backgrounds.

GFP-LOR1 and GFP-LURP1 localized to plasma membrane and nucleus

LURP1 and LOR1 belong to a fifteen member protein family in Arabidopsis termed LOR (LURP one-related) family featuring a domain of unknown function-(DUF567) (Figure 1.2). The crystal structure of LOR1 (At5g01750) has been solved as part of the structural genomic project CESG (http://www.uwstructuralgenomics.org/). The three dimensional structure of the Arabidopsis LOR1 protein was found to resemble human Phospholipid scramblase1 (PLSCR1) (Bateman, Finn et al. 2009). PLSCR1 is known to be imported into the nucleus in response to cytokinin where it acts as a transcription factor (Ben-Efraim, Zhou et al. 2004). A known target gene of PLSCR1 is *IP3R1*. (Zhou, Ben-Efraim et al. 2005). An alignment of human PLSCR1 with the LOR1 from *A. thaliana* (Soding 2005) highlighted conserved sequence features of the PLSCR family. As PLSCR1, PLSCR3 and PLSCR5 were found to be related to LOR1, the latter was used to model possible structural features of PLSCR family members. The structure of LOR1 is a 12-stranded *β*-barrel that encloses a central C-terminal *α*-helix. LOR1 was also found to show structural similarity with the C-terminal domain of the Tubby protein (Boggon, Shan et al. 1999).

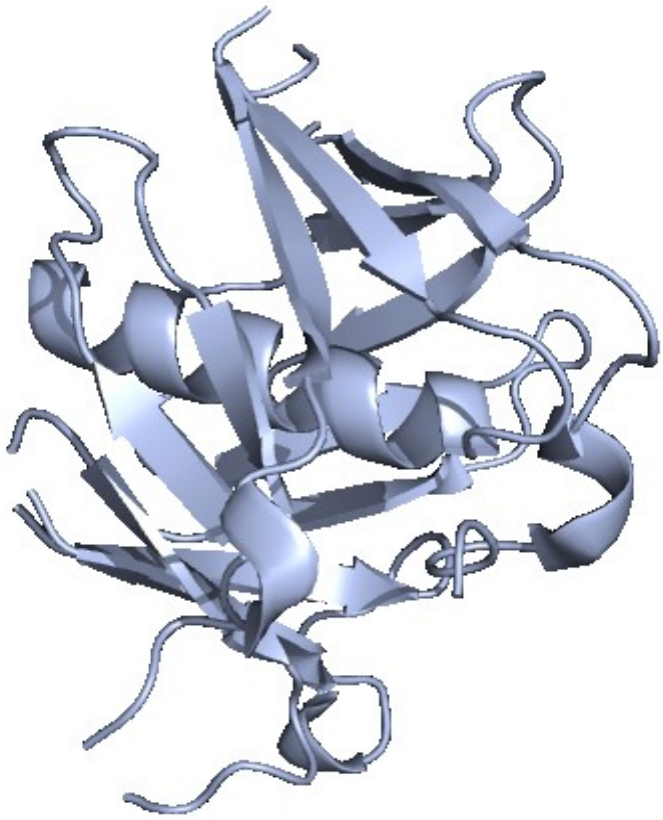

Figure 1.10: The PLSCR family of proteins is related to LOR family. LOR1 provides the first structural model for the Tubby/PLSCR/LOR1 super family (From Bateman, Finn et al. 2009).

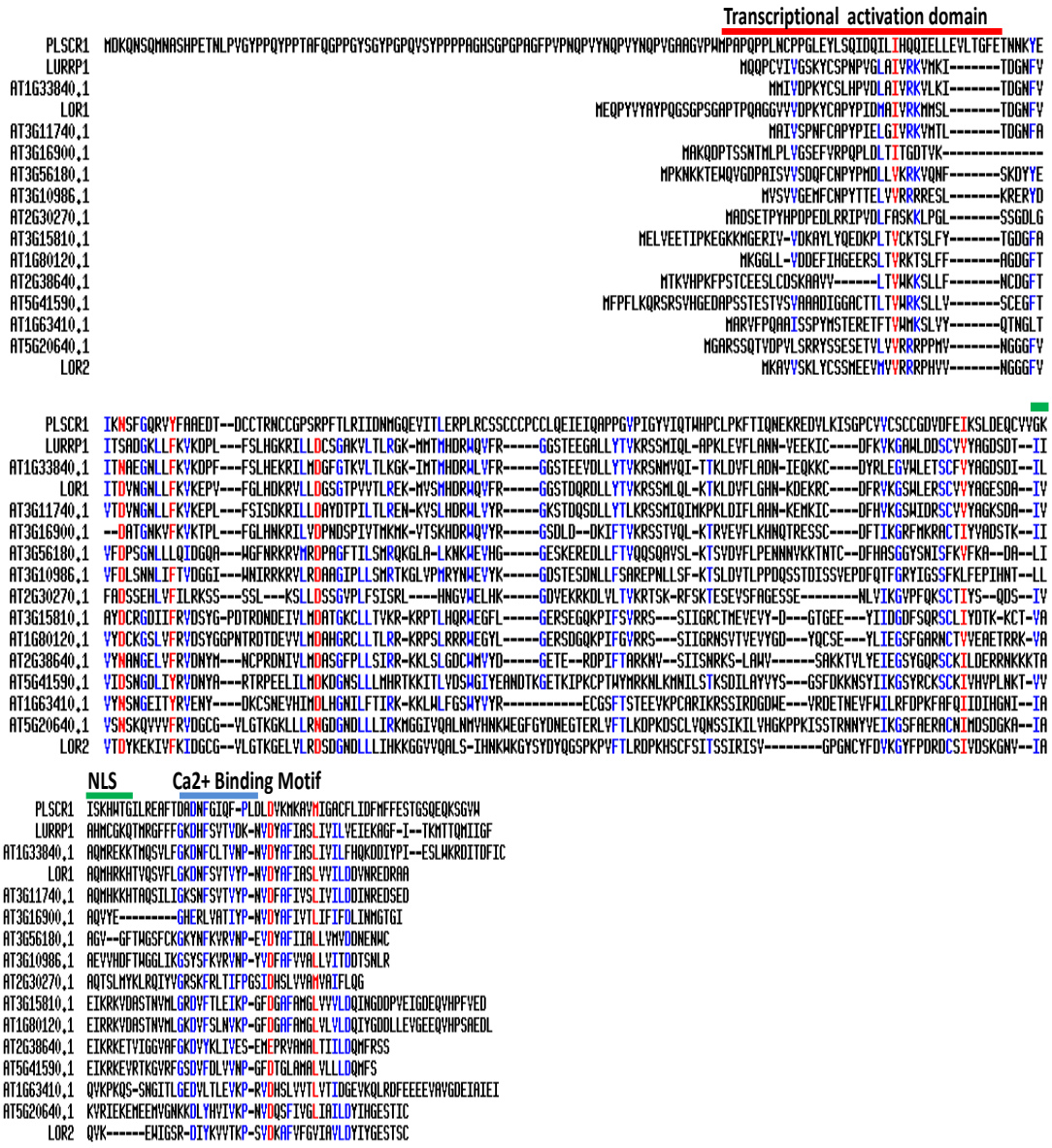

Figure1.11: Amino acid sequence alignment of the Arabidopsis LOR family with human PLSCR1. The transcriptional activation domain (red), nuclear localization signal (NLS, green) and Ca^{+2} binding motif (blue) of PLSCR1 are marked by colored bars (Corpet 1988).

The PLSCR protein family, thus, was found structurally related to the LOR family and LOR1 provided the first structural model for this family solved by X-ray crystallography (Figure 1.10; Bateman, Finn et al. 2009). The experimentally determined PLSCR1 transcriptional activation domain, non-classical nuclear localization signal and Ca^{2+}-binding motif along with its site binding to the promoter of the inositol 1,4,5-trisphosphate receptor are highlighted The transcriptional activation domain (red), nuclear localization signal (NLS, green) and Ca^{+2} binding motif (blue) of PLSCR1 are marked by colored bars (Figure 1.11; Bateman, Finn et al. 2009). As PLSCR1 has been suggested to act as a partially nuclear-localized transcription factor (Zhou, Ben-Efraim et al. 2005), I designed experiments to determine the subcellular localization of LURP1 and LOR1 through GFP fusions of these proteins (Figure 1.12).

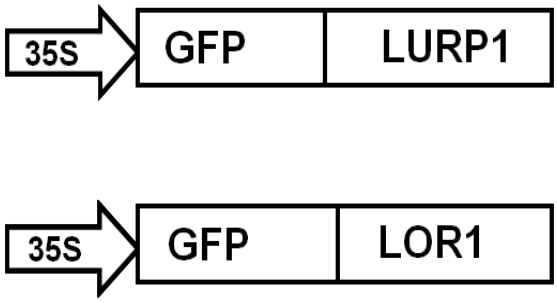

Figure 1.12: Schematic presentation of binary *CaMV35S:GFP-LURP1* and *CaMV35S:GFP-LOR1* expression constructs. 35S: Two directly repeated copies of the full length *CaMV35S* promoter ((Curtis, et al. 2003). The *CaMV35S:GFP-LURP1* and *CaMV35S:GFP-LOR1* constructs were transformed into Arabidopsis and the transgenic Arabidopsis seedlings homozygous for GFP-LURP1 and GFP-LOR1 were analyzed for fluorescence.

The coding regions of LURP1 and LOR1 were fused to that of green fluorescent protein (GFP) by in the binary *CaMV35S:GFP-LURP1* and *CaMV35S:GFP-LOR1* expression constructs. *CaMV35S:GFP-LURP1* and *CaMV35S:GFP-LOR1* were stably transformed in to *lurp1-2* and *lor1-1* mutant

background. Homozygous *CaMV35S:GFP-LURP1* and *CaMV35S:GFP-LOR1* lines were selected based on their segregation on ½ strength MS medium with 50mg/ml hygromycin and confirmed through PCR. It was found that both GFP-LURP1 and GFP-LOR1 were localized to the plasma membrane and other parts of the cells including nuclei (Figure 1.13 & 1.14).

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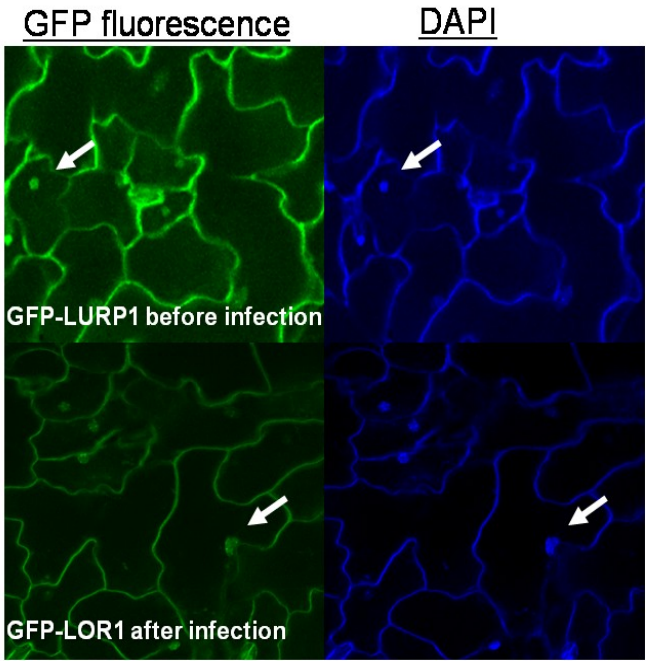

Figure 1:13: GFP-LURP1 is localized in to the nucleus and other parts of the cell. Sub-cellular localization of GFP-LURP1 in transgenic Arabidopsis plants. Fluorescence was observed by confocal microscopy 48 hrs in the absence of *Hpa*Cala2 or after infection of one weeks old Arabidopsis seedlings 2 days after spray-inoculation with $3x10^4$ spores/ml of the virulent *Hpa*Cala2. DAPI staining was performed to specify locations of Nuclei. The arrows point to the nuclei of a leaf epidermal cell.

Sub-cellular localization of GFP-LURP1 and GFP-LOR1 in transgenic Arabidopsis plants was observed by confocal microscopy 48 hrs in the absence of *Hpa*Cala2 or after infection of one week old Arabidopsis seedlings 2 days after spray-inoculation with $3x10^4$ spores/ml of the virulent *Hpa*Cala2. DAPI staining was performed to specify locations of nuclei in GFP-LURP1 and GFP-LOR1

transgenic plants. It was also found that both LURP1 and LOR1 maintained the same localization before and after infection with virulent *Hpa*Cala2.

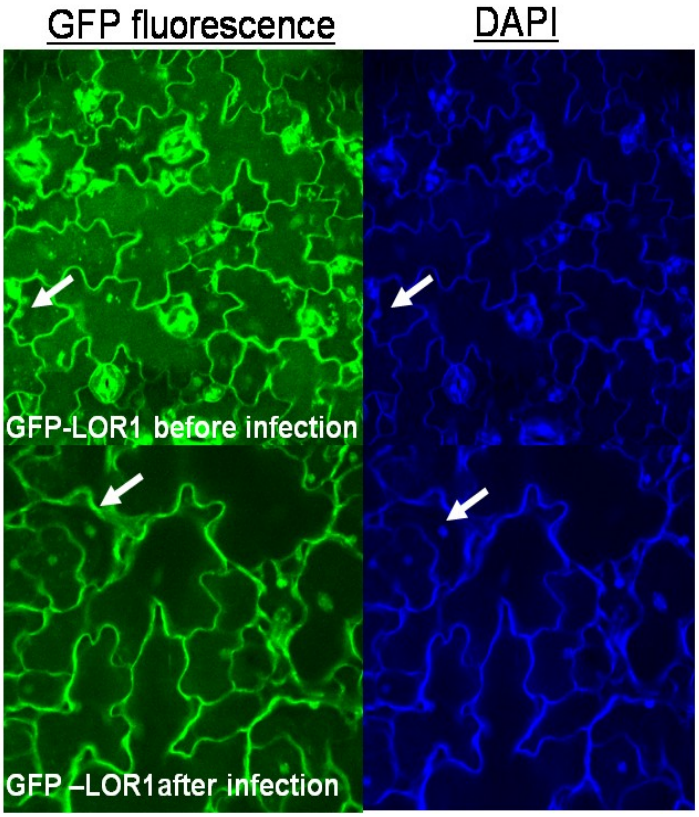

Figure 1.14: GFP-LOR1 is localized in to the nucleus and other parts of the cell. Sub cellular localization of GFP-LOR1 in transgenic Arabidopsis plants. *GFP-LOR1* was stably transformed in to Arabidopsis. The fluorescence was observed by confocal microscopy 48 hrs after infection of one weeks old Arabidopsis seedlings 2 days after spray inoculation with $3x10^4$ spores/ml of the virulent *Hpa*Cala2. DAPI staining was performed to specify locations of nuclei. The arrow showing the nuclei of aleaf epidermal cell.

GFP-LURP1 and GFP-LOR1 restore wild type resistance during compatible and incompatible interaction

The GFP-LURP1 and GFP-LOR1 lines were also tested for *lurp1-2* and *lor1-1* complementation by *Hpa* defense assays. GFP-LURP1 and GFP-LOR1 expressing lines evaluated for basal defense showed the same response as wild ecotype Ler when plants were sprayed with virulent *Hp*Cala2 ($3x10^4$ spores/ml)

(Figure 1.15). Thus, basal defense was fully restored in the *lor1-1* background by expression of GFP-LOR1 confirming that loss of basal defense in this mutant is due to lack of proper *LOR1* function. Furthermore these results showed that GFP-LOR1 is functional in mediating basal defense. Thus the subcellular localization of GFP-LOR1 is likely to accurately mimic that of wild type LOR1.

