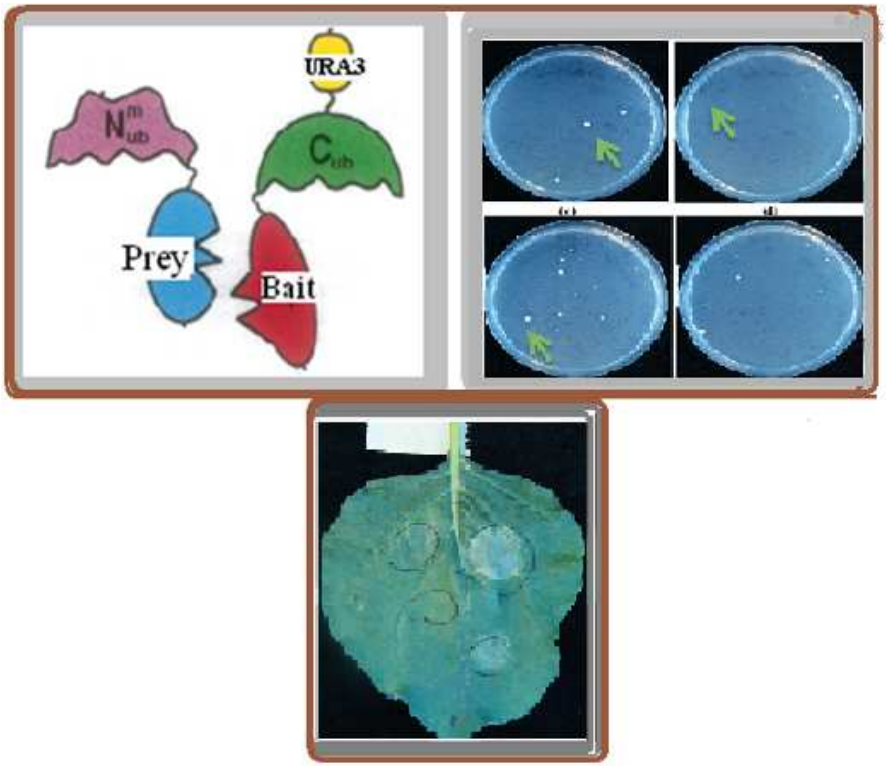
Identification of Tomato Cf-4 Interactors using the Split-Ubiquitin Yeast Two-Hybrid System and Preliminary Characterization of Proton Pump Interactor-1 (PPI-1)



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Master Program of Plant Science

Master Thesis report, (April, 2011)
Laboratory of Phytopathology, Wageningen University, Wageningen

Supervisor: T.W.H. Liebrand

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Abstract

Cf-4-mediated Avr4 recognition in the *Cladosporium fulvum*-tomato interaction was shown to require different signaling proteins to induce HR and mount resistance. Different studies have revealed the identity of some of these proteins and their signaling mechanisms towards resistance development. Nevertheless, the Cf-signaling pathway is poorly understood. To uncover novel Cf-4 interactors, screening of a cDNA library derived from Cf-4/Avr4 dying tomato seedlings was performed earlier using the split-ubiquitin yeast two-hybrid system. This resulted in the identification of previously known Cf-4 interactors and some promising new candidates.

To identify new Cf-4 interactors, the split-ubiquitin yeast two-hybrid technique was employed to screen a cDNA library of tomato dying seedlings. Cf-4 was used as bait and the proteins encoded by the cDNA library as preys. 75 positive colonies were picked out of 2.3 million screened clones. Subsequent confirmation of Cf-4 interactors with different techniques revealed 56 sequences of putative interactors. Among them 17 putative interactors were found in a correct reading frame with NuI- N terminal ubiquitin moiety of the prey vector. Three independent clones show strong homology to a proton pump interactor-1. This protein, which is referred to as Proton Pump Interactor-1 (PPI-1) has, coiled coil and transmembrane domains. Other candidate interactors also identified including Metallothionein-like protein, an ethylene receptor, pyridoxine biosynthesis protein isoform B, spermidine synthase-1 and others.

Even though they were not found in a correct reading frame, some more interactors were also depicted. The previously identified heat shock molecular chaperone (Hsp90-2) was identified with HATPase_c and coiled coil domains. Silencing studies were performed to unravel whether the proton pump interactor-1 (PPI-1) protein is required for Cf-4 function. The result showed in the reduction of hypersensitive response (HR) on *PPI-1* silenced *N. benthamiana:Cf-4* leaves up on agroinfiltration of Avr4. This suggests that PPI-1 might be required in Cf-4-mediated defense response.

List of Abbreviations

CC	Coiled Coil
Cub	C-terminus of ubiquitin protein
DO	Drop-Out media
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
eLRR	extracellular LRR
FLS2	Flagellin Sensing 2
5'FOA	5'Fluoro-orotic acid
HR	Hypersensitive Response
LRR	Leucine-Rich Repeat
MAMP	Microbe Associated Molecular Pattern
MAPK	Mitogen Associated Protein Kinase
Min	Minimal media (for yeast)
NB-LRR	Nucleotide Binding-Leucine-Rich Repeat
Nub	N-terminus of ubiquitin protein
PAMP	Pathogen Associated Molecular Pattern
pCup-NuI	pCup-NuI-GWY-CYC1 (split-ubiquitin prey vector)
PGIP	Polygalacturonase-inhibiting protein
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
RLK	Receptor-Like Kinase
RLP	Receptor-Like Protein
ROS	Reactive Oxygen Species
R protein	Resistance Protein
SAR	Systemic Acquired Resistance
TIR	Toll-Interleuking1 Receptor
TTSS	Type Three Secretion System
VIGS	Virus-Induced Gene Silencing
UBP	Ubiquitin Specific Protease
URA3	Orotidine-5´-phosphate decarboxylase (ODCase)

1. Introduction

1.1. Defense Mechanisms of Plants against Pathogens

Plants are exposed to a wide range of pathogens in nature and are under a continuous struggle to survive. Those plants with adequate defense mechanisms hinder pathogen growth and disease. In contrary, successful pathogens suppress the defense and facilitate their infection. Long time interaction and co-evolution between plants and pathogens result in various complex defense mechanisms in plants and diverse means of circumventing the defense by pathogens (Chisholm *et al.*, 2006).

1.1.1. PAMPs and PAMP Triggered defense responses

Plants have basal defense which recognizes highly conserved structures of pathogens called Pathogen-Associated Molecular Patterns (PAMPs; also known as Microbe-Associated Molecular Patterns, MAMPs) (Jones and Dangl, 2006; Nürnberger *et al.*, 2004). Such defense, referred as PAMP-triggered immunity (PTI), is mediated by recognition of PAMPs through Pattern-Recognition Receptors (PRRs) (Chisholm *et al.*, 2006; Jones and Dangl, 2006). PTI triggers defense response to inhibit pathogen growth and survival such as callose deposition, cell wall enforcement, non-specific necrosis, accumulation of pathogenesis related proteins like chitinases, glucanases and proteases (Deising *et al.*, 2009). Bacterial flagellin and chitin, a major component of fungal cell walls, are well known microbial PAMPs. Flagellin is recognized by Leucine rich repeat (LRR) domain of the Receptor-Like Kinase Flagellin-Sensing 2 (FLS2) in Arabidopsis (Gómez-Gómez and Boller, 2000). Chitin is believed to bind to the LysM domains of LysM receptor-like kinases in Arabidopsis (Miya *et al.*, 2007).

1.1.2. Effectors and Effector Triggered Immunity (ETI)

Pathogens evolve mechanisms to circumvent recognition by the basal defense of plants through secretions of proteins called effectors. These proteins manipulate host defense and result in susceptibility to the pathogen, also referred as Effector-Triggered susceptibility (ETS) (Chisholm *et al.*, 2006; Jones and Dangl, 2006). Plant pathogenic bacteria are known to use Type Three Secretion System (TTSS) to translocate their effectors to the host cells. Yet in fungi the exact mechanism is not understood. In nature, certain plant species have capacity to recognize effectors through their resistance proteins (RP). This recognition activates defense signaling and leads to hypersensitive response (HR) and resistance. Hypersensitive response is a programmed cell death response at the site of infection to restrict the growth and spread of pathogens that leads to starvation and death of the pathogen. Generally, R protein-mediated resistance through recognition of effectors is called Effector triggered Immunity (ETI) (Chisholm *et al.*, 2006; Jones and Dangl, 2006).

Recognition and perception of effectors is followed by downstream signaling to end up with localized cell death or associated defense response. Posttranslational modification of signaling proteins is a known mechanism in signal transfer which alters the activity and localization of signaling proteins. Of this Mitogen activated protein kinase (MAPK) cascade is shown to involve in signal transmission from recognition to the defense response (Pedley and Martin, 2004).

Through a co-evolutionary arms race among plant and pathogen for survival, pathogens are believed to avoid or decrease recognition by mutation or complete removal of their effector in their genome. Alternatively, they suppress ETI by developing novel effectors. On the other hand novel R proteins are developed in plants to recognize these new effectors. This natural selection enables the development of new set of effectors and R proteins in plant-pathogen interaction (de Wit, 2007; Jones and Dangl, 2006).

Generally based on domain organization and composition, R proteins are classified as Nucleotide Binding-Leucine-Rich Repeat (NB-LRR) and extracellular LRR (eLRR) proteins. The former is the most abundant and best characterized class which in turn subdivided in to Coiled-Coil (CC) - NB-LRR and Toll-interleukin-1 receptor (TIR) - NB-LRR subclasses according to their amino terminus (Chisholm *et al.*, 2006). LRR seem to be involved in protein-protein interaction while N- terminus functions as signaling domain (Feys and Parker, 2000). The eLRR class consists of Receptor Like Proteins (RLPs) and Receptor Like Kinases (RLK). Generally, RLP composed of extracellular LRR domain and transmembrane domain. RLK consists of extracellular LRR domain, transmembrane domain and cytoplasmic kinase domain. RLK vary RLP with its cytoplasmic kinase domain, which is crucial for downstream signaling (Fritz-Laylin *et al.*, 2005).

Effector recognition by R proteins could be directly or indirectly. Direct recognition involves the direct binding of R and effector proteins which initiate subsequent downstream signaling. However, indirect recognition employs virulence target proteins from the plant as an intermediate proteins, this is also named as the guard model. The guard model is observed more frequently than the direct recognition model (Chisholm *et al.*, 2006; Jones and Takemoto, 2004). As of guard hypothesis, effector proteins believed to bind with the virulence target by which R proteins monitor the virulence proteins (Dangl and Jones, 2001; van der Hoorn *et al.*, 2002). Indirect recognition enables a single R protein to detect several unrelated effectors, while effectors with common structural motifs are recognized by a single R protein in case of direct recognition (Chisholm *et al.*, 2006).

Beside the above mentioned defense response, plants have also alternative way of tackling pathogens called systemic acquired resistance (SAR), which enables the defense of subsequent secondary infection at a distance from the site of infection. This resistance involves plant hormones

such as salicylic acid, jasmonic acid, ethylene, or abscisic acid which is effective against wide range of pathogens (Grant and Lamb, 2006).

1.2. The *Cladosporium fulvum* - Tomato Interaction

Cladosporium fulvum is a biotrophic, asexual, extracellular fungal pathogen which causes leaf mold in tomato (Deising *et al.*, 2009; Stergiopoulos and de Wit, 2009; Thomma *et al.*, 2005). The interaction between *C. fulvum* and tomato is well studied and follows the gene-for-gene concept (Flor, 1942) which explains the complementation of an avirulence gene in pathogens with its cognate resistance gene in the host plant leading to a Hypersensitive Response (HR) and resistance. HR is a programmed cell death which is highly effective against biotrophic pathogens like *C. fulvum.* Generally the fungus infects leaves of tomato, penetrates through stomata and resides in the extracellular space. Although in rare case *C. fulvum* also attacks stems, blossoms, petioles and fruit. The typical symptoms include initial pale yellow spot in the upper leaves which in severe infection lead to wilting, partial defoliation and death of infected tissue. (Thomma *et al.*, 2005).

Four avirulence proteins *(*Avr2, Avr4, Av4E and Avr9*)* were cloned from *C. fulvum*, which are small cysteine-rich proteins secreted during infection (Thomma *et al.*, 2005). These effector proteins contains even numbered cysteine residues. The cysteine residues are linked to each other with disulfide bonds that gave stability to effectors against degrading enzymes in apoplast (Rooney *et al.*, 2005; van den Burg *et al.*, 2003; van Esse *et al.*, 2008). Further researches came up with the identification of cognate resistance proteins in tomato which recognize the respective effector proteins (Cf-2.2, Cf-4, Cf-4E and Cf-9) reviewed in (Deising *et al.*, 2009). Tomato Cf proteins are from the well characterized RLP class which lacks signaling domain and believed to interact with other protein for downstream signaling (Kruijt *et al.*, 2005).

Among *C. fulvum* effectors, Avr4 is shown to bind and protect the fungal cell wall against plant chitinase. It is also required as a virulence factor (van den Burg *et al.*, 2006; van Esse *et al.*, 2007). Avr2 inhibit cysteine protease (Rcr3) secreted by the plant resulting to its detection by Cf-2, which is a good example for indirect recognition. Although the exact mechanism of this perception is not yet known (Rooney *et al.*, 2005). The role of Avr4E and Avr9 is not understood till now. In case of Cf-9-mediated Avr9 recognition, it is hypothesized that the Avr9 High affinity binding site (HABS) is believed to be required (Kooman-Gersmann *et al.*, 1998). For Avr4 and Avr4E, their recognition mechanism is not elucidated and efforts are undergoing (M. Joosten and T. Liebrand, personal communication). Following Avr4 recognition by Cf-4, signaling cascades are initiated and passed down to the defense responses. Based on the current understanding, proposed signaling pathways required in Avr4-triggered Cf-4-mediated defenses are described (Figure 1) (Stergiopoulos and de Wit, 2009)

In addition to the Avrs, six extracellular proteins (Ecp1, Ecp2, Ecp4, Ecp5, Ecp6 and Ecp7) were characterized from *C. fulvum.* Ecps *are* secreted during infection and suggested to be virulence factors. For Ecp1, 2, 4 and 5, tomato lines carrying cognate *Cf-Ecp* genes were identified though the respective gene was not cloned yet. On the other hand, no tomato lines were found to induce HR for Ecp6 and Ecp7 (Bolton *et al.*, 2008; de Kock *et al.*, 2005; Laugé *et al.*, 2000). Ecp6 is a LysM domain containing effector. Recently Ecp6 has been shown to bind chitin oligosaccharides released from fungal cell wall during invasion and prevent chitin-triggered host immunity (de Jonge *et al.*, 2010).