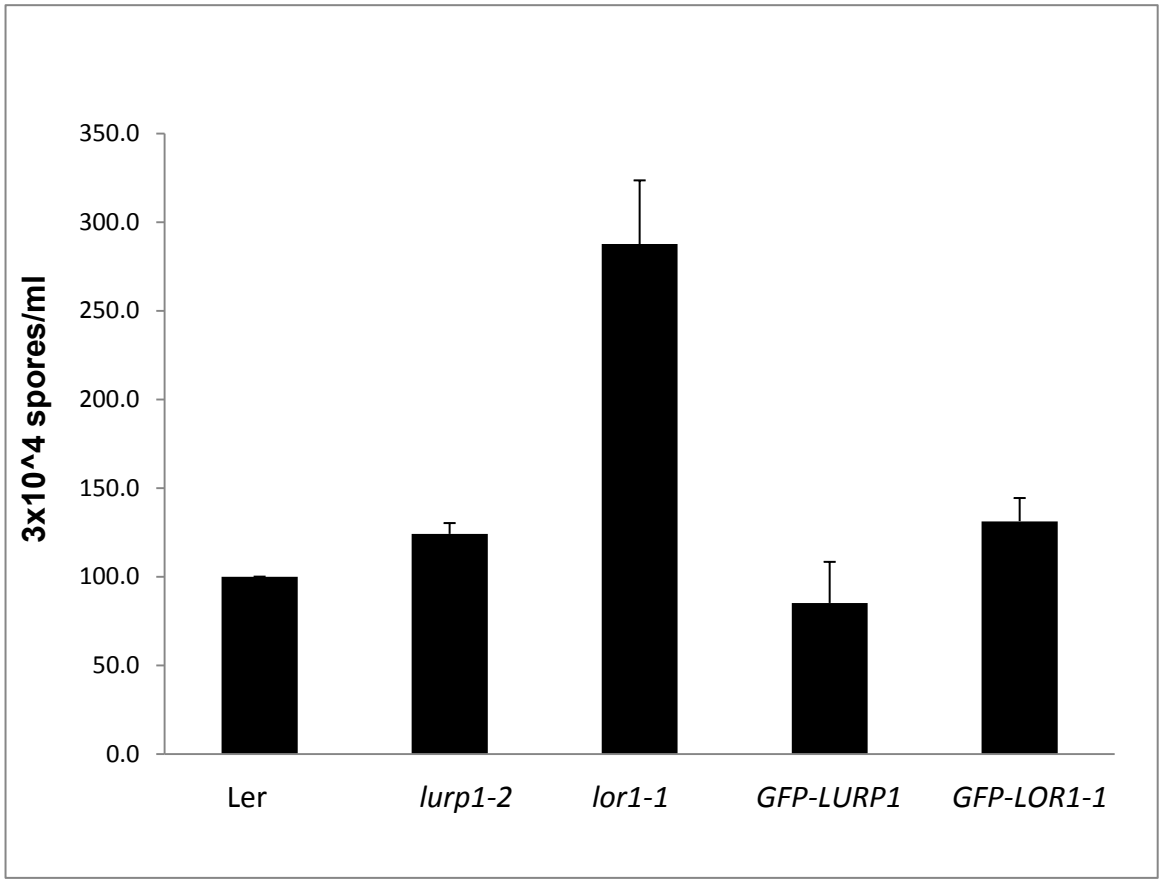

Figure 1.15: Expression of *GFP-LURP1* or *GFP-LOR1* in the *lurp1-2* or *lor1-1* mutants restores wild type basal defense. Complementation of the *lurp1-2* or *lor1-1* mutants with *GFP-LURP1* or *GFP-LOR1* respectively, resulted in spore counts comparable to wild type Ler plants. Ler**,** *lurp1-2*, *lor1-1, GFP-LURP1* and *GFP-LOR1* plants were sprayed with virulent *Hpa*Cala2 ($3x10^4$ spores/ml). Spores were counted 7dpi. Error bars represent standard error calculated from three individual experiments. Spore numbers significantly different from those in Ler are marked by an asterisk (t-test, $p<0.05$).

Plants were also spray-infected with avirulent *Hp*Noco2 ($3x10^4$ spores/ml) and sporangiophores were counted 7 dpi during incompatible interaction (Figure 1.16). Clearly *RPP5*-mediated gene-for-gene resistance was restored in the *lurp1-2* mutant background by expression of GFP-LURP1. This result confirmed that loss of RPP5-mediated immunity in *lurp1-2* plants is due to lack of proper *LURP1* function. Furthermore these results showed that GFP-LURP1 is functional in mediating resistance to *Hpa*Noco2. Thus the subcellular localization of GFP-LURP1 is likely to accurately mimic that of wild type LURP1. It was overall concluded that both GFP-LURP1 and GFP-LOR1 were localized to the plasma membrane, the nucleus and possibly other parts of the cell before and after *Hpa* infection.

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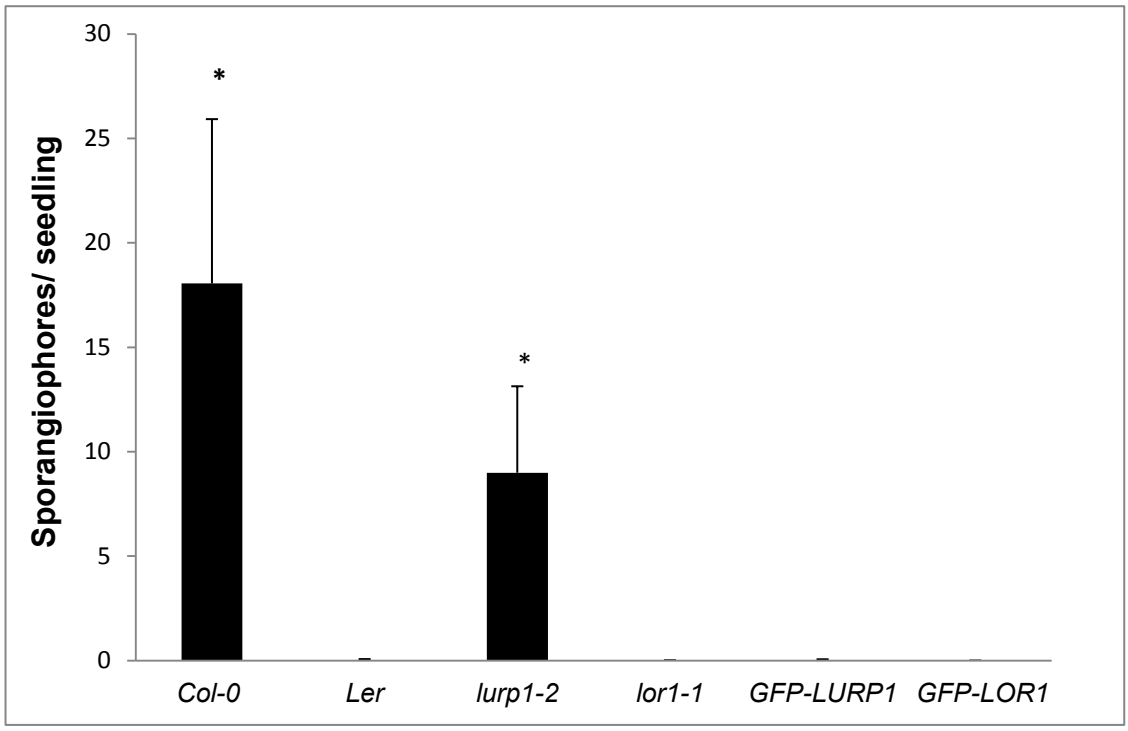

Figure 1.16: Incompatible interaction of GFP-LURP1and GFP-LOR in response to *Hpa*Noco2.
Mutant *lurp1-2* and *lor1-1* lines complemented by GFP-LURP1 and GFP-LOR1 showed spores counts comparable to wild type control Arabidopsis Ler ecotype. Ler, *lurp1-2, lor1-1*, *GFP-LURP1* and *GFP-LOR1* were sprayed with avirulent *Hpa*Noco2 ($3x10^4$ spores/ml). Spores were counted 7 days post infection. Error bars represent standard error calculated from three separate experiments. Spore numbers significantly different from those in Ler are marked by an asterisk (t-test, $p<0.05$).

Design of a Yeast Two-Hybrid screen for LOR1 and LURP1 interacting proteins

The molecular roles of LOR1 and LURP1 are enigmatic at this point. Known molecular functions of proteins interacting with LOR1 or LURP1 may shed light on details of the roles of these two LOR family members. Therefore, a yeast two-hybrid screen system was designed to screen for proteins interacting with LOR1 or LURP1. Yeast expression vectors encoding LOR1 and LURP1 fusions to the GAL4 DNA binding domain (BD) were constructed and transformed into the yeast strain AH109 (Figure 1.17). Full length LURP1 and LOR1 fused to BD proved not to activate transcription in yeast in the absence of a prey protein and were used as bait proteins for performing the screen. For interaction screens, LURP1-BD or LOR1-BD expressing yeast strains were transformed with a cDNA library representing pooled RNAs from 2-week-old Col-0 seedlings either untreated or infected with one of several *Hpa* isolates in the HybriZAP-2.1 vector (Stratagene) (Tsuchiya and Eulgem 2010). Transformants were screened on –TRP-HIS-ADE-LEU selective media (Clontech, http://www.clontech.com/) for the activation of *HIS* and *ADE* markers, which reveals positive bait-prey interactions. Preliminary screening of 2.3 x 10^5 library clones did not result in the identification of candidates (Figure 1.18). This screen will be continued by other members of our laboratory.

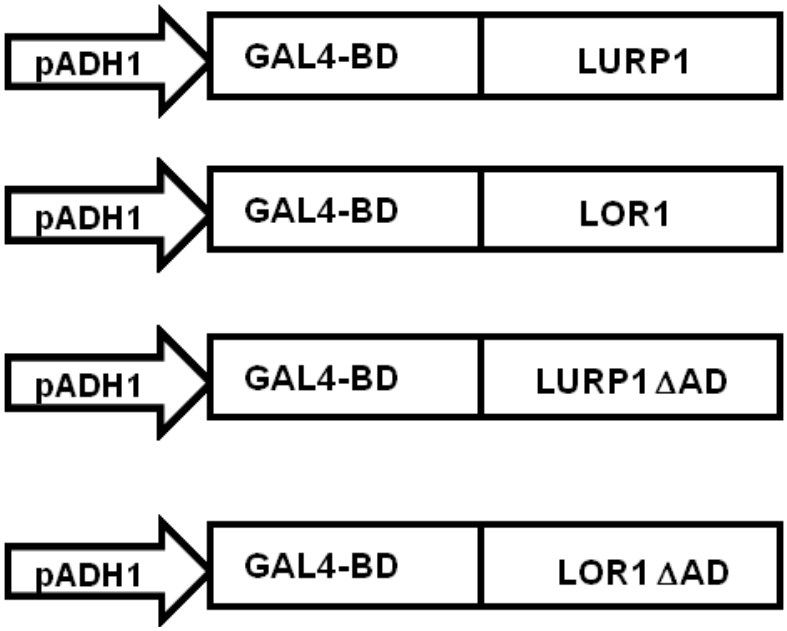

Figure 1.17: Schematic representation of LURP1 and LOR1-BD constructs. Full length LURP1 and LOR1 were used as bait proteins for interaction with the kAD-library encoded prey proteins. LURP1 and LOR1-BD constructs lacking the putative activation domain (AD) of LOR family members were also constructed (LURP1 ΔAD; Δ LOR1 AD). Yeast cells expressing LURP1-BD or LURP1-BD Δ AD, LOR1-BD or LOR1-BD Δ AD grown on synthetic dropout (SD) media lacking tryptophan, leucine, histidine and adenine will indicate interaction between bait constructs and AD-library encoded prey proteins leading to the activation of the HIS3 and ADE2 reporter genes.

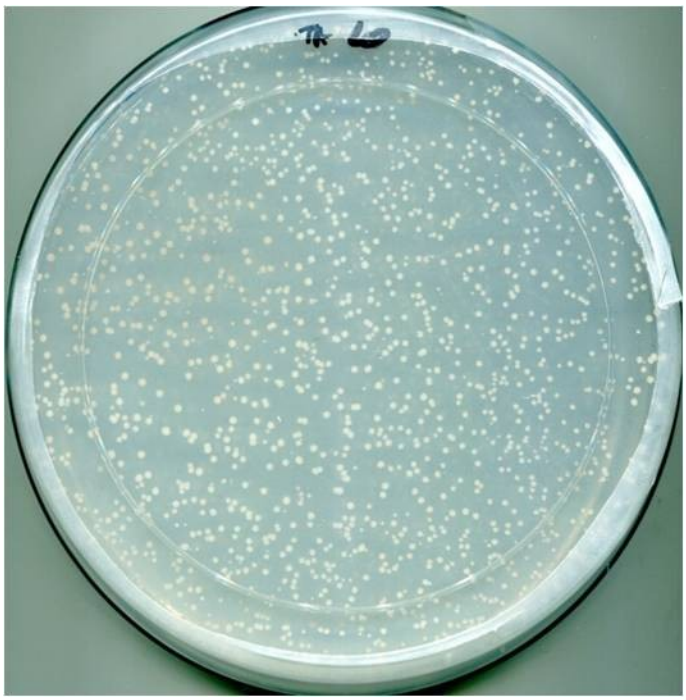

Figure 1.18: Yeast cells expressing AD Hybri-ZAP (Stratagene) AD cDNA library along with LOR1 GAL4-BD fusion protein. Yeast cells were grown on synthetic dropout (SD) agar plates lacking tryptophan and leucine indicating cDNA library AD plasmid and bait LOR1-BD incorporated in to yeast AH109 cells.

Discussion

Transcripts of *LURP* (Late up-regulated in response to *Hpa* recognition) genes exhibit a pronounced increase after *Hpa* recognition by the *R* genes *RPP4* or *RPP7. RPP4* mediates *PAD4* and SA-dependent resistance to the *Hpa* *Hpa*Emoy2 whereas *RPP7* triggers resistance to the *Hpa*Hiks1independently of *PAD4* and SA (Eulgem, Weigman et al. 2004). *LURP1* of this gene cluster is important for SA-dependent defense pathways mediating *R*-triggered immunity and basal defense against *Hpa* (Knoth, Ringler et al. 2007). The *lurp1-2* transposon mutant showed defense-related effects indicating a role of *LURP1* during both compatible and incompatible interactions against the *Hpa* Cala2 and Noco2 isolates respectively. In *lurp1*-2 mutants basal defense as well as *RPP5*-mediated disease resistance were reduced, but not completely abolished. Due to the partial nature of this phenotype, the significance of *LURP1* in defense is not fully clear.

LURP1 is a member of a fifteen members Arabidopsis protein family, we termed LOR (LURP-one related). LOR members are defined by the presence of a conserved DUF567 (domain of unknown function 567). Due to their structural similarity, LURP1 and other LOR proteins may have partially overlapping or redundant biological roles. Of the LOR family members only *LOR1* shows strong constitutive expression based on microarray data, whereas transcripts of other family members are not reliably detectable by microarrays. *LURP1* is the only family member showing a transcriptional induction in response to *Hpa*.

My experiments with a *lor1-1* transposon mutant line revealed a significant role of *LOR1* in basal defense against *Hpa*Cala2, but not in *RPP5*-mediated immunity against *Hpa*Noco2. Conversely, analysis of the *lurp1-2* transposon *mutant* indicated a clear role of *LURP1* in *RPP5* mediated immunity against *Hpa*Noco2, but not basal defense against *Hpa*Cala2. The latter observation is in conflict with results reported previously (Knoth and Eulgem 2008). While previously a significant reduction of basal defense against *Hpa*Cala2 was observed in *lurp1-2*, I could not fully reproduce this finding. In my experiments I only observed a statistically insignificant trend of this mutant to allow for a slightly enhanced production of *Hpa*Cala2 spores (Figure 1.4). A possible explanation for this discrepancy is that, since the experiments described by Knoth & Eulgem (2008), the pathogenicity of the *Hpa*Cala2 culture maintained in our lab has decreased. We observed similar effects with other *Hpa* cultures before. A likely reason for this is that, due to the continuous propagation of *Hpa* on highly susceptible Arabidopsis lines, *Hpa* alleles contributing to pathogenicity are lost.