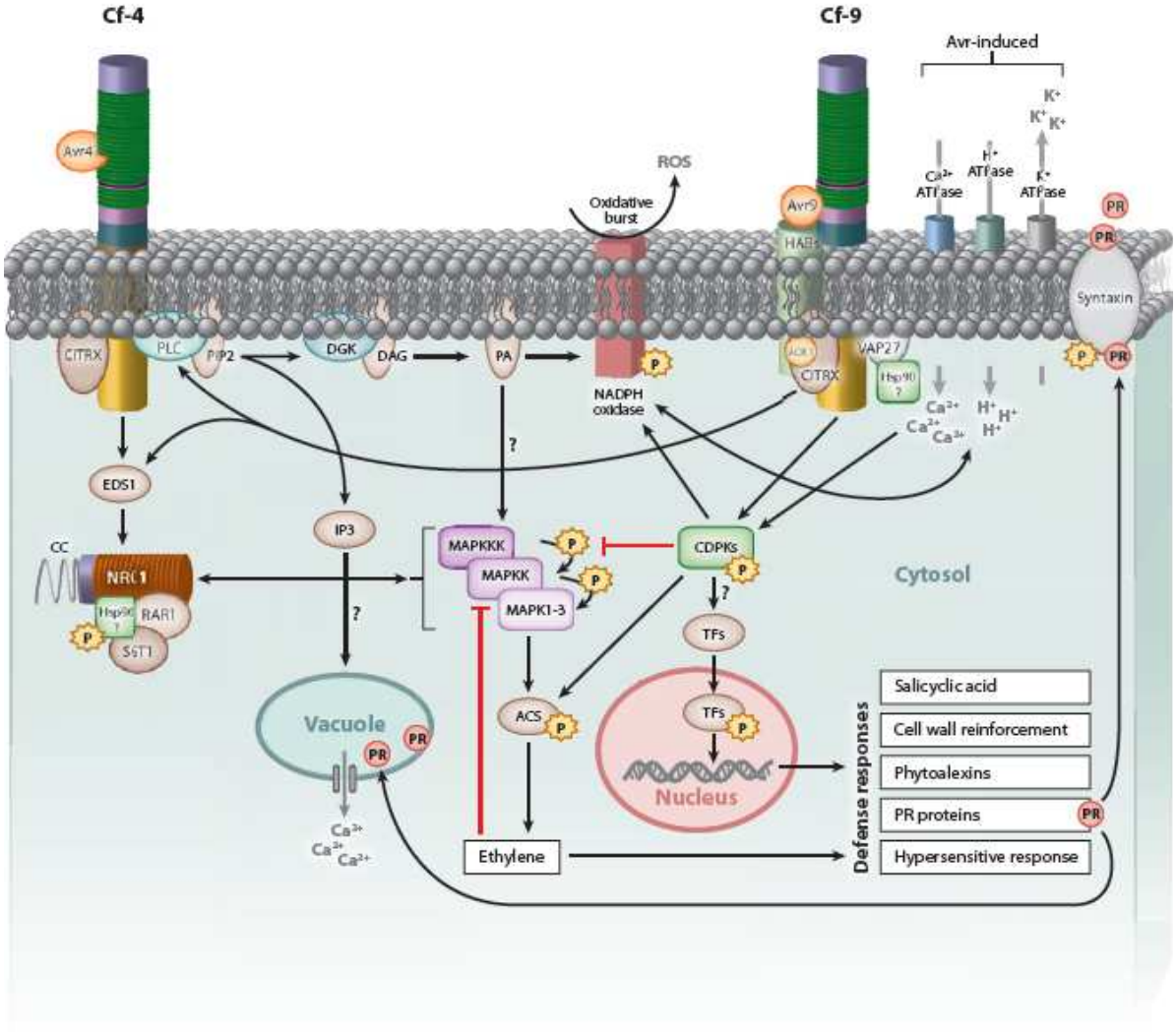

Figure 1. This picture was taken from the review of (Stergiopoulos and de Wit, 2009). It describes the downstream signaling pathways and defense responses of Cf-4 and Cf-9-mediated resistance up on perception of Avr4 and Avr9, respectively. Cf-4-mediated resistance requires EDS1, NRC1, SGT1, RAR1 and Hsp90. MAPK involved in signaling cascades. PM H^+-ATPase are activated and suggested to pump in H^+ to the cytoplasm. **Abbreviations**: DAG, diacylglycerol; DGK, diacylglycerol kinase; EDS1, enhanced disease susceptibility-1; IP3, inositol triphosphate; MAPK1-3, mitogen activated protein kinase-1-3; NADPH-oxidase, nicotinamide adenine dinucleotide phosphateH-oxidase; NRC1: NB-LRR protein required for HR-associated cell death-1; PA, phosphatidic acid; PLC, phospolipase C; PIP2, phosphatidyl inositol disphosphate; PR, pathogenesis-related; RAR1, required for Mla12 resistance-1; ROS, reactive oxygen species; SGT1, suppressor of G-two allele of Skp-1; TFs, transcription factors .

1.3. Aim of the Study

Direct recognition of Avr4 by Cf-4 has never been shown till now in *C. fulvum*-tomato interaction. Regardless of its undiscovered recognition mechanism, Cf-4-mediated resistance to Avr4 recognition was shown to require different signaling proteins to induce HR and mount resistance. Different studies revealed identities of the signaling proteins and their mechanisms in Av4 triggered Cf-4-mediated resistance, yet the Cf-signaling pathways is poorly understood (Deising *et al.*, 2009). To uncover new Cf-4 interactors, cDNA screening of dying tomato seedling was done earlier with split-ubiquitin yeast two-hybrid. The result showed in identification of previously known Cf-4 interactors and promising candidate interactors (T. Liebrand, personal communication).

Thus, this study aims to identify new tomato Cf-4 interactors and characterize Proton Pump Interactor-1 (PPI-1), which might be required in downstream defense signaling upon Avr4 recognition. Identification of Cf-4 interactors may reveal further explanation to the current knowledge of Avr4 triggered Cf-4-mediated defense response. Split-ubiquitin yeast two-hybrid system was employed for the elucidation of the interactor proteins. An unbiased screen was performed using cDNA library derived from Cf-4/Avr4 crossed dying tomato seedling. For the characterization of *PPI-1*, Virus-Induced Gene Silencing was performed. In general, in this study we aim to understand the perception and subsequent downstream signaling mechanisms of plants to extracellular pathogens like *C. fulvum*.

2. Materials and Methods

2.1. The Split-Ubiquitin Yeast Two-Hybrid System

Split-ubiquitin system is an alternative to the conventional yeast two-hybrid technique, which utilizes reassembling of N-terminus (Nub) and C-terminus (Cub) of ubiquitin protein through interaction of bait and prey proteins (Johnsson and Varshavsky, 1994). Ubiquitin is a conserved protein of 76 amino acids found in many eukaryotic cells mainly involve in breakdown of proteins in the cell (Sharp and Li, 1987).

In general, this system functions with the expression of Nub as fused to prey protein and Cub fused to bait and reporter proteins in a single yeast cell. In this study, URA3 was used as a reporter protein with arginine in position 1 (R-URA3). The URA3 is a gene encoding Orotidine-5´-phosphate decarboxylase, an enzyme involved in biosynthesis of uracil. It also converts the non-toxic 5'-fluoroorotic acid (FOA) into a toxic 5'-fluoro-uracil compound. Interaction between bait and prey proteins results reconstruction of the ubiquitin protein. Subsequently URA3 is cleaved by ubiquitin specific protease (UBP). The arginine N- terminus leads the degradation of URA3 with the principle of N-end rule (Varshavsky, 1996). During bait and prey protein-protein interaction, yeast cells will be uracil auxotroph and insensitive to FOA. Protein-protein interaction is monitored through the growth of yeast on FOA containing media. Stable bait expression can also be confirmed by evaluating yeast growth on uracil deficient medium (Johnsson and Varshavsky, 1994; Müller and Johnsson, 2008).

In this study, Cf-4 (bait) is fused to Cub and URA3 and expressed using the pMET-GWY-Cub-URA3-CYC1 vector, which contains a Histidin selection gene. Nub was N-terminally fused to cDNA of dying tomato seedling (prey) and expressed using the pCup-NuI-GWY-CYC1 vector possessing the tryptophan selection gene. Both vectors fused with their respective bait and prey sequences were obtained from T. Liebrand (personal communication).

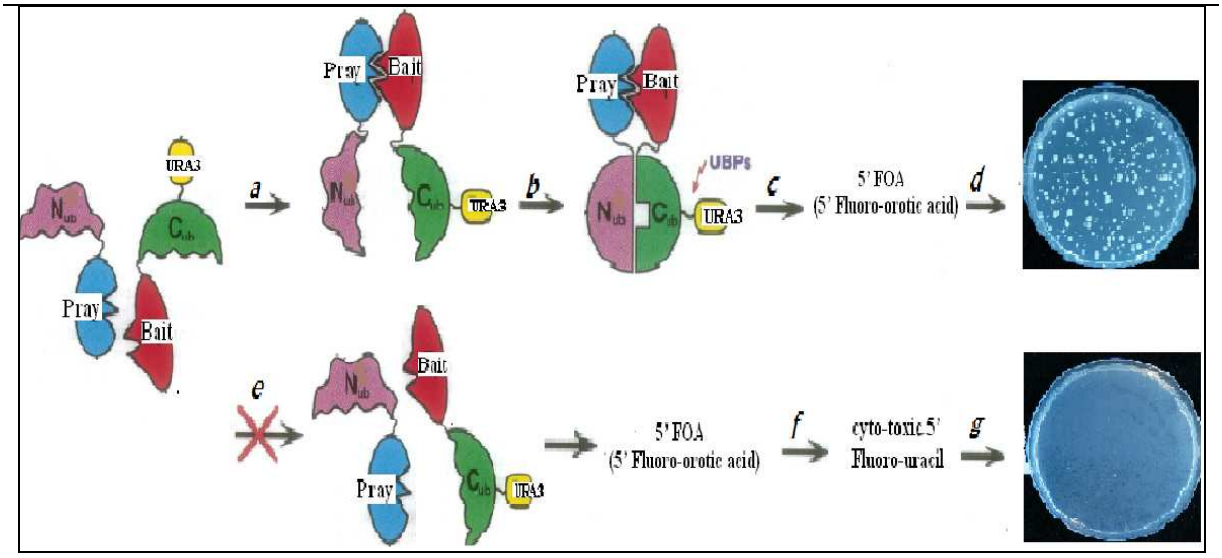

Figure 2. Schematic presentation of Split- Ubiquitin Yeast Two-Hybrid System, Ubiquitin N terminus (Nub) fused to prey protein, Ubiquitin C-terminus (Cub) fused to bait and Reporter protein (URA3). (a) when bait and prey proteins interact, (b) Nub and Cub reconstitute, Ubiquitin specific proteases (UBPs) recognize and cleave URA3, subsequently URA3 will be degraded (c) FOA (non-toxic to yeast) remain unconverted in the media (d) yeast colonies survive, (e) when bait and prey proteins does not interact (f) URA3 remain intact, functional and convert FOA to cyto-toxic 5Fluro -uracil, (g) yeast cells will be killed by the toxic (taken from (Johnsson and Varshavsky, 1994) with slight modification).

2.2. LR Gateway® Cloning of *pMET-peru2* Yeast Two-Hybrid Bait Vector

LR Gateway® cloning of *pMET-peru2* was done between pDonor201-peru2 (SOL2518) and pMet-Gwy-cub-URA3 (SOL2590). The following LR Gateway® reaction mixture was made; 4µl entry/donor vector, 1µl destination vector, 0.5µl 10X TE buffer pH 8 (10mM Tris and 1 mM EDTA), and 0.3µl LRII (LR clonase enzyme mix II). It was incubated at 25^{0}C for 4 hours. Then 0.5µl protinase K was added and incubated for 15 minutes at 37^{0}C to stop the reaction. 2µl of LR Gateway® reaction mix was transformed to electrocompetent DH5α *E. coli* cells (Appendix D). Then, it was plated on L- medium containing 100µg/ml Ampiciline and incubated overnight at 37^{0}C. Plasmid was isolated from three *E. coli* colonies based on Qiagen miniprep kit protocol (Appendix E).

2.3. Miniprep of Bait Vectors, Digestion and Gel Electrophoresis

DH5α strain of *E. coli* expressing plasmids (*pMET-Cf-4(1), pMET-peru2*, *pMET-Cf-2.2* and *pMET-AtFLS2*) and *Saccharomyces cerevisiae* (strain JD53) were obtained from T. Liebrand (personal communication). Each *E. coli* was cultured overnight in 4 ml liquid L- medium containing 100µg/ml ampicillin at 37^{o}C. Plasmid was isolated based on Qiagen miniprep kit protocol (Appendix E). 500µl of overnight *E. coli* culture was mixed with 500µl glycerol (50 %) and put on -80 ^{0}C for later use and long term storage.