Despite this apparent discrepancy, we can clearly conclude that *LURP1* and *LOR1* differ sharply in their roles during *Hpa* defense. While *LURP1* appears to be required for both basal and *RPP5*-mediated immunity against *Hpa*, *LOR1* does only contribute to *Hpa* basal defense. This observation is interesting because the LURP1 and LOR1 proteins are structurally closely related and belong to the LOR family.

However, the regulation of their expression appears to differ substantially. *LURP1* transcript levels are very low in the absence of *Hpa*. In response to *Hpa* recognition transcripts of this gene are massively upregulated. This induction is initiated very early in response to *Hpa* recognition mediated by various *R* genes (Eulgem, Weigman et al. 2004). *R*-gene independent *Hpa* recognition during compatible interactions results in delayed upregulation of *LURP1* transcripts, while they still reach high levels at later time points. In contrast based on microarray analyses (Eulgem, Weigman et al. 2004) *LOR1* transcripts are expressed constitutively.

Assuming that the LURP1 and LOR1 proteins have identical molecular (or similar roles in defense), the early *R*-mediated up-regulated LOR function may be a feature required for successful defense during incompatible *Hpa* interactions, while moderate levels of constitutive LOR activity are insufficient for strong immunity, but still confer basal defense. To test, if differences in the regulation of LURP1 and LOR1 are indeed of critical importance for their disparate functions, *"*promoter swap" lines were created where *LURP1* upstream sequence reaching from the translation start site +177 to position -1004 bp was fused to LOR1 coding sequence resulting in the p^{-1004}*LURP1::LOR1* binary vector construct. Similarly *LOR1* upstream sequence reaching from the translation start site +269 to position -1083 bp was fused to the LURP1 coding sequence resulting in the binary vector construct p^{-1083}*LOR1::LURP1*. The p^{-1004}*LURP1::LOR1* and p^{-1083}*LOR1::LURP1* constructs were separately transformed into both *lurp1-2* and

lor1-1. T_1 lines (except for *p*$^{-1004}$*LURP1::LOR1* in *lor1-1* background that has T_0 lines available) are ready for *Hpa* infection assays by another lab member. The result will further show if either the promoter sequence or the subtle difference in the protein sequence of LURP1 or LOR1 is responsible for the differences in defense functions between LURP1 and LOR1.

A 39-bp *LURP1* promoter region -85 to -46 was found to important for defense-associated *LURP1* expression). It contains one W-box (W^{LURP1}) and two TGA box motifs (Figure 1.8). W^{LURP1} strongly interacted with nuclear DNA binding factors, creating two distinct shifts in electrophoretic mobility shift assays (EMSAs) (Knoth and Eulgem 2008). The interaction causing the upper shift is unaffected by defense-related stimuli, the lower shift is suppressed by *Hpa*Emoy2, SA and the *wrky70* mutation. EMSAs indicated that W^{LURP1} binding proteins can also interact with $W^{PR1\text{-}LS4}$ a negative element suppressing *PR1* gene expression in non induced plants (Lebel, Heifetz et al. 1998). The sequences of $W^{PR1\text{-}LS4}$ and W^{LURP1} motifs suggested interactions with members of the WRKY family, which are known to bind to W boxes (Eulgem, Rushton et al. 2000; Eulgem and Somssich 2007).

The three dimensional structure of LOR1 was recently found similar to that of human Phospholipid Scramblase1 (PLSCR1) (Bateman, Finn et al. 2009). PLSCR1 belongs to a family of plasma-membrane bound proteins. The originally proposed function of PLSCR1 is that of a phospholipid scramblase mediating the transbilayer redistribution of plasma membrane phospholipids. PLSCR1 was

shown translocate into the nucleus in response to interferon and cytokinin where it acts as a transcription factor. PLSCR1 is imported into the nucleus in an importin α/*β* importin-dependent manner and binds directly with importin α. The NLS of PLSCR1 consisting of peptide sequence GKISKHWTGI was found sufficient for its nuclear import. In the nucleus PLSCR1 activates the expression of the inositol 1,4,5 triphosphate receptor type1 (*IP3R1*) gene. PLSCR1 specifically binds to *IP3R1* promoter sequence and mediates transcriptional activation of this gene (Zhou, Ben-Efraim et al. 2005). The DNA binding domain, the nuclear localization signal, a Ca^{2+} binding motif of PLSCRs are conserved in LOR1 and other Arabidopsis LORs suggesting that LORs and PLSCRs could have related molecular roles and exhibit similar subcellular localization. In stably transformed *lurp1-2* and *lor1-1* Arabidopsis lines expressing GFP-tagged LURP1 or LOR1, respectively, both GFP-LURP1 and GFP-LOR1 were localized in to the nucleus and other parts of the cell. This is not in accordance with PLSCR1 where GFP-PLSCR1 fusion was found predominantly at the plasma membrane in T-lymphoid cells but a consistent fraction was also observed in an intracellular membrane compartment which largely co-localized with Golgi complex (Ben-Efraim, Zhou et al. 2004). Complementation of the *lurp1-2* or *lor1-1* mutants with *GFP–LURP1* or *GFP-LOR1,* respectively resulted in restoration of wild type Ler phenotypes in response to virulent *Hpa*Cala2. Mutant *lurp1-2* and *lor1-1* lines complemented by *GFP-LURP1* and *GFP-LOR1* also showed wild type Ler phenotype against avirulent *Hpa*Noco2 during incompatible interaction,

confirming that the resulting trans-genes restore wild type basal defense and *R*-mediated resistance to *Hpa*, and, therefore, accurately mimic function of the respective endogenous proteins.

Knowledge of proteins interacting with LOR1 and LURP1 will shed light on molecular processes they are involved in. Therefore, I designed a yeast two hybrid screening system for LURP1 and LOR1 interacting proteins. Both LURP1 and LOR1 were used as baits to screen a library which represented transcripts from untreated and *Hp*-infected Arabidopsis plants (Tsuchiya and Eulgem 2010). I found that the full length LURP1 and LOR1 GAL4-BD fusion proteins did not self activate transcription of the *HIS3* and *URA2* reporter genes, which contain GAL4 biding sites in their promoters and hence were used for screening. Using LURP1 and LOR1 GAL4-BD fusion proteins, I did not find any interacting partner with 2.3×10^5 library clones in yeast two hybrid screening.

LURP1 and LOR1-BD constructs lacking a putative activation domain (AD) conserved between LOR members and PLSCR-type proteins (Bateman, Finn et al. 2009) were also constructed (LURP1 ΔAD; LOR1 ΔAD). The yeast two hybrid system is ready to be used by other lab members for screening. The biological significance of protein interactions with LOR1 or LURP1 can be confirmed using Arabidopsis T-DNA mutants.

The identity of LURP1 or LOR1 interacting proteins will give new insights into the molecular roles of the LOR-PLSCR related type proteins. PLSCR1 directly interacts with CD4, the main receptor for HIV-1 entry into T-lymphocytes

and macrophages. It was found that CD4 and SLPI (Secretory Leukocyte Protease Inhibitor) involved in antiviral activity against HIV-1 bind to the same region of PLSCR1 showing that SLPI competes with CD4 and disrupt the interaction of CD4, the main receptor of HIV-1 with the PLSCR1 membrane protein at the cell surface of CD4-expressing cells. The interaction between SLPI and PLSCR1 analyzed in the yeast two-hybrid system showed that the full length 132 amino acid long SLPI was not able to interact with PLSCR1 whereas a specific interaction could be detected with SLPI lacking the first 25 residues. This form of SLPI with amino acid sequence from 26–132 corresponded to the mature secreted protein after cleavage of the N-terminal signal sequence (Py, Basmaciogullari et al. 2009).

In barley, CC-type receptor MLA10 conferred resistance to the powdery mildew fungus *Blumeria graminis* f sp *hordei* (*Bgh*) expressing specific effectors AVRA10. Only MLA truncated receptor was found interacting with *Hv*WRKY1 and its homolog *Hv*WRKY2, WRKY transcription factors in a yeast two hybrid screen. Full-length MLA6 bait failed to interact with *Hv*WRKY1 or *Hv*WRKY2 prey variants. This indicated requirements for intra- and intermolecular interactions *in vivo*. Based on these observations, the use of constructs expressing truncated versions of LOR1 or LURP1 may allow for the identification of protein/protein interactions that cannot be detected using the respective full length proteins (Shen, Saijo et al. 2007).

Taken together, my results clearly established LOR1 as a new component of the plant immune system. The fact that LOR1 is only required for basal defense against *Hpa*, but not *RPP5*-mediated immunity, suggests that differences in the regulation between the structurally closely related *LURP1* and *LOR1* genes are critical for their distinct roles in defense. Both LOR1 and LURP1 appear to be localized to nuclei (besides other parts of the cells). While details of their molecular roles are unclear at this point, the structural similarity between LOR and PLSCR proteins suggest a role of the former family in signaling processes. The yeast-two hybrid screen, I designed will likely reveal LOR protein interactors that may shed light on the molecular function of this protein family.

Materials and Methods

Plants and Growth Condition

Arabidopsis thaliana ecotypes and mutants were grown in soil under fluorescent lights (14h day, 10h night, 21C, 100 µEinstein/m^2s). Wild type ecotypes Columbia-0 (Col-0), Landsberg erecta (Ler) and the *lurp1-2* (Knoth and Eulgem 2008) and *lor1-1* mutants which are in the Ler background were used in this study.

Selection of *lurp1-2* and *lor1-1* transposon insertion mutants

Due to inconsistent results with the original *lurp1-2* line described previously (Knoth and Eulgem 2008), I selected a new homozygous line for this insertion. The Arabidopsis transposon mutants, *lurp1-2* (SGT4080) and *lor1-1* (GT 11546), were obtained from the Arabidopsis Biological Resource Centre (ABRC) at Ohio State University. Genomic DNA was extracted from five-week old soil grown seedlings using the Quick DNA Prep for PCR (Weigel and Glazebrook 2002). T3 individuals homozygous for the insertion lines were selected by polymerase chain reaction (PCR) (Alonso, Stepanova et al. 2003) and selfed. The respective T4 progeny for the mutant lines were used for all experiments. Homozygous *lurp1-2* and *lor1-1* lines were selected by PCR using a transposon specific primer and a pair of gene-specific primers flanking the insertion site as described previously (Alonso, Stepanova et al. 2003). For

LURP1-2 (SGT4080) a transposon-specific primer (Ds5'-1, 5'-ACGGTCGGGAAACTAGCTCTAC-3') and two gene specific primers (FP 5'-AACTTCGTGATAACGAGTGC-3') and (RP 5'-TCTTATCAACAGTGACGGAG-3') were used. For *lor1-1* (GT11546) a transposon-specific primer (Ds3'-3, 5'-CGGTCGGTACGGGATTTTCC-3') and two gene specific primers (FP 5'-AGTGAATCAATTTCGGTGGAG-3)' and (RP 5'-GGATGGGCCCTTAATGAAGG-3') were used.

Pathogen infections and tissue staining

Hyaloperonospora arabidopsidis (*Hpa*) was grown, propagated and applied to Arabidopsis as previously described (McDowell, Cuzick et al. 2000). Two-week-old seedlings were spray-inoculated with *Hpa* spore suspensions (3 x 10^4 spores/ml of water) using Preval sprayers. *Hpa* growth was determined 7 days post infection by trypan blue staining, visual sporangiophore counts, or by counting spores/seedlings. A hemicytometer was used to determine the spore density of a suspension of approximately 20mg fresh weight of infected tissue in 10ml water. Trypan blue staining was performed as previously described (McDowell, Cuzick et al. 2000; Torres, Dangl et al. 2002). The student's t-test was used to determine if the effects of the respective mutation on sporulation were statistically significant.

Generation of 5' promoter constructs and transgenic Arabidopsis lines

The 5' promoter constructs of the *LURP1-2* and *LOR1-1* were generated by PCR using Ler genomic DNA as a template. The forward primers for *LURP1-2* and *LOR1-1* contained a *Pst*I site at their 5' ends. The respective reverse primers contained a *Kpn*I site at their 5' ends. The sequences of the primers used and the end-points of the generated 5' promoters were:

LURP1-2 FP, 5'-AAAAGGTACCACTTTGTTTTCCCCTCC-3';
LURP1-2 RP, 5'-AAAACTGCAGGAAGGAATTTGTAAGTTACCAAA-3';
LOR1-1 FP, 5'-AAAAGGTACCTCTTCTCTTTCTCCACCG-3';
LOR1-1 RP, 5'-AAAACTGCAGGTAGATGACGATAGCTCCTTTTA-3'.

The PCR products generated from *LURP1-2* and *LOR1-1* promoter stretches were purified using QIAquick PCR purification kit (Qiagen). The purified PCR products were digested with *Pst*I and *Kpn*I and these inserts were ligated into the *Pst*I and *Kpn*I sites in frame with the ATG start codon of the gene creating translational fusions in pMDC43 expression vector (Curtis and Grossniklaus 2003). The *LURP1-2* promoter was fused to *LOR1* coding sequence and *LOR1-1* promoter was fused to *LURP1-2* coding sequence to create promoter swap constructs. The constructs were transformed in to *E. coli* DH5α (Sambrook, Fritsch et al. 1989). Their insert sequences and the

correctness of vector insert borders were confirmed by sequencing prior to transformation into the *Agrobacterium tumefaciens* strain GV2101 by electroporation (Sambrook, Fritsch et al. 1989). *A.tumefacians*-mediated transformation of Ler (T_0) was performed by the floral-dip method Clough and Bent 1998. The p^{-1004}*LURP1::LOR1* and p^{-1083}*LOR1::LURP1* constructs were transformed in to both *lurp1-2* and *lor1-1* plants. Transgenic plants were selected on 0.5 MS and 0.8% agar media containing 50 ug/ml kanamycin.

Generation of LURP1-GFP and LOR1-GFP transgenic Arabidopsis plants

For the preparation of LURP1-GFP and LOR1-GFP lines, full-length *LURP1* and *LOR1* cDNAs were amplified through PCR using Gateway compatible primers *LURP1-GFP* forward (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCAGCAGCCCTGTGTGAT-3'), *LURP1-GFP* reverse (5'GGGGACCACTTTGTACAAGAAAGCTGGGTCAAAACCTATTATCATTTGTGTTGTCATTT-3'), *LOR1-GFP* forward (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGCAGCCGTACGTGTACGCATACCC- 3') and *LOR1-GFP* reverse primer (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCGGCGCGGTCTTCGCC-3'). The *LURP1* and *LOR1* PCR products were recombined into the pDONR/Zeo plasmid (Invitrogen, http://www.invitrogen.com/) to produce Gateway entry

clones. The cloned ORFs were then transferred into the GFP-expression vector pMDC43 (Curtis and Grossniklaus 2003). The *LURP1-GFP* and *LOR1-GFP* sequence were confirmed by sequencing. The constructs were transformed into their respective mutant background by *A. tumefaciens* strain GV3101 (Sambrook, Fritsch et al. 1989). *A.tumefaciens*-mediated transformation of *lurp1-2* and *lor-1* (T_0) were performed by the floral-dip method (Clough and Bent 1998). Transgenic plants were selected on 0.5 MS/.0.8% agar media containing 50 mg/L Hygromycin. T3 plants homozygous for *LURP1-GFP* and *LOR1-GFP* were selected on 0.5 MS and 0.8% agar media containing 50ug/ml Hygromycin and homozygous plants were confirmed through PCR.

The *CaMV35S:GFP-LURP1* and *CaMV35S:GFP-LOR1* constructs transformed into transgenic Arabidopsis seedlings homozygous for GFP-LURP1 and GFP-LOR1 were analyzed by confocal microscopy. The GFP-fluorescence was observed in untreated GFP-LURP1 and GFP-LOR1 before and after 48 hrs infection of one-week-old Arabidopsis seedlings spray inoculated with $3x10^4$ spores/ml of the virulent *Hpa*Cala2. DAPI staining was performed to specify locations of nuclei in leaf epidermal cells.

Yeast two-hybrid assays were performed using the GAL4 system.

The LURP1 and LOR1 full length coding sequence were amplified using the *LURP1-BD* forward primer (5'-CCGTCGACATGCAGCAGCCCTGTGTG-3') and the *LURP1-BD* reverse primer (5'-

CCGACGTCCTAAAAACCTATTATCATTTGTGTTGTC-3') as well as the *LOR1-BD* forward primer (5'-CCGTCGACATGGAGCAGCCGTACGTGTACGCAT-3') and the *LOR1-BD* reverse primer (5'-CCGACGTCTCAGGCGGCGCGGTCTTC-3'). Two additional *LURP1-BD* and *LOR1-BD* constructs with truncated N-terminal parts were generated by using *LURP1ΔAD-BD* forward primer (5'-AAAAGTCGACGACGGTAACTTCGTGATAACG-3'), *LOR1ΔAD-BD* forward primer (5'-AAAAGTCGACGGCAATTTCGTGATACGGACG-3') and same *LURP1-BD* and *LOR1-BD* reverse primers for PCR amplification. The proteins encoded by the respective vectors lacked their 30 and 50 N-terminal amino acids, respectively. The PCR product was cloned in each case between the *Sal*I and *Pst*I sites of pBD-GAL4 Cam (Stratagene, http://www. stratagene.com/) to generate a DNA binding domain (BD) bait protein fusion constructs. These bait constructs, were transformed in yeast strain AH109 (Clontech, http://www.clontech.com/), which contained the *ADE2* and *HIS3* reporter genes as well as mutations in the endogenous HIS3, ADE2, LEU2 and TRP1. Absence of growth of the resulting yeast lines on –HIS, ADE, LEU and TRP medium indicated that the respective baits do not activate transcription by themselves and can be used for a two-hybrid screen. In each case the LURP1 and LOR1 bait construct were grown on –TRP medium. These bait constructs, contained the TRP1 as a selectable marker.

The correctness of the LURP1 and LOR1 bait constructs was verified through yeast growth harboring only the bait plasmid on medium lacking

Tryptophan. A cDNA library constructed previously (Tsuchiya and Eulgem 2010) using pooled RNAs from 2-week-old Col-0 seedlings either untreated or infected with one of several *Hpa* isolates (including *Hpa* Hiks1) in the HybriZAP-2.1 vector (Stratagene), which encodes fusions with the GAL4 activation domain (AD), was used. Library cDNAs were screened after transformation of library vectors into yeast strain AH109 containing the respective bait vectors (Stratagene). The transformation efficiency for LURP1 and LOR1 yeast two hybrid screen was calculated by counting the yeast colonies on –TRP –LEU medium lacking the two marker genes present on bait and library constructs respectively. After I set up the system, screens for LOR1 and LURP1 interactors are still ongoing and are being performed by other lab members. Interacting proteins will be selected for complementation of histidine and adenine auxotrophy on selection plates lacking tryptophan, leucine, histidine and adenine. Plasmids from the positive clones either from full length or truncated LURP1 and LOR1 bait constructs will be isolated and introduced into *E. coli* DH5a strain for sequencing and further analysis.

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Chapter 2: Role of tomato *Sl*WRKY70 orthologs in defense induction by chemical genomics.

Summary

In Arabidopsis defense-associated up-regulation of the *LURP* gene cluster has been shown to be partially dependent on the *At*WRKY70 transcription factor. The *CaBP22*$^{-333}$*::GUS* reporter gene mimics *LURP* gene expression characteristics in Arabidopsis. A transgenic VFNT Cherry tomato line containing this reporter gene responded to SA, BTH and DCA suggesting the existence of a conserved mechanism of *LURP* regulation in Arabidopsis and tomato. Phylogenetic analysis with all Arabidopsis group III WRKY sequences along with *Sl*WRKY70 protein sequence revealed that Arabidopsis WRKY70 has two orthologs in tomato termed *SlWRKY70a* and *SlWRKY70b*. Three silencing constructs were made to individually or co-silence *SlWRKY70a* and *SlWRKY70b* in cherry tomato plants homozygous for the *pCaBP22*$^{-333}$*::GUS* gene construct. Tobacco rattle virus (TRV) virus-induced gene silencing (VIGS) of *SlWRKY70* genes reduced *pCaBP22*$^{-333}$*::GUS* expression in response to BTH or DCA suggesting that WRKY70-type transcription factors are required for BTH- or DCA-responsiveness of *LURP* gene expression in tomato.

Introduction

Chemical genomics is the use of small organic molecules that interfere with the function of cellular proteins and thereby, induce defined phenotypes. In many cases small molecules affect protein targets or modulate the activity of receptors or enzymes that are fundamental to the understanding of the molecular mechanisms of signal transduction in plants (Surpin, Rojas-Pierce et al. 2005; Kawasumi and Nghiem 2007; Hicks and Raikhel 2012). Chemical genomics can also lead to the identification of compounds that target multiple protein families resulting in a clear phenotype by simultaneously altering their function. These chemicals can further allow real-time control of biological processes by their application in a controlled manner (Kaschani and van der Hoorn 2007; Noutoshi, Ikeda et al. 2012). By interfering with regulatory proteins that control plant immune responses bioactive small organic molecules can therefore act as defense activators and induce plant disease resistance (Knoth, Salus et al. 2009). Synthetic elicitors are drug-like small molecules that induce plant immune responses, but are structurally distinct from natural defense-inducing compounds (Knoth, Salus et al. 2009).

It has been shown that the natural defense signaling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are involved in partially synergistic or antagonistic signal transduction crosstalk (Petersen, Brodersen et al. 2000; Kunkel and Brooks 2002; Glazebrook, Chen et al. 2003; Spoel, Koornneef et al. 2003). In some cases, disease resistance mediated by SA is

dependent on NPR1, a nuclear transported transcriptional cofactor. It has been shown recently that the NPR1-like proteins, NPR3 and NPR4 directly bind SA. These two SA-receptors regulate NPR1 protein turnover (Fu, Yan et al. 2012). NPR1 acts downstream from SA and interacts with TGA-bZIP transcription factors (TFs). Additional TFs, including WRKYs and ERFs, are involved in the regulation of the defense transcriptome (Eulgem, Rushton et al. 2000; Maleck, Levine et al. 2000; Dong, Chen et al. 2003; Ulker and Somssich 2004; Eulgem 2005; Wu, Guo et al. 2005).

While TGA-bZIP TFs form only a relatively small gene family in plants, with only 10 members in Arabidopsis (Jakoby, Weisshaar et al. 2002), WRKY and ERF TFs constitute extremely expanded gene families (Riechmann, Heard et al. 2000 Eulgem, Rushton et al. 2000; Eulgem and Somssich 2007). For example, in Arabidopsis there are 74 different members of the WRKY family (Eulgem and Somssich 2007). Based on conserved structural features, they are categorized into seven groups or subgroups (Eulgem, Rushton et al. 2000). A similar diversity of ERF TFs has been described (Nakano, Suzuki et al. 2006).

Pathogenesis-related (*PR*) gene expression and disease resistance cannot be induced by the SA analog 2,6-dichloroisonicotinic acid (INA) or avirulent pathogens in the *tga6, tga2* and *tga5* triple knockout mutant highlighting important roles of TGA TFs in disease resistance (Zhang, Tessaro et al. 2003). It was found that either TGA2 or TGA5 is sufficient for INA-induced *PR* gene expression and pathogen resistance and further demonstrated that *TGA2*, *TGA5*,

and *TGA6* act redundantly and play a positive role in regulation of disease resistance (Zhang, Tessaro et al. 2003).

Differences in transcript profile between *npr1* mutant and wild type plants showed that nearly all genes responsive to the SA analog acibenzolar- S-methyl benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) are NPR1-dependent indicating a critical role of NPR1 in BTH-mediated transcriptional reprogramming (Wang, Amornsiripanitch et al. 2006). BTH-mediated induction of the *WRKY18*, *WRKY38*, *WRKY53*, *WRKY54*, *WRKY58*, and *WRKY70* genes was either abolished or markedly reduced in the *npr1* mutant (Wang, Amornsiripanitch et al. 2006). It was shown that *wrky18* mutant was partially impaired in BTH-induced resistance to *Pseudomonas syringae pv. maculicola* (*Psm*) and was found to be a positive regulator of plant defense. After BTH treatment, *wrky58* mutant was clearly more resistant to *Psm* than wild type indicating WRKY58 to be a negative defense regulator. The enhanced disease susceptibility phenotype of *wrky18* was abolished in *wrky58/wrky18* double mutant. The double mutant *wrky53 wrky70* showed enhanced disease phenotype to the bacterial pathogen *Pseudomonas syringae* which is absent or only weakly detectable in the respective single mutants indicating that both WRKY53 and WRKY70 are positive regulators of defense responses that act redundantly.

WRKY70 and WRKY54 play redundant roles both as negative regulators of SA synthesis and as positive regulators of SA signaling (Besseau, Li et al. 2012). This shows that WRKY TFs are involved in critical functions in the intricate

signaling network induced by SA and play important roles in plant defense (Eulgem, Rushton et al. 2000; Eulgem and Somssich 2007). It was found that WRKY11 and WRKY17 act as negative regulators during compatible and incompatible interactions against *P. syringae* (Journot-Catalino, Somssich et al. 2006). Specific and redundant functions were assigned to these two structurally related TFs where WRKY11 and WRKY17 negatively regulate *WRKY70* and *WRKY54* in Arabidopsis.

In parsley (*Petroselinum crispum*) WRKY1 also acts as a negative regulator where it binds to its own promoter likely suppressing its own expression after defense induction (Eulgem, Rushton et al. 1999; Turck, Zhou et al. 2004). This differential regulation of promoters by activation or repressing WRKY TFs is regulated by posttranslational modifications of individual family members and their *de novo* synthesis or degradation (Turck, Zhou et al. 2004). Depending on the balance between suppression and activation function of WRKY TFs, the transcriptional output of downstream target genes may be either positively or negatively regulated (Journot-Catalino, Somssich et al. 2006).

Defense gene expression regulated by WRKY70 is dependent on SA and JA immune signaling (Glazebrook 2001; Kunkel and Brooks 2002; Spoel, Koornneef et al. 2003). Two *WRKY70* orthologs are induced by SA and pathogen infection in tobacco (*Nicotiana tabacum)* (Chen and Chen 2002). Overexpressing *WRKY70* in Arabidopsis resulted in enhanced resistance to the two virulent bacterial pathogens *Erysiphae carotovora* and *P. syringae pv. tomato* DC3000,

whereas antisense suppression of *WRKY70* led to enhanced susceptibility to *E. carotovora* (Li, Brader et al. 2006). Increased SA levels activate *WRKY70* gene expression, while an increased JA level represses its expression. Hence, the levels of WRKY70 transcripts represent the SA and JA balance in defined defense conditions. *WRKY70* activity level is further reflected in the expression of downstream target genes of this TF (Li, Brader et al. 2006). High *WRKY70* levels are known to promote expression of a subset of SA-responsive *PR* genes, while low *WRKY70* levels favor expression of JA-responsive genes (Li, Zhao et al. 2004). This suggests that WRKY70 plays a pivotal role in SA and JA defense signals acting as a key node of interaction between these pathways (Li, Zhao et al. 2004; Li, Xie et al. 2006).

Arabidopsis *wrky70* mutant has opposite effect on resistance to the fungal pathogens *Alternaria brassicicola* and *Erysiphae cichoracearum*. Resistance to the biotroph *E. cichoracearum* is triggered by SA dependent pathways whereas resistance to the necrotroph *A. brassicicola* requires JA-mediated defense responses. (Dewdney, Reuber et al. 2000). Considering the role of WRKY70 in cross-talk between SA and JA and the antagonistic and synergistic effects of these two defense hormones, use of synthetic elicitors for a comparative analysis of such defense mechanisms in Arabidopsis and tomato may lead to the identification of critical conserved regulatory nodes.

It was found that in Arabidopsis, *WRKY70* is involved in gene-for-gene disease resistance and basal defense against the oomycete pathogen

Hyaloperonospora arabidopsidis (Hpa). Arabidopsis *WRKY70* is included in the *LURP (late up-regulated in response to Hpa*) gene cluster (Eulgem, Weigman et al. 2004) and it acts downstream from SA accumulation in *Hpa*-induced defense signaling. WRKY70 controls transcript levels of *LURP1* and *CaBP22* suggesting a role for WRKY70 in transcriptional reprogramming (Knoth, Ringler et al. 2007). The main goal of this study is to dissect the *LURP* defense pathway through chemical and molecular genetics. Key tools used in this study were the new synthetic elicitors 3,5-dichloroanthranilic acid (DCA) and BTH in Arabidopsis and tomato.

The organic compounds SA, INA, and BTH are known defense elicitors that trigger certain aspects of plant immunity (Métraux, Ahl Goy et al. 1991; Ward, Payne et al. 1991; Uknes, Mauch-Mani et al. 1992; Schob, Kunz et al. 1997). INA and BTH act as functional analogs of SA because they induce the expression of known SA responsive genes. BTH is also commercially available under the names Actigard and Bion, from Syngenta (www.syngenta.com/). Application of such synthetic elicitors to field crops offers an attractive alternative to the use of conventional pesticides which rely on direct antibiotic activity often leading to undesirable environmental side effects (Kessmann, Staub et al. 1994) Other than acting as crop protectants, these compounds can also play an important role in pharmacological analyses of plant defense mechanisms. In this study BTH and the new defense elicitor DCA were used for such analyses in transgenic cherry tomato line homozygous for *CaBP22::GUS* construct to

understand defense mechanisms in tomato and also to draw an analogy between Arabidopsis and tomato defense responses.

Phylogenetic studies of the tomato (*Solanum lycopersicum*; *Sl*) WRKY family based on the recently completed genome sequence of this species (Consortium 2012) had shown that the *WRKY70* gene has duplicated in an ancestor of tomato and that there are two tomato orthologs of this gene (Hagop Atamian and Isgouhi Kaloshian, personal communication). Defense-related roles of the two *WRKY70* ortholog genes in tomato termed *SlWRKY70a* and *SlWRKY70b* were examined by virus-induced gene silencing (VIGS). For this reason BTH or DCA were applied to transgenic tomato lines containing a *pCaBP22^{-333}::GUS* reporter construct (Knoth, Salus et al. 2009). The effect of individually silencing or co-silencing of *SlWRKY70a* or *SlWRKY70b* on the expression of *pCaBP22^{-333}::GUS* reporter gene showed that both *SlWRKY70a* and *SlWRKY70b* contribute to the expression of *pCaBP22^{-333}::GUS* in response to BTH.

Real time qRT PCR analysis of *CaBP22::GUS* expression in TRV-VIGS *SlWRKY70a or SlWRKY70b* silenced and *SlWRKY70ab* co-silenced plants in response to DCA showed reduced expression of *pCaBP22^{-333}::GUS* compared to non-silenced plants. Taken together these results suggest the existence of a defense mechanism in tomato that involves the *Sl*WRKY70a and *Sl*WRKY70b TFs and the ability of DCA and BTH to activate expression of defense-related genes likely depends on these two TFs.

Results

Response of GUS reporter gene to defense elicitors SA, BTH and DCA in transgenic cherry tomato.