Plasmid digestion and gel electrophoresis was done to confirm the correctness of the plasmid. Using vector NTI version 8 software, *Nhe I* and *AfI II* restriction enzymes were selected and used together with FD digestion buffer for all plasmid digestion. 1µl restriction buffer (10X), 8.4µl each vector (155ng/ul), 0.3µl *Nhe I* and 0.3µl *AfI II* restriction enzymes were mixed, spin down shortly and incubated at 37^{o}C for 2 hours. The digestion mixture was loaded and run on 1% Agarose gel. The bands were compared with the expected number and size of band from the vector NTI software. Plasmids with the correct fragment size will be used for subsequent experiments.

2.4. Yeast Transformation with Bait Vectors and Bait Stability Test with Dilution Spot

JD53 yeast strain were grown overnight on 10 ml liquid YEPD media at 28^{0}C. 1µl (Suppl. Table 4) of plasmids (*pMET-Cf-4(1), pMET-Cf-2.2*, *pMET-AtFLS2* and *pMET-peru2*) were used for yeast transformation following the protocol on Appendix B. Then it was plated on DO-H media (Drop-Out media without Histidine) and incubated at 30^{0}C for 2-3 days. Untransformed yeast was plated on the same media as a negative control.

Five colonies from each transformation were streaked to new DO-H media and grow for 1-3 days. Evaluation of transformed yeast bait stability was performed with the dilution spot test. Each colony were grown overnight on 4 ml liquid DO-H media at 30^{o}C with 210 rpm. Dilution series of (undiluted, 10X, 10^{2}X, 10^{3}X, 10^{4}X and 10^{5}X) were made. 3µl of each dilution series was spotted on min-HU media (minimum medium without histidine and uracil) and incubated at 30^{o}C. Colony growth was monitored under different dilution series and picture was taken.

2.5. Screening of a Tomato cDNA Library

For the cDNA screen, Split- Ubiquitin Yeast Two-Hybrid System with Cf-4 as a bait and proteins encoded by tomato cDNA library as prey was used. cDNA library was prepared from crossed Cf-4/Avr4 dying tomato seedling (T. Liebrand, personal communication). Stable Cf-4 yeast transformant from section 2.2 were grown overnight in 10ml liquid DO-H media at 30 ^{0}C. We followed the protocol for library scale yeast transformation (Appendix C). OD_{600} of overnight culture was measured (2.7) and 50ml YEPD media was inoculated with 3.6ml overnight culture. Then it was incubated at 28 ^{0}C for 4 ½ hours. The OD_{600} was measured again to check the proper cell density (1.0).

Nine independent cDNA transformations were done with 1 µl of cDNA plasmid (1 µg/µl). As a negative control, *pCup-NUI-HA-GWY-CYCI* (SOL2591) transformation was performed with 1.5 µl (100 ng/µl) plasmid. Each transformation mixture was plated on separate min-HT (without Histidin and tryptophan) plate with FOA (1 gm. FOA/ L of min-HT media, appendix A). To calculate the transformation efficiency, 2 µl of cDNA transformation mixture was mixed with 198 µl water and plated on DO-HT media. All the plates were incubated at 30 ^{0}C. Colony count was taken for the plates with 2 µl of cDNA transformation mix. Colonies from the cDNA screening plates were picked and streaked to new DO-HT media. Then it was grown for 3-5 days to be used for subsequent experiments.

2.5.1. Confirmation of Positive Colonies with Dilution Spot Test, Yeast Miniprep and Transformation to *E. coli*

All colonies picked from the cDNA screening were grown overnight on 4 ml liquid DO-T (Drop-Out media without Tryptophan) media at 30 ^{0}C. To confirm positive colonies, dilution spot test was done and plated on min-HT media with FOA as mentioned on section 2.4. Positive colonies from the dilution spot test were grown overnight in 4ml liquid DO-T media under 30 ^{o}C. Plasmid isolation was done from the yeast colonies with Zymoprep™ II -Yeast Plasmid Miniprep kit (Appendix F). Each plasmid was then transformed to electro competent DH5α *E. coli* cells and plated on L medium containing 100µg/ml Ampiciline (Appendix D). the plates were incubated overnight at 37^{o}C.

2.5.2. Colony PCR and *E. coli* Miniprep

To differentiate colonies containing cDNA fragment insert and to estimate the cDNA insert size, colony PCR was done. NuIfw and CYC1rev primers was used that binds to NuI and CYC1 sites of the prey vector, respectively (Table 1). The colony PCR was performed for eight colonies from each transformation. The PCR products were run on 1% agarose gel. For each colony, we used 1x PCR mixture of 5 µl green go tag buffer, 0.5 µl fw and 0.5 µl rev primers, 0.5 µl dNTPs, 0.08 Tag Polymerase and 18.4 µl milliQ water. The following program was used for running the PCR; 95^{o}C for 3 minutes, 95^{o}C for 30 seconds, 53^{o}C for 30 seconds, 72^{o}C for 4 minutes, step 2 to 4 was done 25 cycles and lastly 72^{o}C for 30 seconds and 15^{o}C forever. Positive colonies were overnight grown in 4ml liquid L medium containing 100µg/ml Ampiciline and plasmid isolation was done with Qiagen Miniprep kit (Appendix E).

2.5.3. Co-transformation and One to One Interaction of each cDNA fragment containing plasmids with Gus–Cub Bait Vector to asses Autoactivity

To identify cDNA fragment insert with autoactivity, co-transformation and one to one interaction was studied with pMet-GUS-Cub-Ura3. Co-transformation of each plasmid containing candidate cDNA fragment insert (from section 2.5.2) and pMet-GUS-Cub-Ura3 was done to JD53 yeast. 1 µl of both plasmids (Supp. Table 5) were used for the transformation (Appendix B). The transformation mix was plated on DO-HT media and incubated at 30^{o}C for 2 days. Dilution spot of 4 colonies was done from each co-transformation and plated on min-HT containing FOA as described in section 2.4. Yeast growth was monitored and those plasmids that did not show yeast growth were identified. The cDNA fragment insert from those plasmids were sequenced (Eurofins MWG Operon, Germany). Sequences were analyzed with NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome), SGN (http://solgenomics.net/tools/blast/index.pl), expasy translator of nucleotide to protein sequences(http://expasy.org/tools/dna.html), SMART protein domain prediction (http://smart.embl-heidelberg.de/).

2.6. Construction of vectors for Virus-Induced Gene Silencing and Co-Immunoprecipitation assay of *PPI-1*

Full length gene (1884bp) and VIGS fragment (405bp) of proton pump interactor-1 was obtained using standard PCR from tomato cDNA library. For the full length gene, ao1fw and ao2rev primers were used. The 5` end of ao1fw primer contains 4 bases (CACC) which was designed for the directional TOPO® cloning. For the VIGS fragment, ao3fw and ao4rev primers containing *EcoRI* and *BamHI* flanking site were used respectively (Table 1). We used 1x PCR mix of 2 µl fw and 2 µl rev primers, 3 µl tomato cDNA library, 1 µl dNTPs, 0.6 µl phusion enzyme, 10 µl HE buffer and 33.5 µl sterile water. We used the following PCR protocol; 98^{o}C for 3 minutes, 98^{o}C for 30 seconds, 53^{o}C for 30 seconds, 72^{o}C for 130 seconds, step 2 to 4 was done 40 cycles and lastly 72^{o}C for 30 seconds and 15^{o}C forever. Gel electrophoresis was done with the PCR product and a DNA band was isolated from agarose gel with Illustra isolation kit (Appendix G).

Gel isolated DNA for both full gene and VIGS fragment was TOPO® cloned into pENT-D-TOPO® and pCR4-TOPO® vector respectively. TOPO® cloning mixture of 3µl PCR fragment, 0.25µl salt, 1.75µl sterile water, 0.25µl pCR4-TOPO® or pENT-D-TOPO® was incubated for 2-4 hours at room temperature. 2µl of TOPO® cloning mix was transformed to electrocompetent DH5α *E. coli* cells (Appendix D). It was plated on L-medium containing 50µg/ml kanamycin and incubated overnight at 37^{o}C. To identify positive colonies, we did colony PCR for 20 colonies from each transformation. We used ao1fw and to30rev primers for the full gene and wo13fw and ao4rev for the VIGS fragment.

The procedure mentioned in 2.5.2 with 2.10 minutes of extension time was used. Two positive colonies from each transformation were overnight grown on 5 ml liquid L- medium with 50µg/ml kanamycin. Plasmid was isolated using Qiagen Miniprep kit. Both full length gene and VIGS fragment inserts were sequenced to verify its similarity with the original sequence obtained from the cDNA screening (Eurofins MWG Operon, Germany). We used M13-21, T-7 and ao4 primers for the full length *PPI-1* gene and T-3 primers for VIGS fragment.

TOPO® cloned vector containing VIGS fragment (pCR4-TOPO®-VIGS-*PPI-1*) was digested with *EcoRI* and *BamHI* with buffer E to obtain the VIGS fragment. We used digestion mixture of 40µl plasmid, 3µl *EcoRI*, 3µl *BamHI* and 5µl buffer E. DNA band was cut from agarose gel and DNA isolation was done with Illustra isolation kit (Appendix G). Then, the VIGS fragment was ligated to pTRV2 vector which was digested with *EcoRI* +*BamHI*. Ligation mix of 4µl pTRV2 vector, 5µl VIGS fragment, 1µl 10X ligase buffer, 1µl ligase enzyme was used. The ligation mix was incubated overnight at 16^{o}C in a water bath and transformed to electrocompetent DH5α *E. coli* cells. Colony PCR was done for 24 colonies with wo138fw and ao4rev primers as mentioned in section 2.5.2 but 45 second of extension time. Plasmid was isolated from single positive colony. 0.3µl plasmid was transformed to electrocompetent *Agrobacterium tumifaciens* cells, strain C58c1 with helper plasmid pch32 (Appendix D). The transformation mix was plated on L- medium containing 50µg/ml Kanamycin and 5µg/ml tetracycline. The plate was incubated at 28^{o}C for 2-3 days.

TOPO® cloned vector containing the full length *PPI-1* gene (pENT-D-TOPO®-full length *PPI-1*) was linearized by digesting with *PvuI* which cuts the Kanamycin resistance gene. Digestion mix of 30µl plasmid, 4µl Neb3 and 4µl BSA buffer, 2µl*PvuI* was incubated for 3 hours at 37 ^{o}C. The Linearized band was isolated from the gel with Illustra isolation kit (Appendix G). LR Gateway® cloning was done to pGWB20-10xMyc destination vector with both the linearized and the circular pENT-D-TOPO®-full length *PPI-1* vector. LR Gateway® reaction was performed as described in section 2.2. 2µl LR Gateway® reaction mix was transformed to electrocompetent DH5α *E. coli* cells. The transformation mix was plated on L-medium containing 50µg/ml Kanamycin and 100µg/ml Hygromycin. Colony PCR was done with wo17fw and ao2rev primers as mentioned in section 2.5.2.

2.7. Virus-Induced Gene Silencing of *Proton Pump Interactor-1 (PPI-1)*

For VIGS, we used a 1:1 mixture of pYL156:TRV-RNA2 derived constructs and pYL156:TRV-RNA1 vector described by (Liu *et al.*, 2002a; Liu *et al.*, 2002b). The following pYL156:TRV-RNA2 derived constructs were used. *PPI-1*-pTRV2 (cloned as described before in section 2.6). *Cf-4*-pTRV2 (Gabriëls *et al.*, 2006), SGT1-pTRV2 (Peart *et al.*, 2002), GUS-pTRV2, PDS-pTRV2, SOL2730-pTRV2, SOL2731-pTRV2, SOL2732-pTRV2, SOL2733-pTRV2, SOL2734-pTRV2, SOL2735-pTRV2, SOL2769-pTRV2 and SOL2771-pTRV2, obtained from T. Liebrand (personal communication). The target of the following VIGS constructs are mentioned in the bracket. SOL2730 (BiP1), SOL2731 (BiP2), SOL2732 (BiP3), SOL2733 (SDF2), SOL2734 (Erdj3b), SOL2735 (CRT3), SOL2769 (CRT1) and SOL2771 (all BiPs).

Agrobacterium expressing the above mentioned pTRV2 derived constructs and pTRV1 vector were grown overnight in 10 ml liquid L- medium containing 50µg/ml Kanamycin and 5µg/ml tetracycline. It was incubated at 28^{o}C for 1 day. The overnight cultures were spin down at 4000rpm for 15 minutes. Resuspend with 5ml MMAi containing 39.3mg/ml acetosyringone and grown at 28^{o}C for 1 hr. MMAi containing acetosyringone was made of 20g/L sucrose, 5g/L salt mixture MS without vitamins, 10ml/L 1M Mes PH 5-6 and 1ml/L acetosyringone. $OD_{600\ was}$ measured and it was diluted to OD_{600} = 2. Each VIGS construct and pTRV1 was mixed in a 1:1 ratio and agro-infiltrated to two leaves of 18 days old *Nicotiana benthamiana:* Cf-4 plant. The VIGS assay was performed twice. In total, six *N. benthamiana:Cf-4* plants were used for silencing of *PPI-1*. As a control, Cf-4, SGT1, Gus and PDS silencing were done on five, four, three and two *N: benthamiana:* Cf-4 plants, respectively. In addition, silencing of SOL2735 and SOL2771 constructs was done on two *N: benthamiana*: Cf-4 plants. Silencing of SOL2730, SOL2731, SOL2732, SOL2733, SOL2734 and SOL2769 constructs were done on one *N: benthamiana*:Cf-4 plant each.

Three weeks after agro-infiltration, four leaves per plants were challenged with *agrobacterium* expressing pMog808-Avr4 (OD_{600}=0.03), BAX (OD_{600}=0.5), pBin61-RxD460v (OD_{600}=0.1) and apoplastic fluid containing Avr4 and Avr9. *Agrobacterium* expressing pBin61-RxD460v and BAX were overnight grown in 10ml liquid L- medium containing 50µg/ml kanamycin and 10µg/ml rifampicin. For *agrobacterium* expressing Avr4, was grown in L- medium containing 50µg/ml kanamycin and 5µg/ml tetracycline. The apoplastic fluid was derived from compatible interaction of *C. fulvum* race 5 and MM: Cf-0 (M. Joosten, personal communication). Three to four days after agro-infiltration or apoplastic fluid injection, leaves were examined for HR development. HR score and picture was taken.

2.8. Evaluation of Avr4 Expression in Yeast and Double Yeast Transformation with *pZmL-Avr4* and *Cf-4*

2.8.1. LR Gateway® Cloning of *pZmL-Avr4* and *E. coli* Miniprep

For evaluation of *Avr4* expression in yeast, *pZmL-Avr4* was LR Gateway® cloned from pEntry-Avr4 (SOL2646) and pZmL-dest (SOL2718) vectors. LR Gateway® reaction was done with the same procedure in section 2.2. 2µl of LR Gateway® reaction mix was transformed to electro competent DH5α *E. coli* cells (Appendix D). The transformation mix was plated on L- medium containing 50µg/ml spectinomycine and incubated overnight at 37 ^{0}C.

Three *E. coli* colonies were grown overnight in 5 ml liquid L- medium containing 50µg/ml spectinomycine. Plasmid isolation, digestion and gel electrophoresis was done with the same procedure in section 2.3. *BamHI* and *XhoI* restriction enzymes with Buffer B were used for the digestion. Number and size of bands were compared with the expected bands from vector NTI. Plasmid with the correct *pZmL-Avr4* insert was used for the subsequent yeast transformation.

2.8.2. Yeast Transformation and Infiltration Assay

Yeast transformation was carried out to JD53 yeast strain with *pZmL-Avr4* and pZmL-dest. plasmids as mentioned in section 2.4 (Appendix B). The transformation mix was plated on DO-L media (without Leucine) and incubated at 30^{0}C for 2-3 days. Untransformed yeast was plated as a negative control.

A single yeast colony from both transformations was overnight grown in 10ml liquid DO-L media. The overnight culture was spin down at 4000rpm for 5 minutes. The top 5 ml of the supernatant was used for the infiltration assay. As a positive control, *Agrobacterium* expressing pMOG800-Avr4 was prepared and infiltrated as described in section 2.7 (OD_{600}=0.5). Liquid DO-L media was infiltrated as a negative control. The infiltration was done on four *N. benthamiana:Cf-4* and four Wild *N. benthamiana* plants of 4 and 6 weeks old, respectively. Four leaves were infiltrated on each plant and all the four treatments were infiltrated on each leaf. HR score was taken for the first 3 days and a picture was taken. The treatments were (A) *Agrobacterium* transformed with *pMOG800-Avr4*, (B) supernatant of *pZmL-Avr4* yeast culture, (C) supernatant of pZmL-dest. yeast culture and (D) liquid DO-L media.

2.8.3. Double Yeast Transformation with *pZmL-Avr4* and *pMET-Cf-4*

To monitor the possibility of expressing both Avr4 and Cf-4 in a single yeast cell, double yeast transformation was done with *pZmL-Avr4* (SOL3000) and *pMET-Cf-4* (SOL2557) plasmids. The double transformation was done by transforming yeast with *pMET-Cf-4* and subsequently re-transforming with the *pZmL-Avr4* plasmid or vice versa. *pMET-Cf-4* and *pZmL-Avr4* containing yeast was grown overnight in 10ml liquid DO-H and DO-L media respectively. 1µl of *pZmL-Avr4* plasmid was used to re-transform the *pMET-Cf-4* containing yeast and vice versa following yeast transformation protocol (appendix B). The transformation mix was plated on DO-HL media and incubated at 30 ^{0}C. As a negative control, yeast transformed with either *pMET-Cf-4* or *pZmL-Avr4* was plated on DO-HL media. Bait stability of transformed yeast was carried out for 5 positive colonies with the protocol mentioned on section 2.4. 3µl of the dilution series was spotted on min-HLU media and incubated at 30 ^{0}C. Picture of colony growth was taken after 2-3 days.

2.9. Tomato transformation with Cf-4-eGFP

Tomato transformation was done to generate transgenic tomato plants expressing Cf-4-eGFP (done together with T. Liebrand). About 300 tomato seeds of MoneyMaker-Cf-0 (MM-Cf-0 lines) were sterilized by rinsing for 1 minute in 70% ethanol, twice for 15 minutes in commercial thick bleach and 3 times for 10 minutes in sterile water. The sterilized seeds were sown on Murashige and skoog medium (0.5XMS-10). It was germinated at 25^{o}C for 18h light and 6 h dark. About 12 seed were put in each pot. 0.5xMS-10 medium was composed of 0.5xMS including vitamins, 10g/L sucrose, 8g/L Micro agar and PH adjusted to 5.8 with KOH. After 10 days, *Agrobacterium* strain gv3101 containing pBIN-KS-Cf-4-eGFP plasmid was pre cultured in 2ml LB media containing kanamycin at 50µg/ml and rifampicin at 10µg/ml. Next day 200µl of the pre culture was inoculated to 50ml of LB media with the same antibiotic and grown overnight.

After 11 days of sowing, the cotyledon was cut in two pieces (explants) and placed them upside up on standard Petridish with R3B-medium covered by sterile filter paper. Around 50 explants were put on each Petridish and incubated at the same temperature and light condition for 24 hours. The R3B medium was composed of 1XMS, 20g/L sucrose, 2mg/L NAA, 1mg/L BAP, 8g/L micro agar and PH adjusted to 5.8 with KOH. After 12 days of sowing, the 50ml overnight *Agrobacterium* was OD_{600} of around 1.2. The culture was spin down and resuspend in 1XMS-20 to OD_{600} = 0.14. 200µl of acetosyringone at 200mM was added to the resuspend *Agrobacterium.* Then, the tomato explants were incubated for 15 minutes in the *Agrobacterium* suspension by transferring the filter paper to bacterial suspension containing Petridish. The bacterial suspensions was dried with stack of filter paper and put back to the original R3B plates. It was incubated for 2 days at the same temperature and light condition. Around 15 explants were transferred to selective medium and incubated back at

the previous conditions. Selective media was made of (1xMS, 20g/L sucrose, 1mg/L Zeatine Riboside, 100mg/L kanamycin, 200mg/L vancomycin, 200 mg/L Cefotaxime, 8 g/L Micro agar and PH adjusted to 5.8 with KOH).

The explants were sub cultured after 10 days and every two weeks thereafter to fresh plates of the same selective medium. When green calli/shoots were developed, explants were cut and transferred to selective medium. The explants were monitored continuously and grown shoots were transferred to root- inducing medium. Root inducing medium was composed of 1xMS, 20g/L sucrose, 0.5 mg/L IBA, 50mg/L kanamycin, 200mg/L vancomycin, 200 mg/L Cefotaxime, 8 g/L Micro agar and PH adjusted to 5.8 with KOH. Root developing shoots (plantlets) were transferred to Rockwool and grown in a standard greenhouse conditions (done by Bert Essenstam). To verify successful transformation, DNA isolation and PCR was done from the leaves of six transformed plantlets. Untransformed tomato (MM-0) leaf was included as a negative control (done by Thomas). To confirm Cf-4 expression, apoplastic fluid containing *C. fulvum* Avr4 was infiltrated to the young plantlets and HR development was monitored.

2.10. List of Primers and Plasmids used in this Study

Table 1. List of Primers used in this study; ao1, ao2, ao3 and ao4 were designed for this study. ao1 contains CACC at the 5` end designed for the directional TOPO® cloning. A03 and a04 contains *EcoRI* and *BamHI* sites, respectively.

Primer code	Primer name	sequence 5' -3'
ao1	GW-SlPPI-1fw	**CACC**ATGGGTGTAGAGGTGGAAG
ao2	SlPPI-1rev	CTTTTGAAGATACATGTATCCG
ao3	VIGS-*EcoRI*-SLPPI-1fw	A**GAATTC**GATGACTATGAGAGGAGACTC
ao4	VIGS-*BamHI*-SLPPI-1rev	A**GGATCC**CATAGCCTGTTTATTTTTTGCC
to30	pDONRseqrev	GTAACATCAGAGATTTTGAGACAC
to81	NuIfw	GATTTTCGTCAAGACTTTGACCGGTA
to82	CYC1rev	TTTCGGTTAGAGCGGATGTG
wo13	RPM1-NBS-LRR-fw	TATGTCGACTAAACAATGGCTAGTGAAAATAGTCTTGTAGGGATTG
wo17	M13(-48)-Rv	AGCGGATAACAATTTCACACAGGA
wo138	pTRV2-Fw1	CTCAAGGAAGCACGATGAGC
-	M13 uni (-21)	TGTAAAACGACGGCCAGT
-	T-3	AATTAACCCTCACTAAAGGG
-	T-7	TAATACGACTCACTATAGGG

Table 2. List of plasmids used in this study, including Yeast Two-Hybrid prey and bait vectors

Code	Description	Bacterial selection	Yeast selection	Source
SOL2518	*pDonor201-peru2*	Kanamycin	-	Thomas
SOL2557	*pMET-Cf-4(1)*	Ampiciline	Histidin	Thomas
SOL2559	*pMET-peru2*	Ampiciline	Histidin	Thomas and LR Gateway® cloned b/n SOL2518 and SOL2590
SOL2560	*pMET-Cf-2.2*	Ampiciline	Histidin	Thomas
SOL2576	*pMET-AtFLS2*	Ampiciline	Histidin	Thomas
SOL2589	*pCup-GWY-NuI-CYC1 (clone 1)*	Ampiciline	-	Thomas
SOL2590	*pMet-Gwy-Cub-URA3*	Ampiciline	-	Thomas
SOL2591	*pCup-NuI-HA-GWY-CYC1*	Ampiciline	-	Thomas
SOL2592	*pCUp-NuI-Aca11*	Ampiciline	-	Thomas
SOL2646	*pEntry-Avr4*	Kanamycin	-	Thomas
SOL2686	*pMet-GUS-Cub-Ura3*	Ampiciline	-	Thomas
SOL2718	*pZmL-dest*	spectinomycine	Leucine	Thomas
SOL3000	*pZmL-Avr4*	spectinomycine	Leucine	LR Gateway® cloned b/n SOL2718 and SOL2646
SOL3001	*pENT-D-TOPO®-full length PPI*-1	Kanamycin	-	TOPO® cloned b/n pENT-D-TOPO® vector and PCR product of full length *PPI-1*
SOL3002	*pCR4-TOPO®-VIGS-PPI-1*	Kanamycin	-	TOPO® cloned b/n pCR4-TOPO® vector and PCR product of VIGS-*PPI-1* fragment
SOL3003	*pTRV2-VIGS-PPI-1*	Kanamycin	-	Ligation of VIGS-*PPI-1* fragment in to pTRV2 vector

3. Results

3.1. Yeast stably expresses Cf-4-, peru2- and AtFLS2-Cub-URA3 but not Cf-2.2-Cub-URA3

To screen cDNA library of dying tomato seedling with split-ubiquitin yeast two-hybrid system, yeast that stably expresses Cf-4 was needed as bait. Thus, we started our study with Miniprep of plasmids containing *Cf-4* and other control genes (*Peru2, Cf-2.2* and *AtFLS2*) from *E. coli* (supp. Table 1). Plasmid digestion and gel electrophoresis was performed to confirm the correctness of the plasmids. With the exception of *Peru2-Cub-Ura3*, the other plasmids containing *Cf-4-*, *Cf-2.2-* and *AtFLS2-Cub-Ura3* match the expected fragments. For *Cf-4* containing plasmid two fragments (7041 and 2082bp), for *Cf-2.2* two fragments (7092 and 2952bp) and for *AtFLS2* four fragments (6037, 1977, 1356 and 857bp) were expected and the result was consistent with our expectation (Figure 3). On the other hand for *Peru2*, two fragments (7182 and 2112 bp) were expected, but the result showed three fragments with two of them less than 1kb (Figure 3). To verify this result, digestion of *peru2* containing plasmid was repeated resulting the same outcome (supp. Figure 1).

Consequently, LR Gateway® cloning was performed to acquire the proper *peru2* containing plasmid. Three colonies were picked from *E. coli* transformation. Plasmid isolation and digestion was performed. Clone number 3 was found to be the correct *peru2* containing plasmid showing two fragments with the expected size (7182 and 2112 bp) (Figure 4).

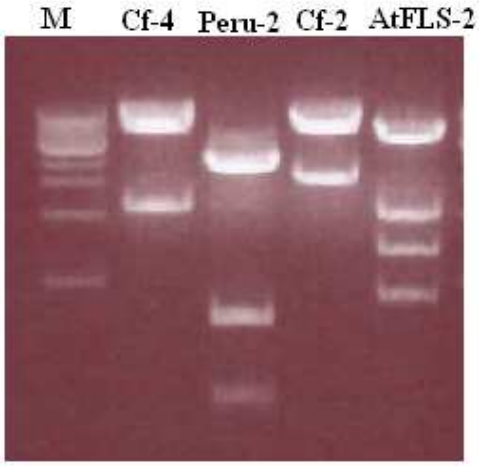

Figure 3. Digestion of plasmid isolated from *E. coli*; M is 1kb marker, *Cf-4* Two fragments (7041 and 2082bp), *Cf-2.2* Two fragments (7092 and 2952bp), *AtFLS2* four fragments (6037, 1977, 1356 and 857bp). For *Peru2* two fragments (7182 and 2112 bp) were expected but 3 fragments (2 of them less than 1 kb) were found.

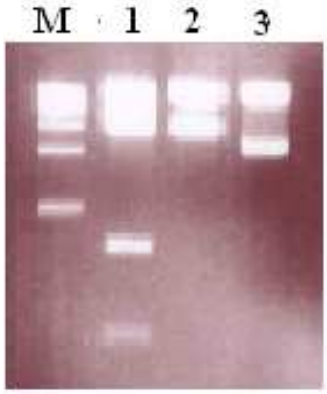

Figure 4. Digestion of 3 LR Gateway® cloned *peru2* plasmids with *Nhe I* and *AfI II* restriction enzymes; M is 1kb marker; plasmid 1 gave 4 bands (2 of them less than 1 kb), plasmid 2 resulted 2 fragments but seems different from expected size, and plasmid 3 matched the expected 2 fragments (7182 and 2112 bp).