A set of Arabidopsis genes identified as the *LURP* gene cluster exhibit late up regulation in response to *Hpa* (Eulgem, Weigman et al. 2004). The WRKY70 TF was found to be required for full *Hpa* responsiveness of at least some LURP genes (Knoth, Ringler et al. 2007). A member of the *LURP* cluster*, CaBP22*, closely matches the average *Hpa*-induced *LURP* expression profile (Eulgem, Weigman et al. 2004). *CaBP22* encodes a putative calmodulin-like calcium-binding protein (McCormack, Tsai et al. 2005). A 5'-deletion analysis of *CaBP22* promoter stretches fused to GUS reporter gene in transgenic Arabidopsis revealed a minimal *Hpa*-responsive region of -333 bp responsible for induced GUS expression (Knoth, Salus et al. 2009). This construct was transformed in cherry tomato and was evaluated for the induction by the defense elicitors SA, BTH and DCA (Figure 2.1a). Both chemicals were able to successfully induce *pCaBP22^{-333}::GUS* expression in cherry tomato (Figure 2.1b-c). Based on this preliminary finding, a transgenic cherry tomato line homozygous *pCaBP22^{-333}::GUS*, line #12, which responded clearly to BTH and DCA was chosen for carrying out a reverse genetic analysis of the role of the WRKY70 transcription factor in the *Hpa*-responsiveness of *pCaBP22^{-333}::GUS* (Table 2.1).

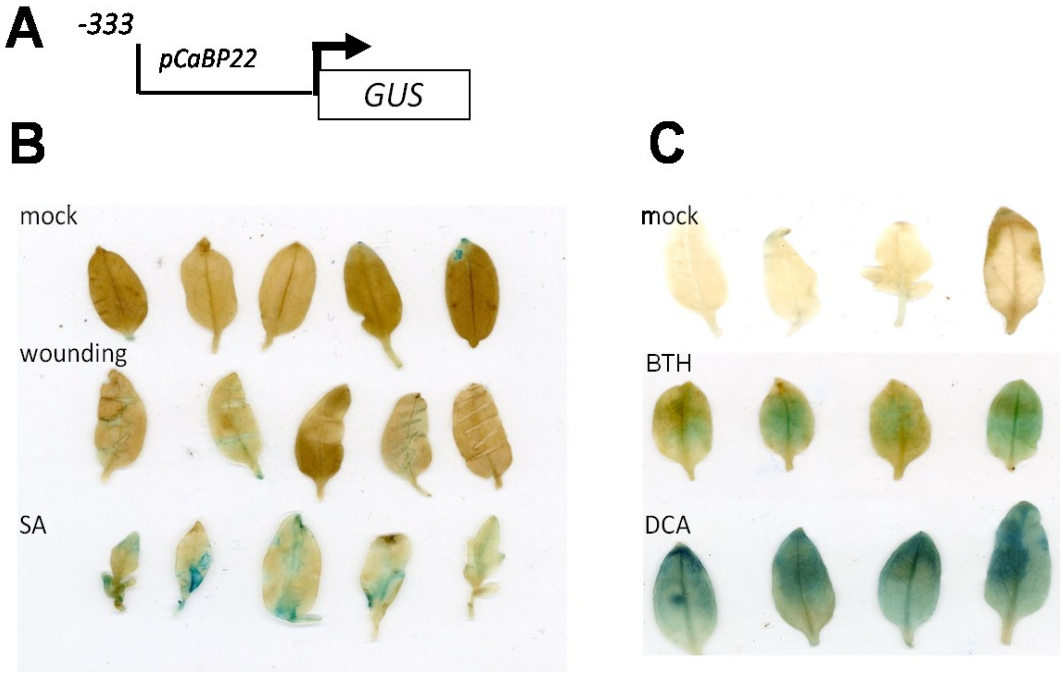


Figure 2.1: Expression of *pCaBP22*$^{-333}$*::GUS* responds to SA signaling in transgenic cherry tomato.
A: Schematic representation of the *pCaBP22*$^{-333}$*::GUS* construct.
B,C: Histochemical GUS staining of cherry tomato plants containing *pCaBP22*$^{-333}$*::GUS* exhibit *GUS* expression in response to SA, SA-analogs or wounding. Two-week old cherry tomato plants treated with mock (0.2%DMSO), 2mM SA, 2mM BTH or 3mM DCA or treated by wounding by cutting with a scalpel blade. Shown are leaves from at least four representative plants. **B** and **C** represent two separate sets of independent experiments each with at least four replicates.

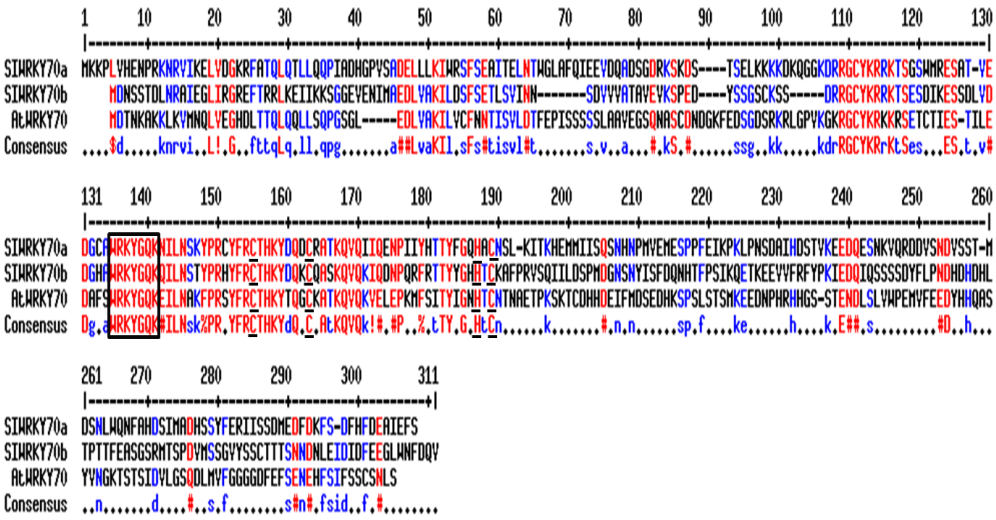
Phylogenetic analysis *of SI*WRKY70a and *SI*WRKY70b

The annotated Arabidopsis genome classification of the WRKY transcription factors was used as reference for the annotation of the tomato *SIWRKY70* gene(s). Sequence analyses using full genome tomato sequence showed that the two *SIWRKY70* genes named *SIWRKY70a* and *SIWRKY70b* encoded proteins with high similarity to *Arabidopsis thaliana* (*At*) member of group III of the WRKY family *At*WRKY70. The amino acid sequence of *SI*WRKY70a and *SI*WRKY70a are shown in Figure 2.2a. As indicated in the figure, *SI*WRKY70a, *SI*WRKY70b shared with *At*WRKY70 a group III-type WRKY domain including the characteristic C_2H_2 Zinc-finger motif of group III WRKYs suggesting that these proteins are indeed orthologs of *At*WRKY70.

A Phylogenetic tree was built based on an alignment of five diverse Arabidopsis Group III and the two WRKY70-related tomato WRKY amino acid sequences (Figure 2.2b). The sequences were aligned based on conserved motifs and domains. The tree was constructed by using the neighbor joining tree program provided on the Geneious website (http://www.geneious.com). Bootstrap values from 1000 replicates are presented at individual node. Bootstrap values of higher than 50 indicate that the respective node is highly significant. The result showed *SI*WRKY70a and *SI*WRKY70b to be closely related to both *At*WRKY70 and *At*WRKY54, which is highly similar to *At*WRKY70 and also clustered in the same group. However, based on a BLASTP run of the *SI*WRKY70a and *SI*WRKY70b protein sequences against all predicted

Arabidopsis proteins (http://www.arabidopsis.org/Blast/index.jsp) both WRKY70-related tomato proteins are more similar to *At*WRKY70 (E-values 2e-28 and 2e-32, respectively) than *At*WRKY54 (E-values 4e-21 and 3e-27, respectively).

A



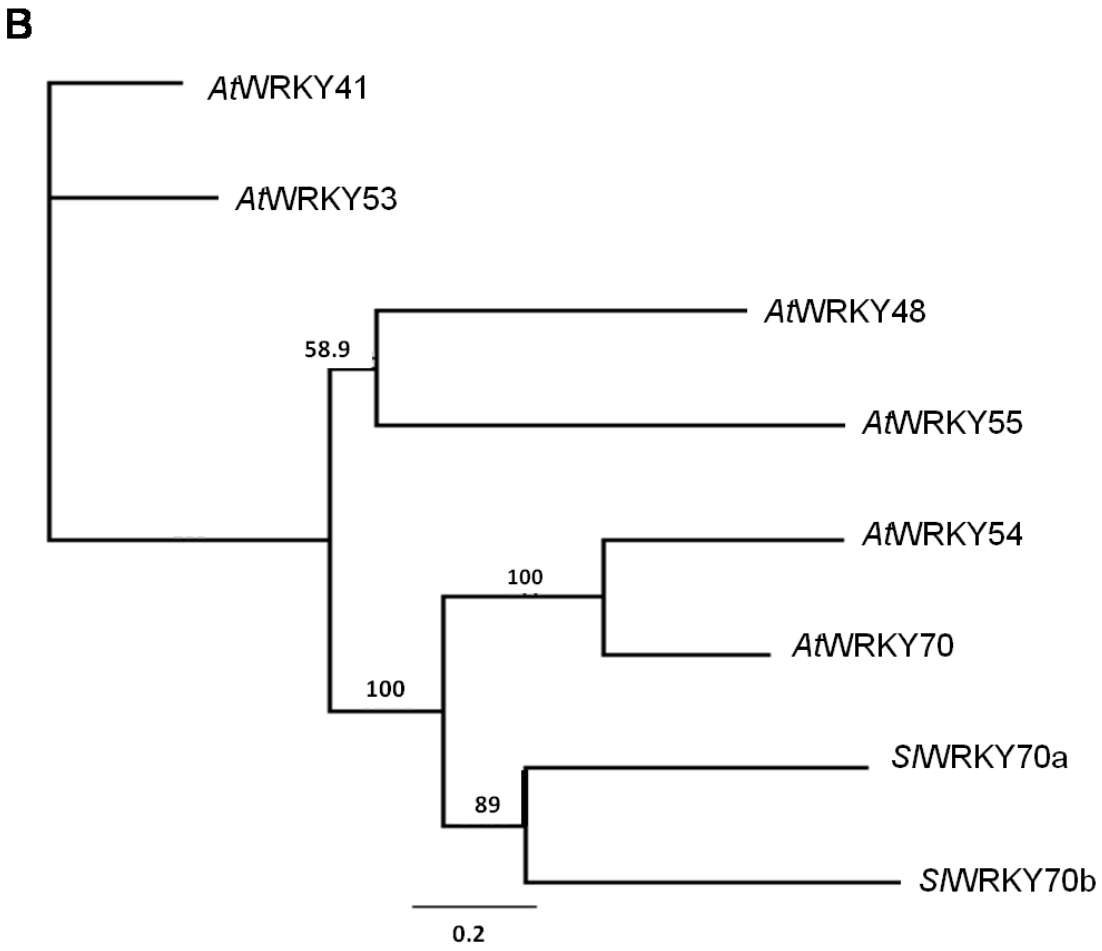
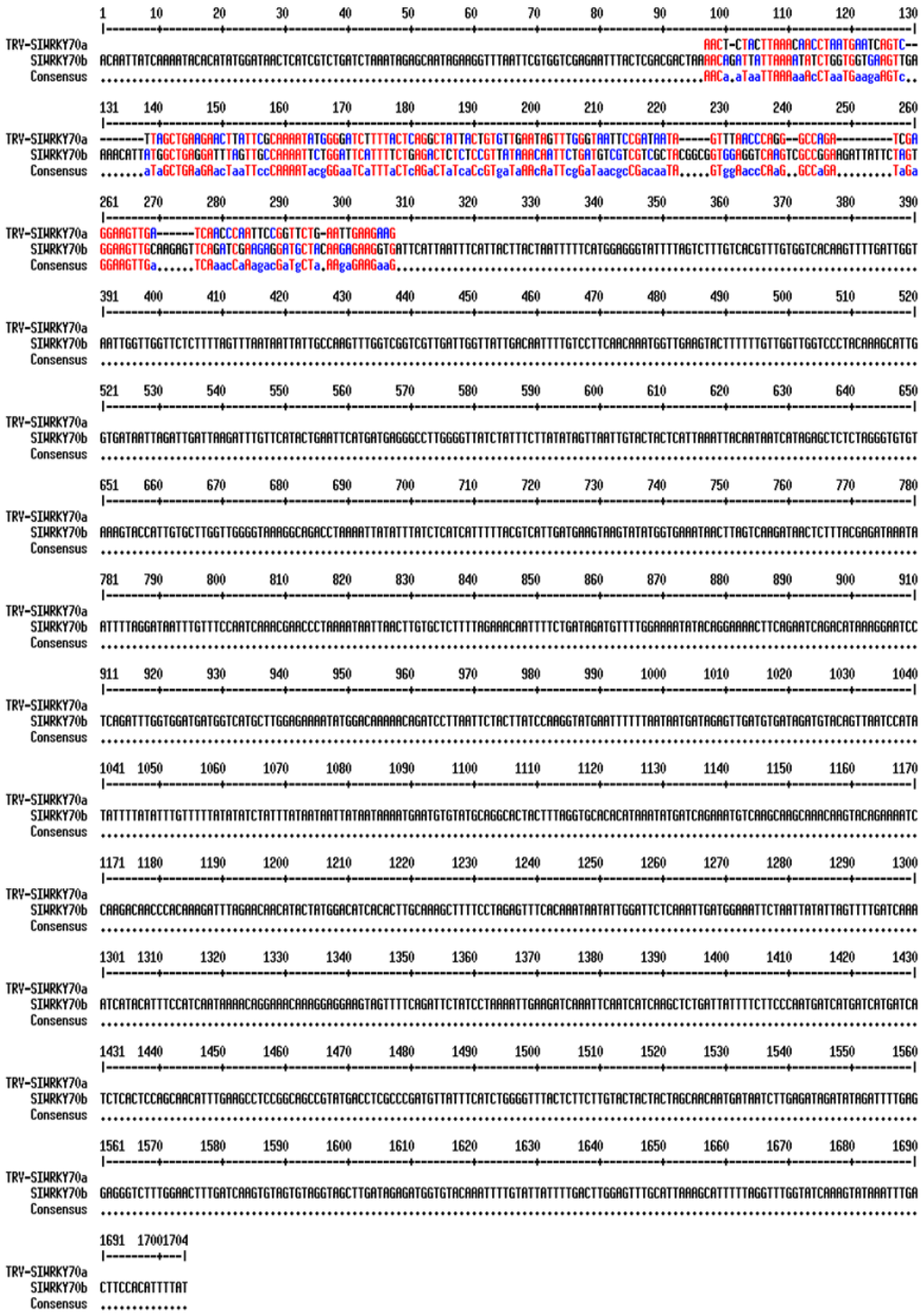


Figure 2.2: Protein sequence alignment of *At*WRKY70, *Sl*WRKY70a and *Sl*WRKY70b and phylogenetic analysis of *Sl*WRKY70a and *Sl*WRKY70b along with Arabidopsis group III amino acid WRKY sequences. A. *Sl*WRKY70a and *Sl*WRKY70b are the two orthologs of *At*WRKY70 of Group III of Arabidopsis WRKY proteins containing a WRKY domain with a invariant N-terminal 'WRKYGQK' motif (boxed) and a C-terminal zinc-finger with conserved cysteine and histidine residues (underlined; multalin.toulouse.inra.fr/multalin/) (Corpet 1988). **B.** The amino acid sequences were aligned and used for phylogenetic tree analysis by the neighbor-joining tree program (http://www.geneious.com). Bootstrap values from 1000 replicates for each amino acid are presented at the individual nodes. The scale bar indicates the number of substitutions per site.

TRV-*Sl*WRKY70a, TRV-*Sl*WRKY70b and TRV-*Sl*WRKY70ab constructs

Tobacco rattle virus (TRV) based VIGS constructs were prepared using sequence information available at SOL genomics network (http://sgn.cornell.edu). The newly available annotated genome sequence resource for tomato provided an opportunity to silence specific tomato genes by VIGS constructs. TRV-*Sl*WRKY70a, TRV-*Sl*WRKY70b and TRV-*Sl*WRKY70ab constructs were designed to individually silence or co-silence *SlWRKY70a* and *SlWRKY70b* (Figure 2.3). For TRV-*Sl*WRKY70ab silencing construct, *SlWRKY70a* and *SlWRKY70b* specific sequences were ligated together for co-silencing.