Plasmid containing *Cf-4, Cf-2.2, AtFLS2* and *Peru2* was transformed to JD53 yeast which result a number of colonies (Figure 5 and supp. Table 2). Five colonies from each transformation were subjected to bait stability test. Except for *Cf-2.2*, the other transformants were observed to be stable showing colony growth at a dilution of 10^{-5} (*peru2*), 10^{-4} (*AtFLS2*), and 10^{-3} (*Cf-4*) Figure 6. Conversely, only mild growth was observed for *Cf-2.2* transformed yeast on the undiluted and 10^{-1} dilution (Figure 6). Yeast transformed with *peru2, AtFLS2* and *Cf-4* containing plasmids stably express their respective protein but not Cf-2.2.

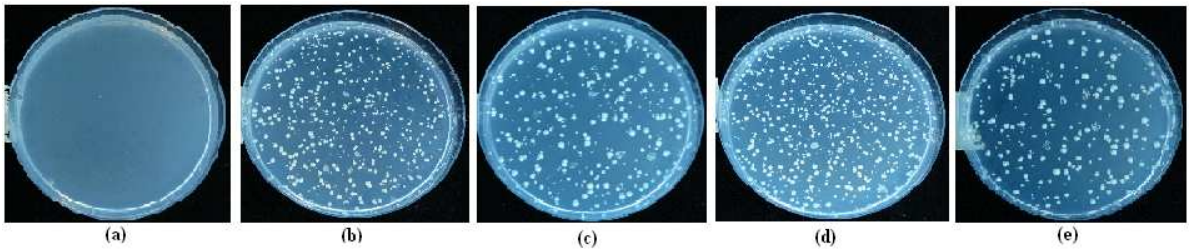

Figure 5. DO-H media plated with untransformed and transformed JD53 yeast colonies
(a) plate with untransformed JD53 yeast strain, (b) *Cf-4* transformant colonies, (c) *peru2* transformant colonies, (d) *Cf-2.2* transformant colonies and (e) *AtFLS2* transformant colonies

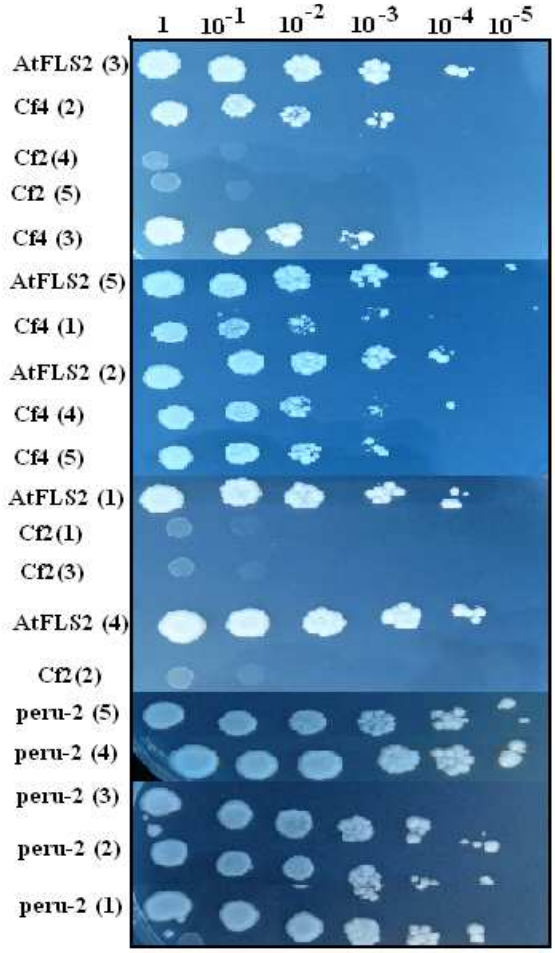

Figure 6. Stability test of *Cf-4, Cf-2.2, Peru2* and *AtFLS2* yeast transformants with a dilution series of 1- 10^{-5}, on min-HU media. Five colonies from each transformants were studied. Except for Cf-2.2, the other bait proteins were found to be stably expressed.

3.2. cDNA library screening of dying tomato seedling revealed previously identified and promising new interactors

To identify new tomato Cf-4 interactors, we employed split-ubiquitin yeast two-hybrid system. Yeast that stably expresses Cf-4 was used as bait and the cDNA library of dying tomato seedling as a prey vector. The cDNA transformation mix was plated on nine min-HT media containing FOA. 75 positive colonies were picked until the 8th day of incubation. After this day, background colonies appeared on the negative control plate. Based on the calculation made, around 2.3 million colonies were screened (Figure 7). Those 75 colonies were subjected to dilution spot test on min-HT media with FOA to confirm the true positives. As a result, 13 colonies were found to be false positive showing no growth at 10^{-3} and more dilution series. The remaining 62 colonies were noticed to grow stably on FOA containing media above a dilution of 10^{-3} (Figure 8 and supp. Figure 2).

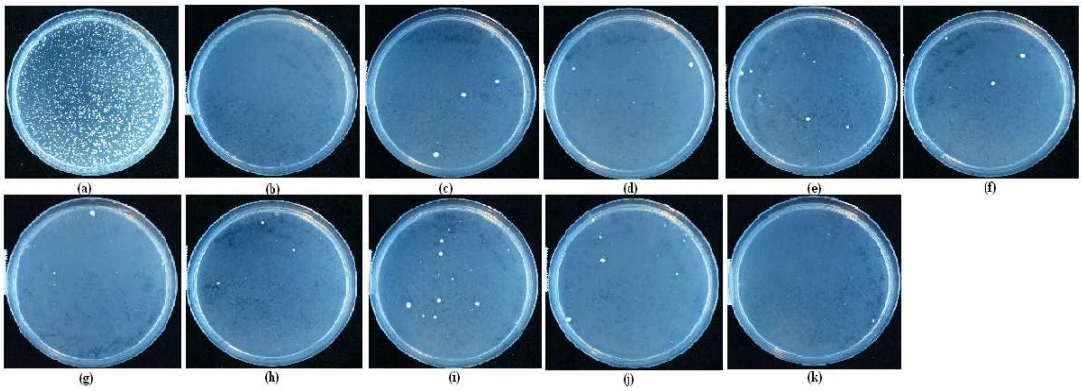

Figure 7. cDNA library screening with Cf-4 (bait) and cDNA (prey), cDNA prepared from Cf-4/Avr-4 crossed dying tomato seedling (a) plated with 2 µl of cDNA transformation mix on DO-HT media (b) plated with *pCup-NUI-HA-GWY-CYCI* transformation mix on min-HT media with FOA (negative control), (e) to (k) plate with 200 µl cDNA transformation mix on min-HT media with FOA. Colony was picked up to 8th days.

Plasmid was Miniprepped from the 62 positive colonies (supp. Table 4). Those plasmids were transformed to electro competent Dh5α *E. coli* cells; resulting high number of colonies in most of the transformation. However, one plasmid could not give any colony which was also repeated three times with 1, 1.5 and 3µl plasmid.

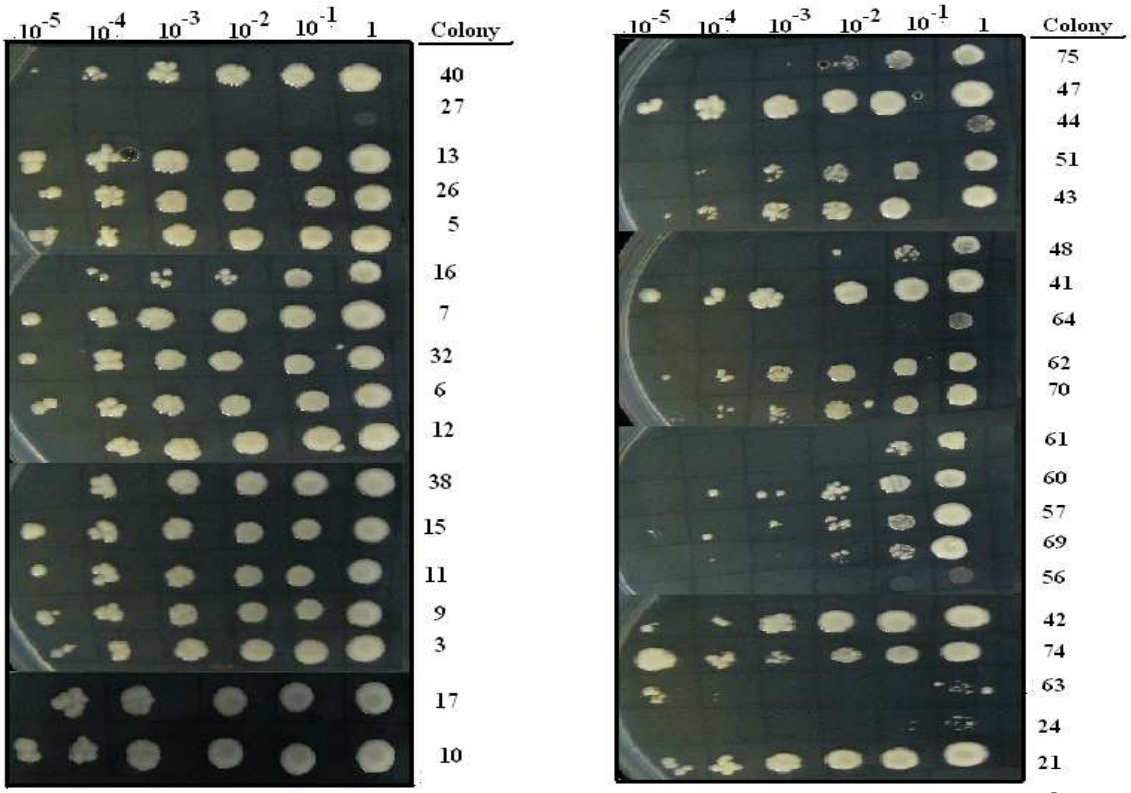

Figure 8. Dilution spot of some positive colonies from cDNA screening (1 refers as undiluted and 10^{-5} as the least dilution). Colonies showing growth at $\geq 10^{-3}$ are considered as positive clones.

Both bait (Cf-4) and prey vectors (cDNA) contain the same bacterial selection gene (Ampiciline). Thus it was not possible to differentiate colonies with prey vector that contains cDNA fragment insert using selective antibiotic. For this, colony PCR was done with NuIfw and CycIrev primers that bind to NuI and CycI sites of the prey vector. Colony PCR was done for 60 positive clones with 8 colonies from each. The result indicated that, apart from one clone which was checked for 24 colonies and result in no positive clone, the other *E. col*i transformants found to contain the cDNA fragment. Out of this, 48 clones contain a single insert with similar fragment size, 9 clones with 2 different insert size and 2 clones with 3 different inserts size (Figure 9 and supp. Figure 3-7).

Plasmids were isolated from 72 colonies of the above mentioned clones (Supp. Table 5). An autoactivity test was carried out with one to one interaction of each 72 cDNA fragment containing plasmids with the bait plasmid *pMet-GUS-Cub-Ura3*. Each cDNA fragment insert containing plasmids and *pMet-GUS-Cub-Ura3* was transformed to yeast. Four colonies from each co-transformation were used for the dilution spot test. Stable yeast growth in the dilution test indicates the autoactivity of this specific candidate interactor. Based on this, it was found that 57 of the candidate interactors did not show autoactivity for all the tested four colonies. However, the rest 15 putative interactors were auto active showing stable yeast growth. Among them four of the candidate interactors showed stable yeast growth for all four colonies. Candidate interactor 54.6 and 67.6 had

shown stable yeast growth for three and two of the tested colonies, respectively. For nine of the putative interactors, only one colony was noticed to grow stably among the tested four colonies.

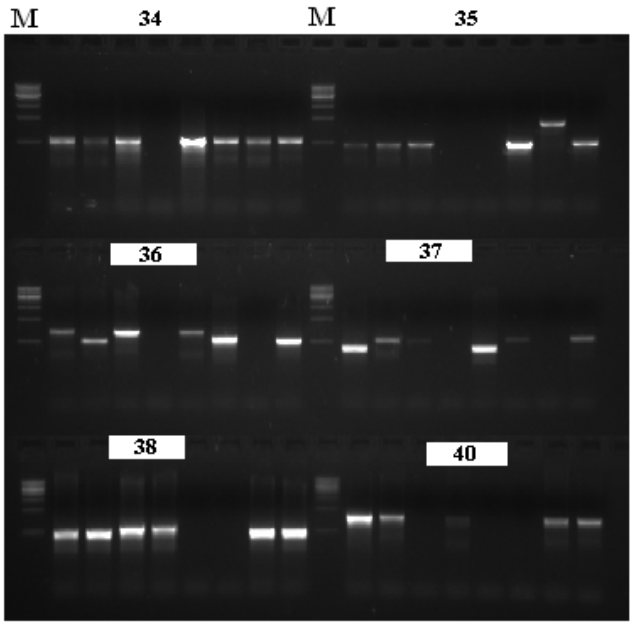

Figure 9. Colony PCR on *E. coli* transformants with plasmid number 34, 35, 36, 37, 38 and 40. Eight colonies were used for the colony PCR from each transformants. A single band of similar insert size was detected from *E. coli* transformants with plasmid 34, 38 and 40. Two bands with different insert size were noticed from *E. coli* transformants with plasmid 35, 36 and 37.

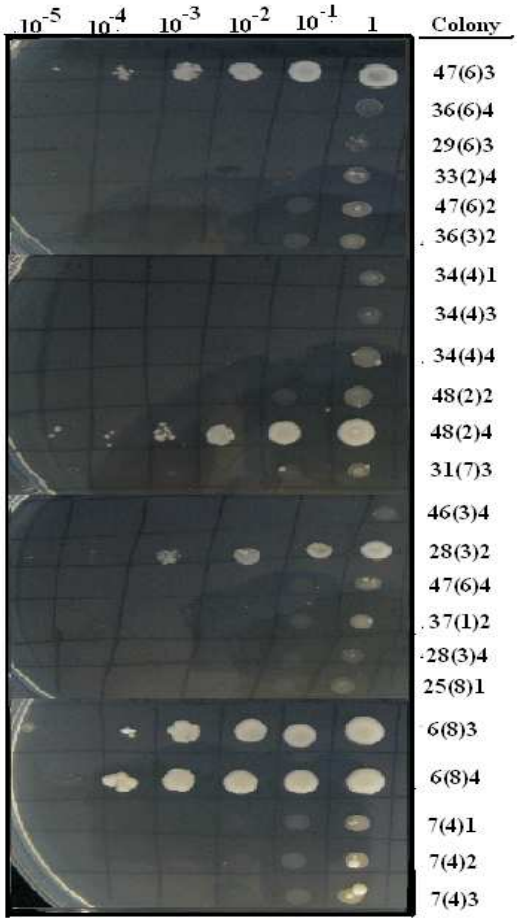

Figure 10. Autoactivity test of candidate interactors with *pMet-GUS-Cub-Ura3* (one to one interaction). Dilution spot test of some co-transformed yeast colonies on min-HT+FOA media. Stable yeast colony growth indicates the Autoactivity of the candidate interactor

Sequencing of non- autoactive putative interactors revealed that, 17 of them were in a correct reading frame based on NuI N-terminal ubiquitin moiety (Table 3). Among the candidate interactors, three independent clones were shown strong homology to proton pump interactor-1 (PPI-1), two of them were exactly identical with 345bp and the third one matches 248bp of the C- terminus of the protein. The PPI-1 protein contains transmembrane and coiled coil domains.

The remaining candidate interactors include, Metallothionein-like protein, spermidine synthase 1, RNA binding protein-like protein, pyridoxine biosynthesis protein isoform B (Pdx1-B), ethylene receptor, ribulose 1,5-bisphosphate carboxylase/oxygenase small, photosystem I (PS I) reaction center protein subunit II, protease inhibitor/seed storage/lipid transfer protein family protein and ALY protein. Moreover three independent clones were found to be conserved hypothetical protein.

Besides, one candidate interactor was found to be the previously identified heat shock molecular chaperon (Hsp90-2) with HATPase_c and coiled coil domains (Gabriëls et al., 2006). Even though they were not found in a correct reading frame to NuI N-terminal ubiquitin moiety, several interactors were identified (supp. Table 9). Such as phosphoglycerate mutase-like protein, proteasome-like protein, phosphoinositide binding protein, protein phosphatase 2C, small heat shock protein (Hsp23.5), DNAJ heat shock N-terminal domain-containing protein and cell death associated protein.

Table 3. Tomato Cf-4 interactors identified from split-ubiquitin yeast two-hybrid screening with a correct open reading frame based on the NuI N-terminal ubiquitin moiety, [(1)] corresponding SGN-unigene based on SGN blast, [(2)] Predicted protein by NCBI protein BLAST including the GenBank accession number [(3)] predicted domains based on SMART domain blast

Cf-4 Interactor	**SGN-unigene [(1)]**	**Predicted protein by BLAST [(2)]**	**Predicted domains [(3)]**
2.7	SGN-U578438	RuBisCo small (*Capsicum annuum*), AF065615.1	low complexity region and pfam:RuBisCO_small
3.1	SGN-U580538	putative ethylene receptor (*Solanum lycopersicum*), U38666.1	3 transmembrane domains, GAF, HiskA, HATPase_c domain
9.2	SGN-U575965	protease inhibitor/seed storage/lipid transfer protein family protein (*Tamarix hispida)*, ACM78616.1	Signal peptide, AAI, low complexity
11.2	SGN-U577360	putative pyridoxine biosynthesis protein isoform B (Pdx1-B), (*Nicotiana tabacum)*, AY532657.1	Pfam:SOR_SNZ; pfam:ThiG
11.3 (identical to 11.2)	SGN-U577360	putative pyridoxine biosynthesis protein isoform B (Pdx1-B), (*Nicotiana tabacum)*, AY532657.1	Pfam:SOR_SNZ; pfam:ThiG
12.2	SGN-U590663	Metallothionein-like protein (560bp) (*L. esculentum)*, Z68310.1	Pfam:Metallothio_2
13.2	SGN-U568906	RNA binding protein-like protein (*Solanum tuberosum)*, DQ241864.1	2 low complexity regions and RRM domain
15.4	SGN-U580167	Tomato photosystem I (PS I) reaction center protein subunit II (psaD), M21344.1	low complexity region and Pfam:PsaD
34.1	SGN-U577948	ALY protein, (*Nicotiana benthamiana*), AM167906.1	3 low complexity regions and RRM domain
36.3	SGN-U580117	spermidine synthase 1 (*D. stramonium*), Y08252.1	Pfam:Spermine_synth, Pfam:DUF752
37.8	SGN-U579523	conserved hypothetical protein (*Ricinus communis*], XP_002533430.1	Pfam:DUF788
58.4	SGN-U562609	hypothetical protein ARALYDRAFT_319835 (Arabidopsis lyrata subsp. Lyrata) XP_002886219.1	pfam:Rer1
60.2	SGN-U578145	proton pump interactor 1 (PPI1), (*Solanum tuberosum*), GU808087.1	Blast: MYSc, Pfam:DUF827, coiled coil and transmembrane
68.4	SGN-U578145	proton pump interactor 1 (PPI1), (*Solanum tuberosum), GU808087.1*	Blast: MYSc, Pfam:DUF827, coiled coil and transmembrane
70.5	SGN-U592593	molecular chaperone Hsp90-2 (Lycopersicon esculentum), AY368907.1	HATPase_c and coiled coil
74.3	SGN-U579523	conserved hypothetical protein (*Ricinus communis)*, XP_002533430.1	pfam:DUF788
75.2	SGN-U578145	proton pump interactor 1 (PPI1), (*Solanum tuberosum*), GU808087.1	Blast: MYSc, Pfam:DUF827, coiled coil and transmembrane

3.3. Virus-Induced gene Silencing of *Proton pump interactor-1 (PPI-1)* showed reduction of HR up on Avr4 Agroinfiltration

From NCBI and SGN blast of sequences obtained from the cDNA screening, three candidate Cf-4 interactors (60.2, 68.4 and 75.2) were shown strong homology to proton pump interactor-1. Candidate interactor 60.2 and 68.4 were exactly identical and 75.2 match the C- terminus of the protein (Table 3).

To investigate the requirement of PPI-1 in Cf-4-mediated resistance, VIGS of *PPI-1* was performed in *N. benthamiana:Cf-4*. 405 bp of *PPI-1* gene was obtained by standard PCR from tomato cDNA library with *EcoRI* and *BamHI* sites (Figure 11). The PCR fragment was TOPO® cloned to pCR4-TOPO® vector and transformed to *E. coli*. Colony PCR revealed a number of positive colonies (Figure 12). Plasmid from the positive colonies were sequenced and found to match with the original sequence of *PPI-1* obtained from cDNA screening. The TOPO® cloned vector was digested with *EcoRI* and *BamHI* and obtained the VIGS fragment (Supp. Figure 6). The VIGS fragment was ligated to pYL156:TRV2 vector, transformed to *E. coli* and with colony PCR positive colonies were identified (Figure 13). Subsequently, the plasmid from *E. coli* was isolated and transformed to *Agrobacterium.* Then agroinfiltration was performed in young *N. benthamina:Cf-4* plants with 1:1 mixture of *Agrobacterium* expressing *pTRV2* derived VIGS constructs and *pTRV1* vector.

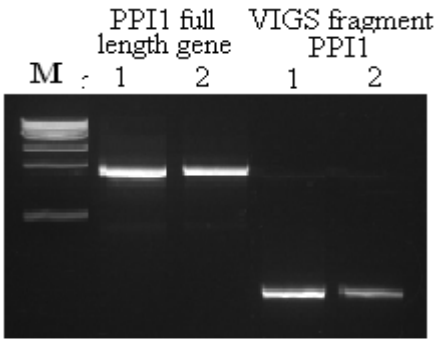

Figure 11. PCR of full length (1884bp) and VIGS fragment (405bp) of *PPI-1* from tomato cDNA library, M is 1kb marker

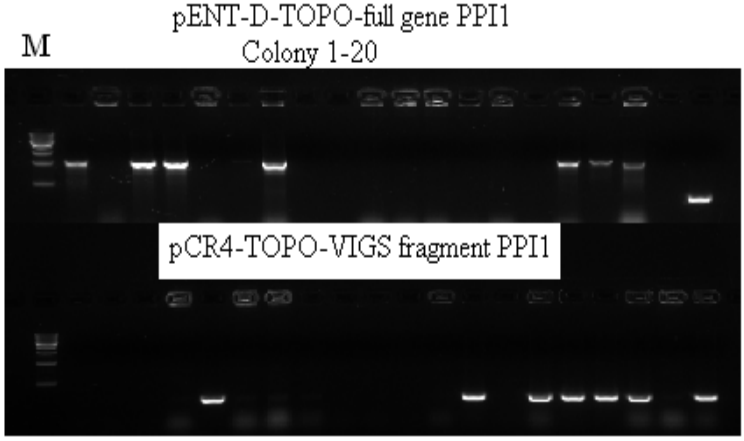

Figure 12. Colony PCR of 20 *E. coli* colonies transformed with *pENT-D-TOPO®-full* gene *PPI-1* (upper lanes) and *pCR4-TOPO®-VIGS fragment PPI-1*(lower lanes), M is 1 kb marker

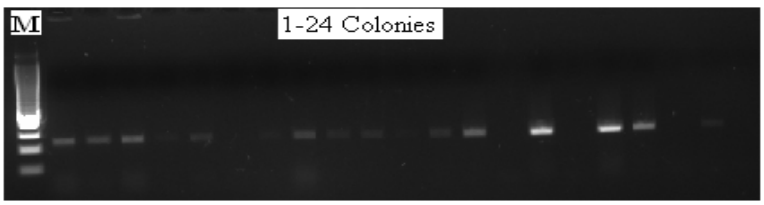

Figure 13. Colony PCR of 24 colonies (*E. coli* transformed with pTRV2-VIGS-*PPI-1*); M is 200bp marker

The following VIGS constructs will not be discussed in this report. They are beyond the scope of this study. SOL2730 (BiP1), SOL2731 (BiP2), SOL2732 (BiP3), SOL2733 (SDF2), SOL2734 (Erdj3b), SOL2735 (CRT3), SOL2769 (CRT1) and SOL2771 (all BiPs).

Reduction of HR was noticed at Avr4 infiltrated spot of PPI-1 silenced leaves compared to GUS silenced leaves. In most of the Cf-4 and SOL2735 silenced leaves, only mild collapsed cell death was noticed with the infiltration of Avr4. For SOL2730 and SOL2734 silenced plants, moderate HR was observed at Avr4 infiltrated spot. Silencing of SOL2731, SOL2732, SOL2733 and SOL2769 resulted an HR comparable to GUS silenced plants (Figure 14).

In all cases of silencing including Gus silenced plants, spot infiltrated with the apoplastic fluid did not show an HR. In SGT1 silenced plants, no HR was observed with infiltration of Avr4, BAX, Rx and apoplastic fluid. Similarly SOL2771 silenced plants did not show an HR with infiltration of Avr4, BAX and Rx. In most of the cases, Infiltration of Rx and BAX resulted very strong and strong HR respectively, except for SGT1 and SOL2771 silenced leaves where no HR was detected (Figure 14).

Photo bleaching phenotype of PDS silenced plants confirmed the proper functioning of the VIGS in this set of experiment (Figure 14). Only SGT1 and SOL2771 silenced plants were shown abnormal phenotype. SGT1 silenced plants were small in size. Their leaves were dark green and the edges of the leaves were curled up. SOL2771 silenced plants were very small and look dying (Picture not shown). HR score after three days of agro-infiltration or protein injection was described (Supp. Table 8).