A



B

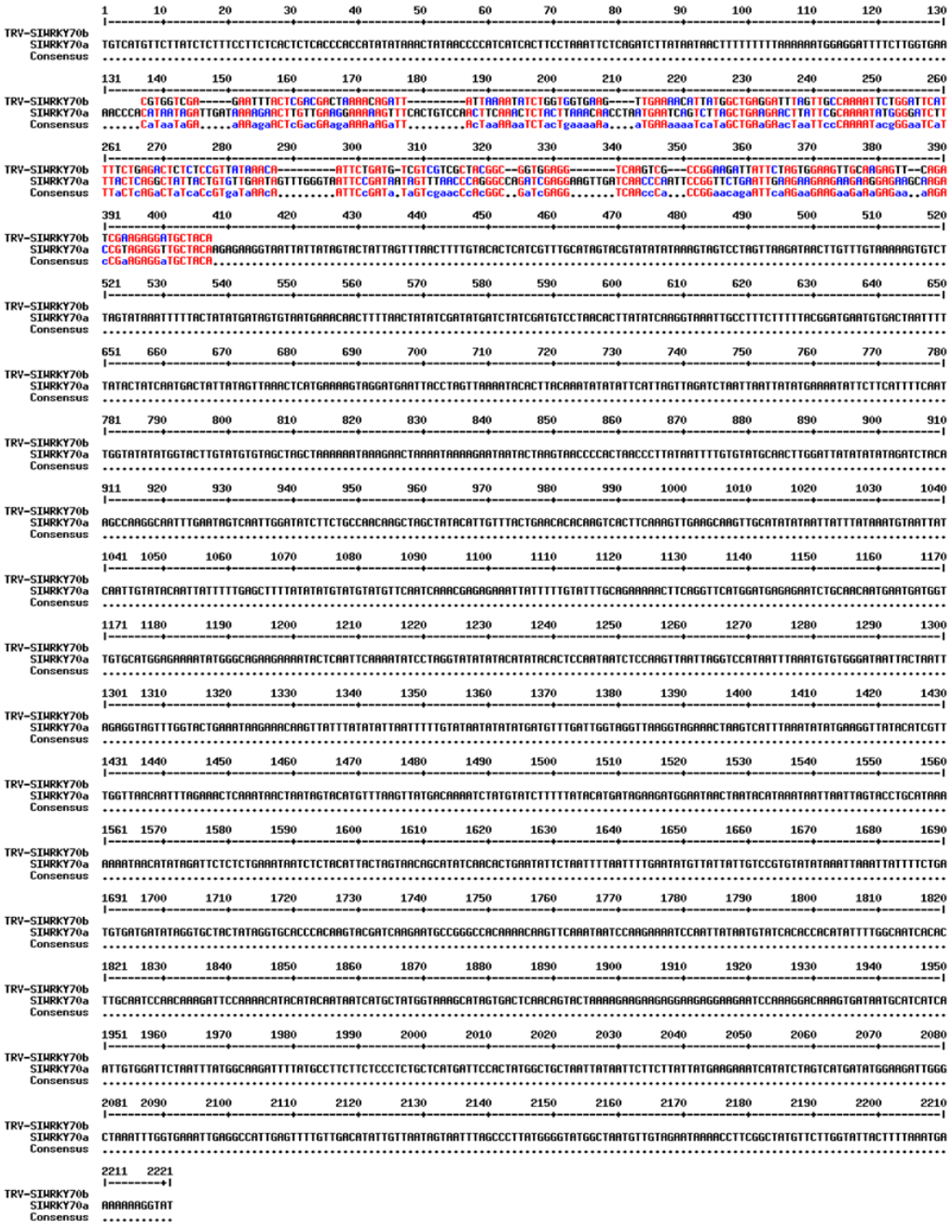


Figure 2.3: Sequences of TRV-VIGS constructs targeted for silencing *SlWRKY70a, SlWRKY70b* and *SlWRKY70ab* in transgenic cherry tomato plants containing *pCaBP22^{-333}::GUS* reporter gene. A. TRV-*Sl*WRKY70a aligned to *SlWRKY70b* sequence. **B.** TRV-*Sl*WRKY70b aligned to *SlWRKY70a* sequence (http://sgn.cornell.edu) (multalin.toulouse.inra.fr/multalin/; Corpet 1988).

***SlWRKY70a* and *SlWRKY70b* are required for BTH-induction of *CaBP22* expression in cherry tomato plants.**

To assess the roles of *SlWRKY70a* and *SlWRKY70b* in BTH-mediated disease resistance, the gene-specific TRV vector-based VIGS constructs TRV-*SM*WRKY70a, TRV-*SM*WRKY70b and TRV-*SM*WRKY70ab were used. While the TRV-*SM*WRKY70a, TRV-*SM*WRKY70b constructs were designed to specifically silence each of these two genes, TRV-*SM*WRKY70ab contains sequences that should co-silence both of them. Plants agroinfiltrated with empty TRV vector showed expression of the *pCaBP22*$^{-333}$*::GUS* reporter gene in response to BTH indicating that TRV did not interfere with BTH-induction of *CaBP22*$^{-333}$*::GUS* reporter gene expression (Figure 2.4). Plants agroinfiltrated with TRV-*SM*WRKY70a showed slightly less BTH-induced GUS expression, whereas in *SlWRKY70b*-silenced plants GUS was expressed at a very low level as compared to non-silenced, BTH-treated TRV control plants (Figure 2.4). In *SlWRKY70ab* co-silenced plants, no BTH-responsive GUS expression was detectable, suggesting that both *SlWRKY70a* and *SlWRKY70b* contribute to the expression of *pCaBP22*$^{-333}$*::GUS* in response to BTH. Overall it can be concluded that *SlWRKY70a* and *SlWRKY70b* together play a role in mediating enhanced expression of the *CaBP22*$^{-333}$*::GUS* reporter gene in response to BTH.

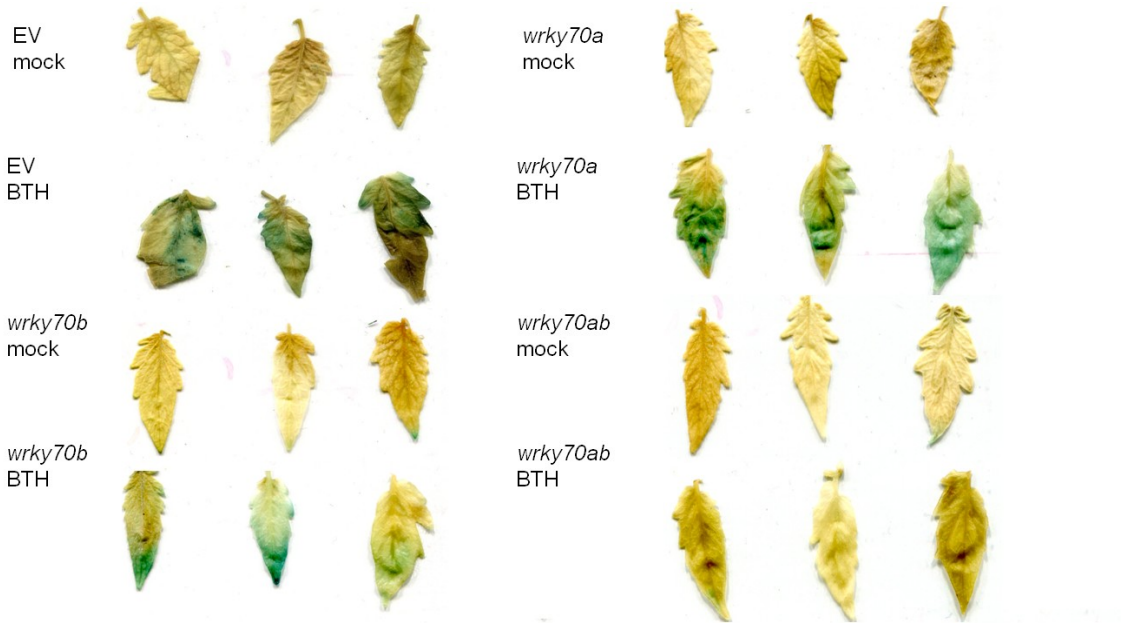


Figure 2.4: Effect of silencing of *SlWRKY70a, SlWRKY70b* or *SlWRKY70ab* on the expression of *pCaBP22^{-333}::GUS* reporter gene in response to BTH. Two week-old plants were agro infiltrated with TRV-VIGS-EV (Empty vector), TRV-VIGS-*Sl*WRKY70a, TRV-VIGS-*Sl*WRKY70b or TRV-VIGS-*Sl*WRKY70ab to examine the effect of silencing of *SlWRKY70* paralogs on the expression of *pCaBP22^{333}::GUS* reporter gene expression in tomato. Two weeks later, plants of the transgenic *pCaBP22^{-333}::GUS* cherry tomato line #12 were treated with 2mM BTH. Each treatment was replicated three times.

Effect of VIGS on *SlWRKY70a* and *SlWRKY70b* transcripts levels

To further assess the role of *SlWRKY70a* and *SlWRKY70b* in defense gene regulation, plants silenced for each of these genes individually or co-silenced for both genes were evaluated after mock or DCA treatment and compared to non-silenced control plants. After DCA treatment, in plants infiltrated with TRV-*Sl*WRKY70a, transcripts of this gene were less abundant compared to control non-silenced plants (Figure 2.5). Similarly, after DCA treatment, *SlWRKY70b* transcripts were less abundant in TRV-*Sl*WRKY70b infiltrated plants as compared to control plants. Furthermore, the transcript levels of both *SlWRKY70a* and *SlWRKY70b* were clearly reduced in TRV-*Sl*WRKY70ab co-silenced as compared to non-silenced plants. Thus in each case TRV-mediated silencing was successful. However, *SlWRKY70a* transcript levels appeared also to be reduced in *SlWRKY70b*-silenced plants, indicating that this silencing construct does not exclusively target one of the two tomato WRKY70 paralogs (Figure 2.5). While *SlWRKY70b* transcript levels were not reduced in untreated *SlWRKY70a* silenced plants, they were reduced after DCA treatment. Thus, none of the used silencing constructs appears to be specific for either *SlWRKY70a* or *SlWRKY70b* and both genes seem to be affected by the TRV constructs meant to silence the respective *WRKY70* member. The alignments in figure 2.3 shows that each of the two TRV-VIGS insert exhibit some sequence similarity with respective paralogus transcript. However if these sequence similarities are sufficient to mediate the observed cross silencing effects is unclear.

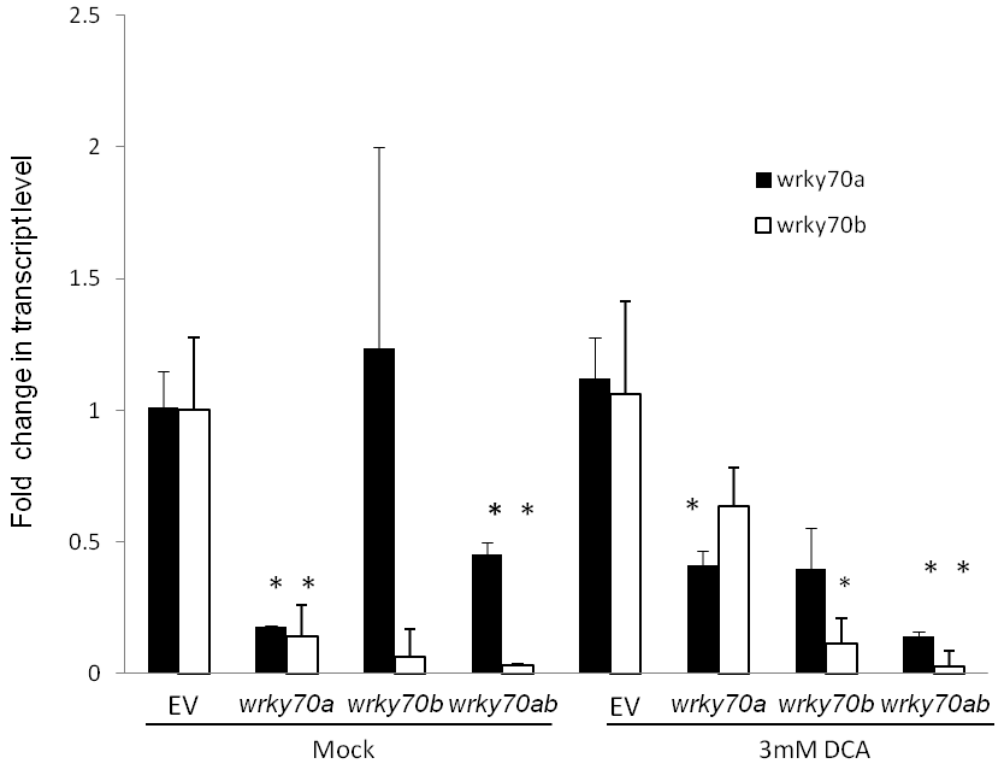


Figure 2.5: Fold change in transcript level of *SlWRKY70a* and *SlWRKY70b* in TRV-VIGS agro-infiltrated cherry tomato plants silenced for *SlWRKY70a*, *SlWRKY70b* or co-silenced for *SlWRKY70a* and *SlWRKY70b* after DCA treatment. Transcript levels were quantified using real-time qRT-PCR. EV represents plants infected with empty TRV vector. Fold-change values are normalized with transcript levels of the endogenous *Ubi3* gene. Data represent two technical replicates. Error bars represent standard error of mean (SEM). Significant levels of transcript reduction based on student's t- test ($P < 0.05$) are marked by an asterisk.

Roles of *Sl*WRKY70a and *Sl*WRKY70b in DCA-induced expression of *CaBP22::GUS*

Chemical pretreatment of Arabidopsis seedlings with DCA prior to *Hpa* infection induced strong resistance 1 hour after treatment (Knoth, Salus et al. 2009). The early defense induction after DCA treatment coincided with a similar induction of *WRKY70* and *CaBP22* expression in response to *Hpa*. DCA-induced resistance began to decline between 3 and 6 d after chemical treatment making it a potent elicitor whose activity is both rapid and reversible in Arabidopsis. It was also found that the interaction of DCA with defense signaling pathways is likely to occur downstream or independently of SA and is partially dependent on WRKY70 in Arabidopsis (Knoth, Salus et al. 2009).

As DCA is a potent defense inducer affecting the SA/WRKY70-dependent branch of defense network in Arabidopsis, its role was examined in transgenic cherry tomato containing *CaBP22::GUS* silenced with TRV-*Sl*WRKY70a, TRV-*Sl*WRKY70b or TRV-*Sl*WRKY70ab. It was found that plants infiltrated with the empty TRV vector showed minimal level of GUS expression after treatment with DMSO, whereas the same type of plants treated with 3mM DCA showed increased GUS expression. TRV-*Sl*WRKY70a and TRV-*Sl*WRKY70b-infiltrated untreated cherry tomato samples containing *CaBP22::GUS* showed significant high level of GUS expression however DCA-treated TRV-*Sl*WRKY70a and TRV-*Sl*WRKY70b samples showed very low expression of the *CaBP22::GUS* reporter gene. Based on these observations it appears that that *Sl*WRK70a and

*SM*WRKY70b can negatively regulate the expression of the *CaBP22::GUS* reporter gene in the absence of defense induction. However, this effect is only obvious in plants infiltrated with either TRV-*SM*WRKY70a or TRV-*SM*WRKY70b construct, as in TRV-*SM*WRKY70ab-infiltrated plants, there was no significant difference in DCA treated and untreated samples. Given that all three TRV-*SM*WRKY70 constructs affect transcript levels of each of the two tomato WRKY70 paralogs, these results are difficult to interpret. Possible silencing with each of the three TRV constructs changes the balance between *SM*WRK70a and *SM*WRKY70b in a specific manner. Thus a certain balance between the levels of these two TFs may be needed for tight silencing of *CaBP22::GUS*.