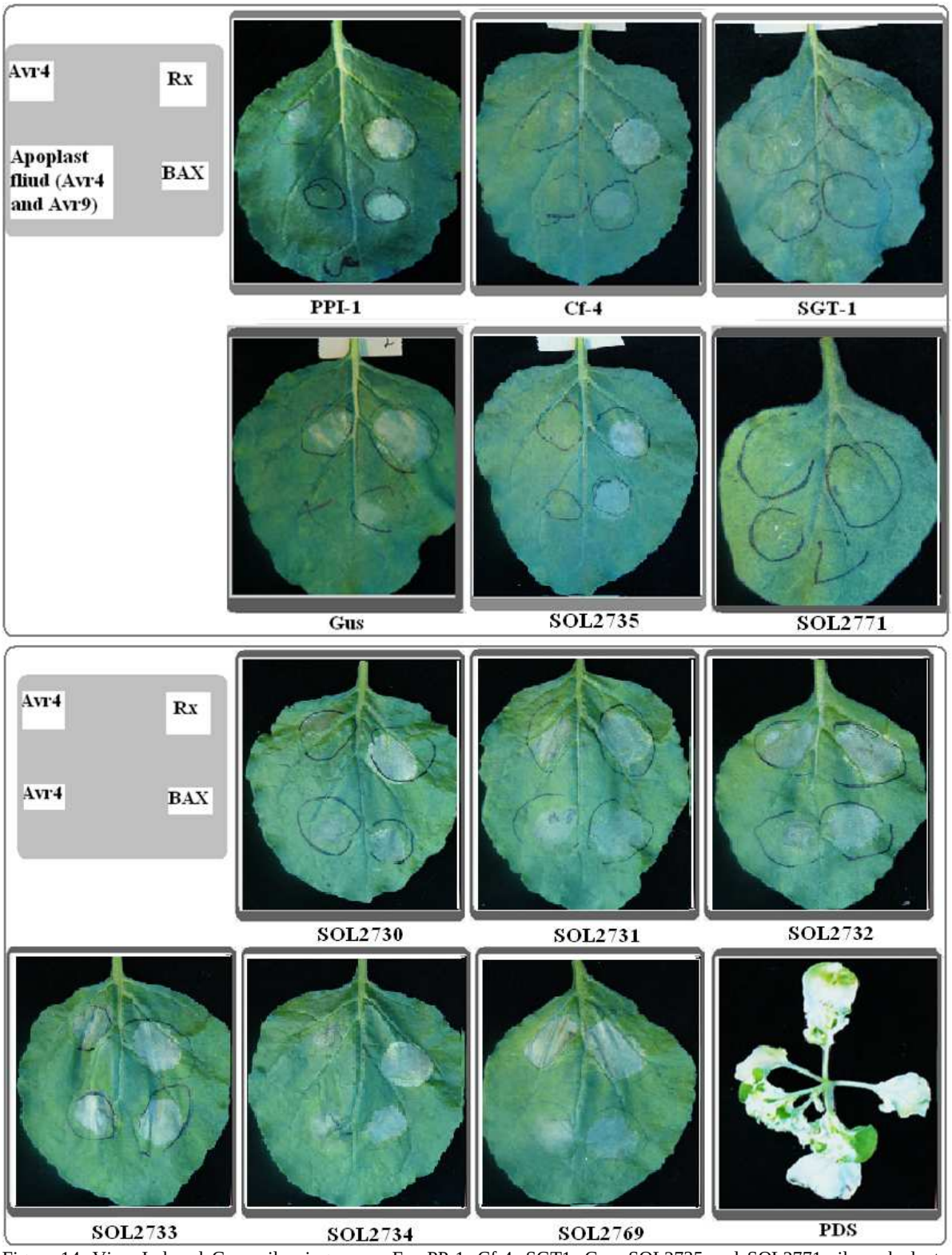

Figure 14. Virus-Induced Gene silencing assay. For PP-1, Cf-4, SGT1, Gus, SOL2735 and SOL2771 silenced plants, *agrobacterium* expressing pMog808-Avr4, pBin61-RxD460v, BAX and apoplastic fluid containing Avr4 and Avr9 were infiltrated as shown in the above left picture. For SOL2730, SOL2731, SOL2732, SOL2733, SOL2734 and SOL2769 silenced plants, *agrobacterium* expressing pMog808-Avr4, pBin61-RxD460v and BAX were infiltrated as shown in the lower left picture. PDS silenced plant were included to show the proper functioning of VIGS in this set of experiment.

In addition, to assess the binding of PPI-1 with Cf-4 protein, we planned to perform co-immunoprecipitation assay. For this, we cloned the full *PPI-1* gene from tomato cDNA library by standard PCR (Figure 11). The PCR fragment was TOPO® cloned to pENT-D-TOPO® vector and transformed to *E. coli.* Colony PCR revealed a number of positive colonies (Figure 12). Plasmid from the positive colonies were sequenced and found to match with the original sequence of *PPI-1* obtained from cDNA screening. The TOPO® cloned vector was digested with *PvuI* that cuts the Kanamycin resistance gene in to two halves (Supp. Figure 6). LR Gateway® cloning of both circular and linearized pENT-D-TOPO®-full length*PPI-1* plasmids was done to pGWB20-10xMyc destination vector. However, we could not get positive *E. coli* colonies from the colony PCR after LR cloning to pGWB20; despite we did the LR cloning and colony PCR several times. The LR Gateway® cloning reaction seems to work properly. A band was observed from a gel loaded with PCR product of LR Gateway® cloning mixture of circular pENT-D-TOPO®-full length *PPI-1* gene (Supp. Figure 7). Thus, we were unable to carry on the co-immunoprecipitation assay.

3.4. Avr4 was transformed to yeast, but its expression was not confirmed yet

To study expression of Avr4 in the yeast, *pZmL-Avr4* was obtained with LR Gateway® cloning. Three *pZmL-Avr4* plasmids were assessed with digestion to verify the correctness of the cloned plasmid. As a result all the three plasmids were shown to contain two fragments with the expected size (429 and 6642bp) (Figure 15).

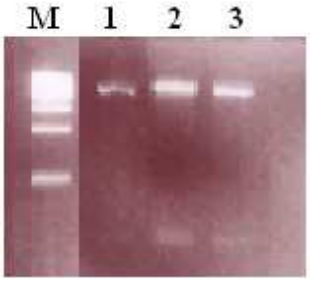

Figure 15. Digestion of 3 LR Gateway® cloned *pZmL-Avr4* plasmids with *BamHI* and *XhoI* restriction enzymes; M is 1kb marker, all 3 plasmids result 2 fragments with expected size (429 and 6642bp)

To study Av4 expression from its yeast transformant, infiltration assay was performed. The supernatant of overnight *pZmL-Avr4* and pZmL-dest. yeast culture was infiltrated to *N. benthamiana:Cf-4* expressing and wild *N. benthamiana* plants. *Agrobacterium* expressing Avr-4 and liquid DO-L media was also infiltrated as a positive and negative control, respectively. Strong HR was observed in Avr-4 infiltrated spot of Cf-4 expressing benthamiana plant, while no phenotypic symptom was detected with *pZmL-Avr4*, pZmL-dest. and DO-L infiltrated spot (Figure 16). On the other hand, infiltration of both *pZmL-Avr4* and pZmL-dest result in a strong HR looking symptom in the wild benthamiana plants. Whereas no phenotypic symptom was noticed on Avr-4 and DO-L infiltrated spots (Figure 16). HR score for the first three days after infiltration of the treatments was summarized in suppl. Table 7.

Figure 16. Infiltration assay; left (N. benthamiana:Cf-4 leaf), right (wild N. benthamiana leaf) infiltrated with (A) *Avr4* expressing *Agrobacterium*, (B) Supernatant of *pZmL-Avr4* yeast culture, (C) Supernatant of pZmL-dest. yeast culture and (D) liquid DO-L media

3.5. Yeast Transformed with both *pZmL-Avr4* and *Cf-4* stably expresses Cf-4

To study the possibility of transforming both *pZmL-Avr4* and *Cf-4* containing plasmids in to a single yeast cell, sequential transformation was performed with both plasmids. Either re-transforming *pZmL-Avr4* containing yeast with *Cf-4* containing plasmid or vice versa. The double transformation in both ways result a number of transformant colonies (Figure 17). From the transformed yeast, bait stability test revealed colony growth to the dilution of 10^{-3} and 10^{-4} (Figure 18). This indicates that Cf-4 stably expressed in the double transformed yeast.

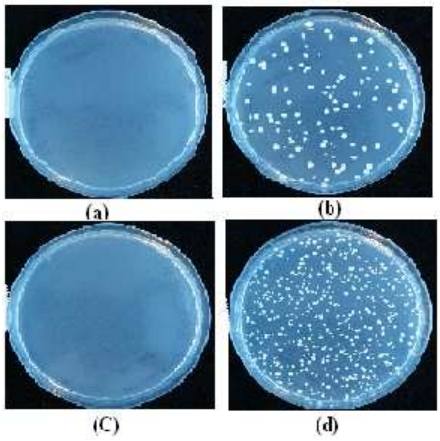

Figure 17. plates cultured with transformed and re-transformed JD53 yeast strain on DO-HL media (a) *Cf-4* transformant, but not *pZmL-Avr4*, (b) *Cf-4* transformant, re-transformed with *pZmL-Avr4*, (c) *pZmL-Avr4* transformant, but not *Cf-4*, (d) *pZmL-Avr4* transformant, re-transformed with *Cf-4*

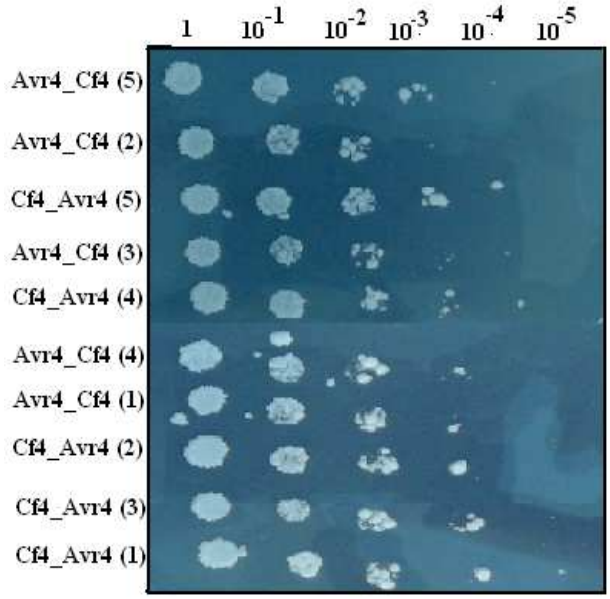

Figure 18. Bait stability test of double yeast transformant on min-HLU media; (Avr4_Cf4) *pZmL-Avr4* transformant re-transformed with *Cf-4* and (Cf4_Avr4) *Cf-4* transformant re-transformed with *pZmL-Avr4*. Five colonies from each with a dilution series of 1-10^{-5} (1 refers as undiluted and 10^{-5} as the least dilution)

3.6. Generation of Cf-4-eGFP transgenic Tomato lines

Agrobacterium tumifaciens-mediated transformation was employed to generate transgenic Money maker-Cf-0 (MM-Cf-0) with Cf-4 C-terminally tagged with eGFP (Cf-4-eGFP). It was started with around 1500 MM-Cf-0 explants incubated on *Agrobacterium* suspension containing Cf-4-eGFP construct. After subsequent transfer of the explants to fresh selective media, more than 120 green shoots/calli were obtained. The green calli/shoot was transferred to rooting media and most of them are currently on this stage. Currently, 12 plantlets (root developed green shoots) were grown in the Rockwool under standard greenhouse conditions. PCR analysis of sample plantlets confirmed that the tomato lines are transgenic (T. Liebrand, personal communication). Infiltration of apoplastic fluid containing Avr4 to the transgenic tomato lines resulted in a clearly visible HR in some plantlets. This confirms the expression of Cf-4-eGFP in the transgenic tomato lines. For the other plantlets, PCR analysis and infiltration of apoplastic fluid containing Avr4 will be done. In addition, Q-RT-PCR will be performed to analyze the number of transgene insert.

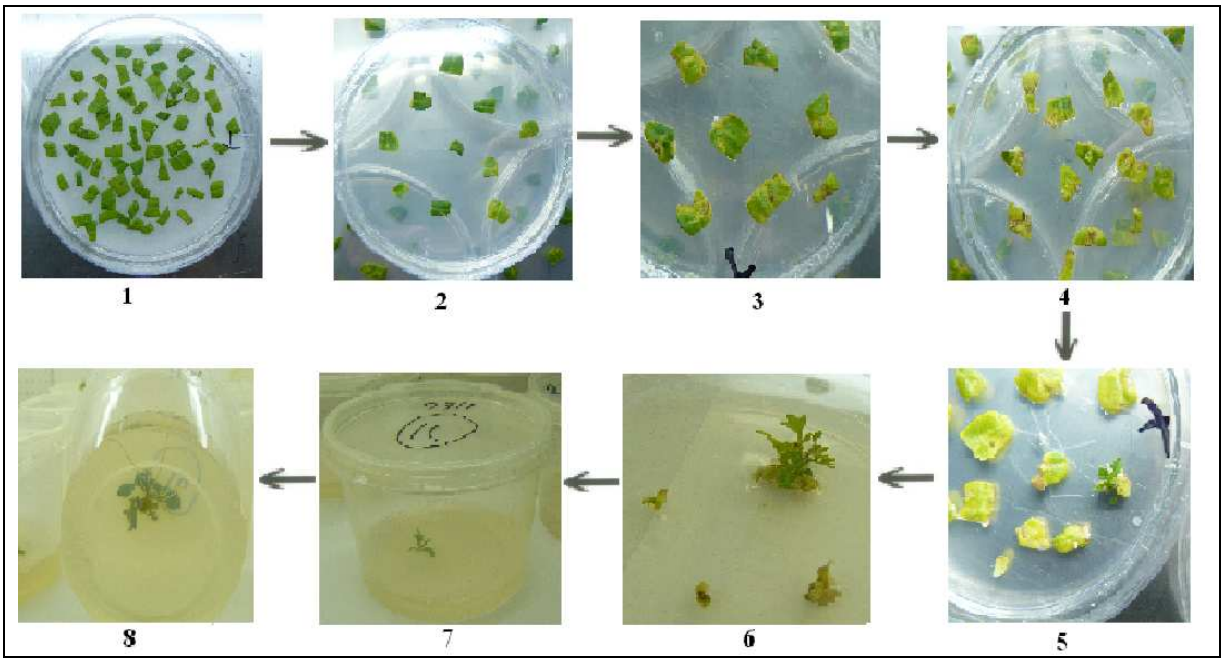

Figure 19. Different stages of tomato transformation with Cf-4-eGFP*;* (1) explants in R3B media after co-culturing with *Agrobacterium*, (2) after 1 week on selective media, (3) callus start to develop after 17 days on selective media, (4) many more callus developed at 5th week on selective media, (5) green shoot start to develop at 8th week, (6) bigger green shoots at 9th week, (7) green shoots on rooting media at 10th week and (8) root developed plantlets at 11th week

4. Discussion

4.1. Cf-4-Cub-URA3 is stably expressed in the yeast

For screening of cDNA library with split-ubiquitin yeast two-hybrid system, stable Cf-4 expression was required in the yeast. Cf-4 stability in the transformed yeast was confirmed by a dilution spot test on media lacking uracil. URA3 is an enzyme involved in biosynthesis of uracil which was fused to Cub as a reporter for the split-ubiquitin yeast two-hybrid system. *Cf-4*, *Cf-2.2*, *AtFLS2* and *Peru2* genes were also fused to *Cub-URA3*. The stable expression of these genes was determined by the expression of URA3, i.e. the ability of the transformed yeast to grow on a media lacking uracil. The result of yeast transformation and their stability test showed that all transformation except for Cf-2.2-Cub-URA3 were stable showing yeast growth. AtFLS2 and Peru2 transformed yeast were relatively more stable compared to Cf-4. This result was consistent with the previous study (T. Liebrand, personal communication). Instability of Cf-2.2 transformed yeast could be due to low expression of the *Cf-2.2-Cub-URA3* gene or the fusion protein might be degraded by proteases in yeast.