The results obtained for *CaBP22::GUS* reporter gene with DCA-treated plants are much clearer and easier to interpret. Clearly DCA-inducibility of the *CaBP22::GUS* reporter gene is reduced after infiltration with either one of the three TRV silencing constructs (Figure 2.6). Thus either *SlWRKY70a*, *SlWRKY70b* or both of these transcription factors are needed for full DCA-inducibility of *CaBP22::GUS*.

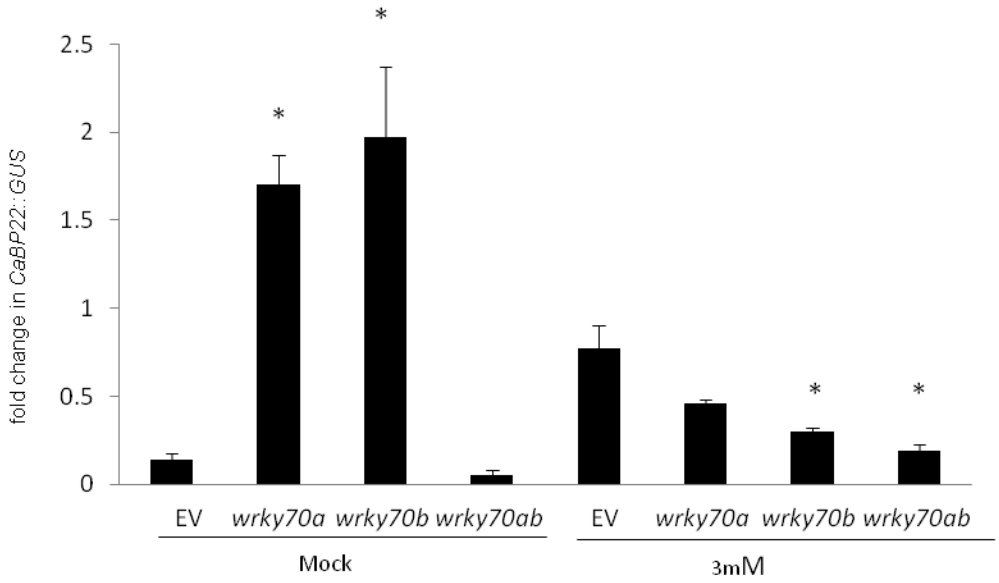


Figure 2.6: Real-time qRT-PCR analysis of *pCaBP22-^{333}::GUS* transcript levels in TRV-VIGS silenced *SlWRKY70a*, *SlWRKY70b* or *SlWRKY70ab* cherry tomato plants before and after DCA treatment. Four-weeks-old cherry tomato plants were treated with 3mM DCA after *SlWRKY70a*, *SlWRKY70b* or *SlWRKY70ab* silencing. Leaves of cherry tomato plants were harvested 24hrs after DCA treatment. cDNAs were synthesized from control and DCA treated cherry tomato plants and were used to determine *pCaBP22^{-333}::GUS* transcript level. Transcripts were assigned fold values after normalization with the internal control *Ubi3*. Significance of transcript level changes relative to the respective empty vector control was determined by student's t- test ($P < 0.05$).

No.	T2 Parental lines in transgenic cherry tomato	*pCaBP22*$^{-333}$*::GUS* reporter line phenotype
1	Line1	Heterozygous
2	Line2	Heterozygous
3	Line3	Heterozygous
4	Line4	Heterozygous
5	Line5	Heterozygous
6	Line6	Heterozygous
7	Line7	Heterozygous
8	Line8	Heterozygous
9	Line9	Heterozygous
10	Line10	Heterozygous
11	Line11	Heterozygous
12	**Line12**	**Homozygous**
13	Line13	Heterozygous
14	Line14	Heterozygous
15	Line15	Heterozygous
16	Line16	Heterozygous
17	Line17	Heterozygous
18	Line18	Heterozygous
19	Line19	Heterozygous
20	Line20	Heterozygous
21	Line21	Heterozygous
22	Line22	Heterozygous
23	Line23	Heterozygous
24	Line24	Homozygous (Not confirmed)

Table 2.1: Transgenic cherry tomato line #12 evaluated as homozygous for *pCaBP22*$^{-333}$*::GUS* reporter gene through Kanamycin selection and PCR based analysis.

Discussion

Microarray experiments identified a cluster of *LURP* genes that operate in an SA-dependent pathway mediating resistance to *Hpa* in Arabidopsis (Eulgem, Weigman et al. 2004). Members of this cluster exhibit a highly coordinated and pronounced increase of their transcript levels after *R*-mediated *Hpa* recognition and basal defense against oomycetes (Eulgem, Weigman et al. 2004; Eulgem and Somssich 2007; Knoth, Ringler et al. 2007; Knoth and Eulgem 2008). *CaBP22* represents the average transcript profile of *LURP* expression in responses to *Hpa.* A functional 5' deletion promoter analysis by GUS reporter gene assays in stably transformed Arabidopsis plants revealed a stretch from the likely transcriptional start site up to position -333 to be the shortest *Hpa*-responsive fragments of the *CaBP22* promoter (Knoth, Salus et al. 2009). Multiple transgenic tomato lines (cultivar VFNT Cherry tomato) containing this *pCaBP22*$^{-333}$*::GUS* construct responded to SA, BTH and DCA indicating that mechanisms of *LURP* regulation are conserved between Arabidopsis and tomato.

The two tomato orthologs of *AtWRKY72*, *SlWRKY72a* and *SlWRKY72b,* were transcriptionally up-regulated after RKN and aphid infestation in the tomato cultivar Motelle, which is resistant to these two pests due to the *R*-gene *Mi-1*. In Arabidopsis, *AtWRKY72* also plays an important role in basal defense against RKN as well as *Hpa*. *AtWRKY72* –dependent defense responses in Arabidopsis appear to be independent from SA (Bhattarai, Atamian et al. 2010). However, so

far no role of *At*WRKY72 in *R*-mediated immunity has been reported. While their contribution to this type of immunity appears so far to be unique to the *Mi-1* pathway in tomato, the basal-defense-related function of WRKY72-type transcription factors seems to be conserved between Arabidopsis and tomato.

A second type of WRKY factors involved in *Mi-1*-mediated immunity are WRKY70-related proteins. In tomato, one of two WRKY70 orthologs *Sl*WRKY70a has recently been reported to be required for full immunity of tomato against aphids and RKN (Atamian, Eulgem et al. 2012). The Arabidopsis ortholog of this gene, *AtWRKY70*, is included in the *LURP* gene cluster (Eulgem, Weigman et al. 2004) and controls transcript levels of at least two other *LURP* genes, *LURP1* and *CaBP22* suggesting a role for *At*WRKY70 in transcriptional reprogramming during plant disease resistance (Knoth, Ringler et al. 2007). A homozygous transgenic cherry tomato line was examined for the expression of *pCaBP22*$^{-333}$*::GUS* in *SlWRKY70* silenced plants in response to BTH and DCA.

WRKY transcription factors are known to regulate transcript levels of their target genes upon binding to the W box (TTGAC/CT) promoter element. These TFs are defined by a conserved DNA binding domain of ~60 amino acids containing the nearly invariant stretch WRKYGQK followed by a unique zinc-finger pattern of Cys and His residues (Rushton, Tovar Torres et al. 1996). WRKYs are sub-divided into three groups in Arabidopsis (Eulgem, Rushton et al. 2000). Group I members have two WRKY domains, while groups II and III have only one WRKY domain. The $Cx_{4-5}CX_{22-23}HXH$ zinc finger pattern is the defining

feature of group I and II WRKY domains whereas group III contains a $Cx_7CX_{23}HXC$ zinc pattern. While there is one WRKY70 TF in Arabidopsis, there appear two related WRKY70 TFs in tomato, hence named *SlW*RKY70a and *SlW*RKY70b. In a phylogenetic analysis based on an amino acid sequence alignment of various group III WRKYs *SlW*RKY70a and *SlW*RKY70b clustered closely together with *At*WRKY70 and *At*WRKY54. BLASTP analysis revealed that both *SlW*RKY70a and *SlW*RKY70b are more closely related to *At*WRKY70 than *At*WRKY54. Both *SlW*RKY70a and *SlW*RKY70b have a typical group III WRKY domain with an invariant N-terminal 'WRKYGQK' motif and a C-terminal zinc-finger with conserved cysteine and histidine residues characteristic for this group.

The conservation of WRKY72-related functions in tomato and Arabidopsis immunity as well as the fact that WRKY70-type TFs are structurally conserved between these two distantly related eudicot species prompted me to investigate whether the defense related roles of this type of TFs are also preserved during evolutionary processes separating Arabidopsis and tomato. I decided to focus on the documented role of *At*WRKY70 in the regulation of the *LURP* member *CaBP22*. As no clear ortholog of this gene could be identified in tomato, I made use of the *pCaBP22*$^{-333}$*::GUS* reporter gene, which had been transformed into cherry tomato cv. VFNT plants by the UC-Riverside plant transformation facility. I selected T1 lines that responded by enhanced GUS expression to treatment with BTH and DCA and selected individuals homozygous for the reporter gene. One

of the resulting lines, line #12, which responded clearly to BTH and DCA was chosen for all further experiments. The fact that *pCaBP22*$^{-333}$*::GUS* exhibited in tomato and Arabidopsis inducibility by DCA and BTH, suggested that the regulatory mechanism controlling this reporter gene is conserved between both tested plant species. In my studies on this conserved mechanism, I used expression of *pCaBP22*$^{-333}$*::GUS* as a "read-out", which likely represents the collective expression state of so far unidentified members of a hypothetical tomato *LURP* cluster.

To address if the WRKY70/CaBP22 regulatory module is also conserved between Arabidopsis and tomato, VIGS analysis by TRV-mediated silencing targeting *SlWRKY70a*, *SlWRKY70b* or both of these genes was performed. Unfortunately all three TRV constructs appeared to target both of these paralogous tomato genes. While my results do not allow to discriminate between *Sl*WRKY70a and *Sl*WRKY70b, they clearly showed that WRKY70-type TFs are required for full inducibility of the *pCaBP22*$^{-333}$*::GUS* reporter by DCA and BTH. Thus, despite the fact that TFs of this type are duplicated in tomato relative to Arabidopsis, the WRKY70/CaBP22 regulatory module seems to be conserved between these two species.

A surprising observation was that in the absence of defense induction, levels of *pCaBP22*$^{-333}$*::GUS* expression were strongly elevated in TRV-*Sl*WRKY70a and TRV-*Sl*WRKY70b infiltrated tomato plants. While this observation may suggest a role of these two WRKY70 proteins in suppressing

pCaBP22^{-333}*::GUS* expression in resting (non defense-activated) cells, this effect was not observed in plants infiltrated with the TRV-*SM*WRKY70ab construct. A possible explanation for these inconsistencies may be that a certain balance of *SM*WRKY70a and *SM*WRKY70b levels is needed for their function as transcriptional suppressors in the absence of defense-inducing stimuli.

WRKY proteins have been shown to form homocomplexes or heterocomplexes (Xie, Zhang et al. 2006; Xu, Chen et al. 2006). Such complex formation could explain why a certain balance between *SM*WRKY70a and *SM*WRKY70b is needed for their proper function as transcriptional suppressors. It was shown that the closely related members of Arabidopsis subgroup IIa, WRKY18, WRKY40, and WRKY60, interact with themselves and with each other to form both homocomplexes and heterocomplexes. The *wrky18/wrky40* and *wrky18/wrky60* double mutants as well as the *wrky18/wrky40/wrky60* triple mutant were more resistant to virulent *P. syringae* but more susceptible to the fungus *Botrytis cinerea* than single mutants (Xu, Chen et al. 2006). While effects of WRKY40 and WRKY60 on disease resistance were only detectable in combined mutants of these genes, single mutation of WRKY18 was sufficient to detect a measurable contribution to the outcome of plant pathogen interactions (Wang, Amornsiripanitch et al. 2006; Xu, Chen et al. 2006; Shen, Saijo et al. 2007)

Although WRKY18 seems to contribute more strongly to defense regulation than, WRKY40 and WRKY60, these three structurally related WRKY

members appear to function partially redundantly and possibly cooperatively. In *in vitro* binding studies using electrophoretic mobility shift assays, WRKY18 and WRKY40 exhibited clear binding to canonical W-box motifs, while WRKY60 showed little binding activity for the same W-box sequences. These differences in their DNA binding activity were consistent with their functional interactions in influencing disease resistance. WRKY18 has a pronounced W box-binding activity and can alone influence plant disease resistance. Interaction of WRKY18 with WRKY40 or WRKY60 results in altered DNA binding activity and specificity that may contribute to the fine tuning of their regulatory functions in plant resistance. The changes in DNA binding activity as a result of physical interactions among these three WRKY proteins further suggested that WRKY40 and WRKY60 antagonized WRKY18 in defense responses. Thus, a defined balance of their levels may be of critical importance for their common contribution to defense signaling.

The allelic rice WRKY proteins *Os*WRKY45-1 and *Os*WRKY45-2 may also be involved in such differential complex formations. While the former seems to negatively regulate resistance against the bacterial pathogen *Xanthomonas oryzae pv oryzae* (*Xoo*) by promoting the accumulation of both SA and JA, the latter positively regulated *Xoo* resistance by only increasing the levels of JA but not of SA (Tao, Liu et al. 2009). Both *OsWRKY45-1* and *OsWRKY45-2* positively regulate rice resistance against the fungal pathogen *Magnaporthae grisea*. It was further found that *OsWRKY13* regulates *OsWRKY45-1* and *OsWRKY45-2* via the

binding to W-box, W-box-like, or other *cis*-acting elements, because *OsWRKY13* was also found bound to the *OsWRKY45-1* promoter region which did not contain a W-box or W-box-like element (Tao, Liu et al. 2009). Differential expression of *OsWRKY45-1* and *OsWRKY45-2* in response to *Xoo* could be partly due to the binding of *Os*WRKY13 to different sites within *OsWRKY45-1* and *OsWRKY45-2* promoters.

In Arabidopsis *CaBP22* transcript levels are reduced in *wrky70* mutants indicating a direct or indirect role of *At*WRKY70 in regulating *CaBP22* transcription (Knoth, Ringler et al. 2007). The W box motifs, which may function as WRKY binding sites, is lacking in the entire stretch of 1230 bp intergenic region upstream from *CaBP22*, as well as in the transcribed region of this gene. Promoters targeted by WRKY factors tend to contain clusters of multiple W boxes (Eulgem, Rushton et al. 1999; Maleck, Levine et al. 2000; Yu, Chen et al. 2001; Chen, Provart et al. 2002; Dong, Chen et al. 2003; Eulgem, Weigman et al. 2004; Turck, Zhou et al. 2004). This suggest that *At*WRKY70 either does not directly interact with promoter elements of *CaBP22* or that this WRKY may interact with W-box related DNA sequences as *OsWRKY45* (Tao, Liu et al. 2009). Binding of a WRKYs to a promoter element distinct from a canonical W boxes has also been reported for tobacco, where *Nt*WRKY12 binds to an element termed WK-box (Sun, Palmqvist et al. 2003; van Verk, Pappaioannou et al. 2008).

Taken together results presented in this chapter strongly support that a regulatory mechanism dependent on WRKY70-type transcription factors is conserved between Arabidopsis and tomato. As members of the families of Brassicaceae and Solanaceae, these two species represent a large evolutionary distance within the clade of eudocots. Thus, the function of WRKY70-type transcription factors appears to be at least partially preserved during the evolutionary processes leading to the diversification of eudicot species. This conserved WRKY70-dependent mechanism is inducible by the SA analog BTH and the new synthetic elicitor DCA, which also triggers the SA-dependent defense signaling branch. BTH and DCA differ in their interference with defense signaling. While the former predominantly induces defense signaling processes dependent on the well characterized transcriptional co-factor NPR1 (Wang, Amornsiripanitch et al. 2006), DCA seems to largely trigger NPR1-independent, but WRKY70-dependent signaling processes (Knoth, Salus et al. 2009). Previous studies already revealed the existence of a bifurcation in defense signaling downstream from SA accumulation. SA can either trigger defense responses in a NPR1/WRKY70-depedent manner or in a NPR1-independent, but WRKY70-dependent manner (Li, Brader et al. 2004). It appears that early and transient signaling processes rather trigger the NPR1-independent mechanism, while NPR1 is important for long-lasing SA-responsive defense induction including systemic acquired resistance (Dong, Li et al. 2001; Dong 2004). In Arabidopsis enhanced *LURP* expression is associated with both early and transient local

immunity as well as long-lasting and systemic defense induction (Knoth and Eulgem 2008; Knoth, Salus et al. 2009). Based on my findings this appears also to be the case in tomato. One possible difference between the WRKY70/LURP regulatory modules in Arabidopsis and tomato may be the regulation of WRKY70-type genes. In Arabidopsis *At*WRKY70 transcript accumulation is clearly inducible by DCA. Based on my findings, this may not be the case in tomato. While LURP induction may be simply mediated in Arabidopsis by a defense-associated increase of WRKY70 levels a different mechanism must be enhancing WRKY70 activity. Such mechanisms may include post-translational modifications or differential binding of WRKY70-type transcription factors with co-factors. Future studies will have to address this interesting possibility.

Materials and Methods

Plant materials and growth conditions

Tomato cv. VFNT cherry tomato and transgenic lines containing *pCaBP22*$^{-333}$*::GUS* derived from it were used in this study. T1 seeds of cherry tomato containing *pCaBP22*$^{-333}$*::GUS* were grown in soil in a mist room . Fifteen days after germination the seedlings were transplanted in to pots and kept in a green house. For the selection of homozygous T2 lines 100 T2 seeds from each of 24 individual T1 individuals were grown on ½ strength MS medium containing 100mg/L Kanamycin. T1 line 12 was selected as potentially homozygous based on a 100% survival rate of T2 seeds on Kanamycin plates. Homozygosity of line 12 was further confirmed by PCR performed on 24 T2 seedlings of this line.

For VIGS, plants were grown in soil at with 16hrs light (200 uEinstein/m^2s) and an 8hrs dark period before treatment with the virus. After virus treatment, plants were incubated at 19^{o}C under the same light conditions until used for chemical treatment performed in the lab. Plants were then moved back to the same conditions at 19^{o}C.

VIGS constructs and Agrobacterium-mediated virus infection

The bipartite TRV vector (TRV1 and TRV2) was used for VIGS (Hayward, Padmanabhan et al. 2011) to individually silence or co-silence the tomato *SlWRKY70a* and *SlWRKY70b* genes in tomato. Inserts representing *SlWRKY70a*

and *SlWRKY70b*-specific transcript sequences were developed from genomic DNA extracted from cherry tomato and amplified by PCR using the following Gateway compatible primers.

SlWRKY70a forward primer:

5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGTTTCACTGTCCAACTTCA-3′;

SlWRKY70a reverse primer:

5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCTTCAATTCAGAACCG-3′;

SlWRKY70b forward primer:

5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTGGTCGAGAATTTACTC-3′ and

SlWRKY70b reverse primer:

5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTGTAGCATCCTTTCGATC-3.

For *SlWRKY70ab, SlWRKY70a* and *SlWRKY70b* specific inserts were amplified using the above mentioned Gateway compatible *SlWRKY70a* forward *as SlWRKY70ab* forward and *SlWRKY70b* reverse as *SlWRKY70ab* reverse primer. For *SlWRKY70ab* reverse primer (5'-AAAAGGATCCCTTCTTCAATTCAGAACCG-3') and *SlWRKY70ab* forward primer (5'-AAAAAGATCTCGTGGTCGAGAATTTACTC-3') were used to produce restricted *BamH*I and *Bgl*II *SlWRKY70a* and *SlWRKY70b* restricted PCR products. The restricted PCR products were ligated together to form *SlWRKY70ab* construct. The inserts representing transcribed sequences from *SlWRKY70a, SlWRKY70b* or *SlWRKY70ab* were then recombined into the pDONR207 vector (Invitrogen, http://www.invitrogen.com/). The clones were recombined using pYL279 Gateway-compatible TRV2 vector (Liu, Burch-Smith et

al. 2004). The identities of the clones were confirmed by sequencing and were transformed into *Agrobacterium tumefaciens* strain GV3101. The resulting vector TRV-*SM*WRKY70a contained 198 nucleotides of *SlWRKY70a* transcript while the resulting TRV-*SM*WRKY70b vector contained 229 nucleotides of *SlWRKY70b* transcript. TRV-*SM*WRKY70ab contained both inserts resulting in a total of 433 nucleotides.

Cultures of *A. tumefaciens* strain GV3101 containing TRV1 and TRV2 or TRV1and TRV-*SM*WRKY70a, TRV-*SM*WRKY70b or TRV-*SM*WRKY70ab were grown using 50ug/ ml Kanamycin and 25ug/ ml Rifampicin (Li, Xie et al. 2006). *A. tumefaciens* cultures were pelleted and resuspended in infiltration buffer at OD600 of 1.0. Cells were incubated at room temperature for 3 h before use. Equal volume of TRV1 Agrobacterium culture was mixed with TRV2 empty vector or TRV-*SM*WRKY70a, TRV-*SM*WRKY70b and TRV-*SM*WRKY70ab before infiltration. The abaxial sides of the cotyledons and leaflets of two to three week old seedlings were infiltrated with TRV-*SM*WRKY70a, TRV-*SM*WRKY70b and TRV-*SM*WRKY70ab using 1ml needleless syringes.

RNA isolation and RT-PCR

Total RNA was isolated using TRIzol (Invitrogen, http://www.invitrogen.com/). Twenty micrograms of total RNA were treated with RNase-free DNase (Fermentas, http://www.fermentas.com/). The DNase treated RNA was subsequently purified by chloroform extraction and precipitation. First-

strand cDNAs were synthesized from 5ug DNase-treated RNA using SuperScript III reverse transcriptase (Invitrogen). For quantitative real-time RT-PCR, transcripts were amplified from 1 ul of 5x diluted cDNA in a 15 ul reaction using gene-specific primers and iQ^{TM}SYBR Green Supermix (Bio-Rad, http://www.bio-rad.com/). The PCR conditions were 94^{0}C for 5 min, then 94^{0}C for 30 sec, 55^{0}C for 30 sec and 72^{0}C for 30 sec (number of cycles 45), and 72^{0}C for 3 min, followed by generation of a dissociation curve. The generated threshold cycle (CT) was used to calculate the transcript abundance relative to the housekeeping gene (tomato *Ubi3*) as described previously (Ginzinger 2002). To measure the relative transcript level of *CaBP22^{-333}::GUS* reporter gene in cherry tomato, the same conditions were used as described above with the annealing temperature of 58^{0}C for *GUS* expression. The following primers were used for *SlWRKY70a*, *SlWRKY70b*, *SlWRKY70a SlWRKY70b* and *GUS* transcripts measurement.

SlWRKY70a FP: 5'-TTGTGGATTCTAATTTATGGCAA-3';

SlWRKY70a RP: 5'- ACCAAGAACATAGCCGAAGG -3'**;**

SlWRKY70b FP: 5'- GACCTCGCCCGATGTTATT -3';

SlWRKY70b RP: 5'- TGTACACCATCTCTATCAAGCTAC-3';

GUS FP: 5'-CGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTC-3';

GUS RP: 5'-GTCGTGCACCATCAGCAGGTTATCGAATCCTTTGCC-3';

Ubi3 FP: 5'- GTGTGGGCTCACCTACGTTT-3';

Ubi3 RP: 5'- ACAATCCCAAGGGTTGTCAC-3'

Chemical treatment

BTH and DCA stock solutions were prepared in water or 100% DMSO respectively. Stock solutions were diluted in water and tomato plants were root-drenched with 35ml of water solution containing 3mM BTH and 3mM DCA. Final DMSO concentrations never exceeded 0.02%. Mock treatments were application of water and 0.02% DMSO in water for BTH or DCA, respectively. DCA was dissolved in 0.02% DMSO in water diluted with few drops of 1N KOH. DCA and BTH were supplied from (Sigma, http://www.sigmaaldrich.com/) and Syngenta (www.syngenta.com/) respectively.

Analysis of GUS Activity

GUS histochemical staining was performed using three to four individual leaflets of 4 weeks old cherry tomato plants stained in a 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc) solution containing 1mg/ml X-gluc, 50mM Na_2PO_4 pH 7.2, 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6$. The leaflets were incubated at 37^{o}C for 48 hours and cleared with 70% EtOH.

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Conclusion

The Brassicaceae plant species *Arabidopsis thaliana* (Arabidopsis) and the oomycete, *Hyaloperonospora arabidopsidis* (*Hpa*) serves as an effective model for host-pathogen interactions in which different *R* genes recognize various *Hpa* isolates. It was previously shown that the *LURP* (Late up regulated in response to *Hpa* recognition) gene cluster show late, pronounced and coordinated transcriptional up-regulation in response to *Hpa* and the related oomycete *Phytophthora infestans* (Eulgem, Weigman et al. 2004; Eulgem, Tsuchiya et al. 2007; Knoth and Eulgem 2008; Knoth, Salus et al. 2009). *WRKY70* which is included in *LURP* gene cluster regulates the expression of other *LURP* genes such as *LURP1* and *CaBP22* (Knoth, Ringler et al. 2007). In view of the importance of the *LURP* gene cluster and its role in disease resistance, the overall goal of my work was to further dissect this defense pathway in Arabidopsis and tomato to get an insight into key regulatory processes controlling the defense transcriptome across plant species.

The first part of my project focused on the biological and molecular characterization of the Arabidopsis gene *LURP1* and its paralog *LOR1* (LURP-one related 1). *LURP1* a member of the *LURP* gene cluster that shows an unusually pronounced up-regulation in response to oomycete pathogens as well as SA and the defense elicitor DCA (Knoth and Eulgem 2008; Knoth, Salus et al. 2009). This up-regulation is partially dependent on the WRKY70 transcription factor (Knoth, Ringler et al. 2007). *LURP1* is a representative of a 15 member

gene family *LOR* (LURP-one related). It is the only family member that shows a transcriptional induction in response to *Hpa*. Based on microarray data from our lab (Eulgem et al., 2007; Bhattarai et al., 2010), of the remaining *LOR* family members only *LOR1* exhibits clearly detectable transcript levels and which are constitutive, whereas other family members do not show any detectable levels of gene expression. Experiments with the *lor1-1* transposon mutant demonstrated a significant role of this gene in basal defense, whereas the *lurp1-2* mutant revealed a significant role of this gene in disease resistance against *Hpa*Noco2 mediated by the *R*-gene *RPP5.* The transgenic Arabidopsis promoter swap lines p^{-1004}*LURP1::LOR1* and p^{-1083}*LOR1::LURP1* were constructed and transformed into both *lurp1-2* and *lor1-1* each to evaluate the differences in the defense related roles of these genes.

The Arabidopsis LOR1 protein resembles human Phospholipid scramblase1 (PLSCR1). This similarity is not well obvious at the level of primary structure. However the 3-dimensional structure of LOR1 shows strong similarity to that of PLSCR1 (Bateman, Finn et al. 2009). PLSCR1 belongs to a novel superfamily of plasma-membrane bound proteins and is translocated into the nucleus where it act as transcription factor. The nuclear localization signal and a Ca^{2+} binding motifs of PLSCR1 are partially conserved in LURP1, LOR1 and other Arabidopsis LORs. Based on this I performed experiments to examine possible roles of LURP1 and LOR1 as transcription factors. This was done by determining their subcellular localization using GFP fusions of these proteins.

GFP-LURP1 and GFP-LOR1 were found by confocal microscopy to be present at the plasma membrane and in the nucleus with or without *Hpa* defense activation. GFP-LURP1 and GFP-LOR1 were stably expressed in their respective mutant backgrounds. The defense phenotype of the *lurp1-2* and *lor1-1* mutants were complemented by stable expression of the GFP-LURP1 and GFP-LOR1 fusion proteins showing that these mimic the wild type function of LURP1 or LOR1, respectively, during compatible or incompatible interactions with *Hpa*.

I also performed a preliminary yeast two hybrid screen to identify potential interacting partners of LURP1 and LOR1 using LURP1-BD and LOR1-BD as bait constructs and a cDNA library representing pooled RNAs from 2-week-old Col-0 seedlings either untreated or infected with one of several *Hpa* isolates in the HybriZAP-2.1 vector, (Stratagene) (Tsuchiya and Eulgem 2010). Transformants were screened on -TRP-HIS-ADE-LEU selective media for the activation of *HIS* and *ADE* markers. LURP1-BD and LOR1-BD constructs lacking a putative conserved activation domain of LOR-related proteins (AD) (Bateman, Finn et al. 2009) were also constructed (LURP1 ΔAD; LOR1 ΔAD) for additional yeast two hybrid experiments. Screening of 2.3 x 10^5 library clones with the LURP1-BD and LOR1-BD full length baits did not result in the identification potential interacting candidates. Further work on this is carried out by other members in the lab.

The second part of the project focused on examining defense mechanisms in tomato that involve WRKY70-like transcription factors and a member of the *LURP* cluster *CaBP22*. It was shown that *CaBP22* represent the

average profile of *LURP* genes expression in response to *Hpa* (Eulgem 2005). The previously constructed *CaBP22* promoter GUS fusion reporter construct *pCaBP22^{-333}::GUS* exhibited expression in response to *Hpa* in Arabidopsis.

Transgenic tomato lines (cultivar VFNT Cherry tomato) containing the *pCaBP22^{-333}::GUS* reporter gene responded in independent tomato lines to SA, BTH and DCA pointing to a mechanism of *LURP* regulation that is conserved between Arabidopsis and tomato. Phylogenetic analysis revealed *SI*WRKY70a and *SI*WRKY70b as the two tomato orthologs of Arabidopsis WRKY70. Three silencing constructs to specifically silence and co-silence *SIWRKY70a*, *SIWRKY70b* were made using Tobacco rattle virus (TRV). TRV-2 engineered-gateway compatible *SIWRKY70a*, *SIWRKY70b* and *SIWRKY70ab* vectors were constructed based on tomato genome sequence from SOL Genomics Network (http://sgn.cornell.edu). Gene specific *SIWRKY70a*, *SIWRKY70b* and *SIWRKY70ab* PCR products were inserted into TRV2 vector and transformed into *Agrobacterium tumefaciens* strain GV3101. TRV-*SIWRKY70a*, TRV-*SIWRKY70b* and TRV-*SIWRKY70ab* along with TRV1 were then infiltrated into two week old cherry tomato line #12 homozygous for *pCaBP22^{-333}::GUS* reporter gene. VIGS-induced silencing of *SIWRKY70a* and *SIWRKY70b* resulted in an incremental reduction of *GUS* expression in response to 2mM BTH as well as 3mM DCA as compared to equally-treated non-silenced plants, indicating that WRKY70-type transcription factors are required for mediating BTH- or DCA-responsive defense gene induction. Results presented in this chapter strongly

support that a regulatory mechanism dependent on WRKY70-type transcription factors is conserved between Arabidopsis and tomato. As members of the families of Brassicaceae and Solanacea, these two species represent a large evolutionary distance within the clade of eudicots. Thus, the function of WRKY70-type transcription factors appears to be at least partially preserved during the evolutionary processes leading to the diversification of eudicot species. This conserved WRKY70-dependent mechanism is inducible by the SA analog BTH and the new synthetic elicitor DCA, which also triggers the SA-dependent defense signaling branch.

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