4.2. Identification of new tomato Cf-4 interactors

From the cDNA library screening, 17 candidate Cf-4 interactors were identified in a correct reading frame in correspondence with NuI N- terminal ubiquitin moiety. Among them, three independent clones were shown strong homology to potato proton pump interactor-1 with 97% identity. The proton pump interactor-1 will be discussed in section 4.3.

Cf-4 interactor 12.2 showed strong sequence homology to a tomato Metallothionein-like protein with 98% identity. This candidate interactor was also found in the previous screening (T. Liebrand, personal communication). Moreover, four members of Metallothionein-like protein were found as MLO interactor (Mildew resistance locus o) in barley using split-ubiquitin yeast two-hybrid (Dr. Chiara Consonni, PhD thesis, 2005). Metallothioneins (MT) are small, ubiquitous, cysteine rich, metal binding proteins found in plant, animal, fungi and cyanobacteria. They are suggested to be involved in metal homeostasis and detoxification. Recently In plants, studies speculated to be involved in reactive oxygen species (ROS) scavenging, despite the exact mechanism is unclear (Hassinen *et al.*, 2011). ROS are central regulator of cell death, which are highly expressed following biotic and abiotic stress (Steffens and Sauter, 2009).

Up regulation of the MT GhMT3 was reported in cotton seedling with high salinity, drought, low temperature, abscisic acid, ethylene, ROS, Zn and Cu. Over expression of GhMT3 in *N. tabacum* increases tolerance to ROS generated stress, suggesting ROS scavenging activity of GhMT3 (Xue *et al.*, 2009). In spite of its unclear co-relation between metal binding and ROS activity of MT, it was hypothesized that metals are released during binding of ROS to MT and the released metal may serves as signalling cascade (Hassinen *et al.*, 2011). MT2b is another group of Metallothioneins, down regulated to allow ethylene and ROS mediated epidermal cell death during normal root growth in rice (Steffens and Sauter, 2009). OsMT2b; a MT is down regulated following rice blast infection. Its over expression in rice resulted susceptibility to rice blast and bacterial blight pathogen with reduced elicitor induced ROS production suggesting its ROS scavenging activity (Wong *et al.*, 2004). Thus down regulation of MT seems to appear during defense response following pathogen infection to allow ROS mediated cell death.

Two Cf-4 interactors (11.2 and 11.3) were shown 88% homology to tobacco Putative pyridoxine biosynthesis protein isoform B (Pdx1-B). In Arabidopsis thaliana Pyridoxine biosynthesis gene (PDX1 and PDX2) were shown to be up regulated following different abiotic stresses, such as high light, chilling, drought, ozone and hydrogen peroxide treatment. This suggests that Pyridoxine might have antioxidant role and involve in oxidative stress responses (Denslow *et al.*, 2007).

Interactor 13.2 and 36.3 were shown 96% homology to potato RNA binding protein-like protein and *D. stramonium* Spermidine synthase-1, respectively. Down regulation of RNA binding proteins and spermidine synthase were reported following heat shock treatment of tobacco cells. This suggested that these proteins might have antioxidant function by which their down regulation contributes to oxidative burst. Addition of exogenous spermidine to the heat shock tobacco cells protect the cells from programmed cell death. This suggested that spermidine might affect the expression or activity of some ROS scavenging enzymes (Marsoni *et al.*, 2010). It should be noted that, the cDNA library was prepared from a dying tomato seedling, where the plant suffer stress condition. Thus, up regulation of oxidative stress related genes were expected from the dying seedling. Consequently, some of the identified interactors were related to oxidative stress responses as was expected. Nevertheless, this oxidative stress related interactors might also be required for defense response.

Cf-4 interactor 3.1 has strong homology to potato and tomato ethylene receptor with 100% identity. The plant hormone ethylene has an important role in linking developmental signals and responses to biotic and abiotic stimuli. Ethylene synthesis was stimulated and negatively regulates MAPK signalling following calcium dependent protein kinases (CDPKs) activation in Avr9 triggered Cf-9 mediated defense response (Deising *et al.*, 2009). Moreover, tomato ethylene receptor gene *LeETR4* is induced during the hypersensitive response activated with the infection of *Xanthomonas campestris* pv. vesicatiria (Ciardi *et al.*, 2001).

Cf-4 interactor 37.8 and 74.3 were found to be a conserved hypothetical protein of *Ricinus communis*. Likewise, interactor 58.4 was shown strong homology to hypothetical protein of *Arabidopsis lyrata* subsp. Lyrata. Although these hypothetical proteins are of unknown function, their interaction with Cf-4 in the yeast two-hybrid is probably significant to study them further. Therefore, it is worthwhile to study the correlation of the above mentioned candidate tomato Cf-4 interactors in *C. fulvum* / tomato interaction.

Interactor 70.5 was 99% homologues to tomato heat shock chaperone (Hsp90-2). This interactor was also identified in earlier screening (T. Liebrand, personal communication). It has been shown that Hsp90 is required in Avr4 triggered Cf-4-mediated defense response (Gabriëls *et al.*, 2006). Furthermore, around 38 Cf-4 interactors were identified from the cDNA library screening, despite they were not in a correct reading frame in correspondence to NuI N-terminal ubiquitin moiety.

4.3. Proton pump interactor-1 could be required in Cf-4-mediated resistance of *C. fulvum*

Three independent Cf-4 interactors were shown strong sequence homology to Proton pump interactor-1 (PPI-1). Two of them have exactly identical sequence and the third differs in its N-terminal from the other two. Virus-Induced gene silencing of tomato *PPI-1* on *N. benthamiana:Cf-4* showed reduction of HR with the infiltration of *Agrobacterium* expressing Avr4. This suggests that, PPI-1 might be required in Cf-4-mediated defense response to *C. fulvum*. Although the VIGS experiment should be repeated and other assays like co-IP are needed to give strong conclusion.

PPI-1 of Arabidopsis thaliana was found to stimulate the plasma membrane proton pump (PM H^+-ATPase) (Morandini *et al.*, 2002). The plasmamebrane H^+-ATPase is an enzyme that pumps out positive charge (H^+) to extracellular medium. This enzyme is found in plasmamebrane of plants and fungi which is required for processes such as import and export of solutes, PH homeostasis and cell growth. The activity of PM H^+-ATPase is influenced by several factors including treatment with an elicitor from a pathogen (Palmgren, 2001). The activity of plasmamebrane H^+-ATPase was shown to increase four fold during treatment of Cf-5 expressing tomato cells with *C. fulvum* Avr5 containing apoplastic fluid (Vera-Estrella *et al.*, 1994a). The pumping out of protons to the extracellular medium leads more acidic condition than the cytoplasm. This creates electrochemical gradient of charges between the cytoplasm and extracellular medium. This in turn activates the flow of solutes to the cytoplasm. In intact leave, acidification of extracellular medium could induce loosening of the cell wall and change calcium distribution across the plasma membrane. This may leads to callose formation that could limit fungal infection or lead to starvation of the fungus (Vera-Estrella *et al.*, 1994a).

Phosphorylation of the penultimate residue, Thr, triggers H^+-ATPase activation. Subsequent binding of regulatory 14-3-3 protein to the phosphorylated site protects the PM H^+-ATPase from being de-phosphorylated (Bobik *et al.*, 2010; Palmgren, 2001). Fungal toxin fusicoccin has been shown to activate H^+-ATPase by a mechanism involving the C terminus domain (Johansson *et al.*, 1993). Conversely, in earlier study it was proposed that *C. fulvum* elicitor recognition by plasmamebrane receptor triggers activation of G proteins. This in turn lead to de-phosphorylation of H^+-ATPase through the induction of membrane bound phosphatase (Vera-Estrella *et al.*, 1994b).

To come up with further evidences of PPI-1 requirements in Cf-4- mediated resistance, we intended to perform co-immunoprecipitation assay. For this, we cloned the full *PPI-1* gene from tomato cDNA library by standard PCR (Figure 11). The cloning of *PPI-1* gene was successful, as shown by sequencing. Next we performed LR Gateway® cloning to tag *PPI-1* C-terminally with a 10xmy-tag. However, we could not get positive *E. coli* colonies from the colony PCR after LR cloning to pGWB20. Despite we did the LR cloning and colony PCR several times. The LR Gateway® cloning reaction seems to work properly. A band was observed from a gel loaded with PCR product of LR Gateway® cloning mix of circular pENT-D-TOPO®-full length *PPI-1* gene (Supp. Figure 7). Thus, we were unable to carry on the co-immunoprecipitation assay. However recently, a positive colony was obtained from the colony PCR and the plasmid was transformed to *Agrobacterium* for the co-IP assay (Z. Belew and T. Liebrand, personal communication).

4.4. Yeast transformed with *Cf-4* and Avr4 stably expresses Cf-4, but Avr4 expression is not confirmed

To investigate expression of Avr4 in the yeast, we performed yeast transformation with *pZmL-Avr4*. In this study, stable Cf-4-Cub-URA3 expression was already shown in the yeast. *pZmL-Avr4* was transformed to yeast either alone or together with *Cf-4* containing plasmid. In both ways transformant colonies were found in a media lacking Leucine and Histidin +Leucine, respectively. This indicates successful transformation of *pZmL-Avr4* to yeast. In case of transformation with both *pZmL-Avr4* and *Cf-4*, stable Cf-4 expression was verified by plating the transformation mix on a min-HLU media (lacking uracil, Histidin and Leucine). This shows the stable expression of URA3 and Cf-4 in the double yeast transformant. However, we were not able to confirm the expression of *Avr-4* in yeast. The Avr4 should be secreted in yeast, as it contains a signal peptide. The result from supernatant infiltration test on *N. benthamiana:Cf-4* plant was not conclusive. This could be due to low expression level of Avr4 in the yeast or it might be unstable in yeast.

When Avr4 expression is verified in yeast, then cDNA screening can be done with the existence both Cf-4 and Avr4 in a single yeast cell. It has been shown that FLS2 receptor form a complex with BRI1-Associated Receptor Kinase-1 (BAK1) during interaction with the ligand flg22 (Chinchilla *et al.*, 2007). Similarly, we thought the existence of both Avr4 and Cf-4 proteins in the yeast cell might influence the identification of Cf-4 interactors in the yeast two-hybrid. Although, the cDNA library was prepared from a dying seedling by which Cf4-mediated resistance was activated after Avr4 recognition.

4.5. Transgenic tomato lines expresses Cf-4-eGFP

We were able to generate transgenic tomato lines expressing Cf-4-eGFP under the 35S promoter using *Agrobacterium*-mediated transformation. Previously, it has been shown the production of transgenic Cf-4 (HCR9-4D) and Hcr9-4E tomato lines using the same transformation technique (Thomas *et al.*, 1997). Similarly, transgenic Moneymaker expressing Avr2 and transgenic Arabidopsis plants producing Avr4 and Avr9 were generated following the same protocol (van Esse *et al.*, 2007; van Esse *et al.*, 2008).

4.6. Conclusions and Recommendations

We were able to transform both *Cf-4-Cub-URA3* and *pZmL-Avr4* containing plasmids into a single yeast cell. Besides, Cf-4 bait stability was confirmed, but Avr4 expression is not verified yet. However, the result of yeast transformation and preliminary infiltration assay will give an indication to study Avr4 expression with different experimental setup. Thus, it is suggested to perform the infiltration assay with a more concentrated supernatant derived from Avr4 transformed yeast culture. Otherwise employing a western blot assay using anti myc antibodies, since the *pZmL-Avr4* vector contains Avr4 fused to a myc tag.

From the yeast two-hybrid cDNA library screening three independent clones were shown to be PPI-1 (proton pump interactor-1). Silencing of tomato *PPI-1* on *N. benthamiana:Cf-4* plants showed reduction of HR upon Avr4 Agroinfiltration. This suggests that, PPI-1 might be required in Cf-4-medaited resistance of *C. fulvum.* It is recommended to perform more assays to proof the hypothesis of PPI-1 requirement in Cf-4-mediated defense. This might be done using *in planta* Co-Immunoprecipitation assay to assess whether PPI-1 will bind to Cf-4 protein. In addition, activation of the proton pumps (H^+-ATPase) can be monitored for specific phosphorylation through application of Avr4 protein to Cf-4 expressing tobacco cell suspension. It is known that plasmamebrane H^+-ATPase involved in pumping out of proton to extracellular medium leading to acidification (Vera-Estrella *et al.*, 1994a). Moreover, further researches are needed to determine the requirement of other identified candidate Cf-4 interactors in Cf-4-mediated resistance.

The experiment of generating Cf-4-eGFP transgenic tomato lines will be continued by T. Liebrand. Upon root development, more plantlets will be transferred to Rockwool in the greenhouse. Confirmation of successful transformation will be done for all plantlets. Expression of Cf-4-eGFP will be evaluated by infiltration of apoplastic fluid containing Avr4 or *Agrobacterium* expressing Avr4. In addition, Q-RT-PCR will be done to analyze the number of transgene insert.

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7. Supplemental Figures

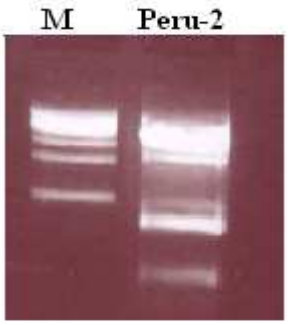

Figure 1. *Peru2* plasmid digestion performed for the 2nd time, two fragments (7182 and 2112 bp) were expected but 3 fragments (2 of them less than 1 kb) were found. We concluded the plasmid from Thomas is incorrect *Peru2* plasmid; M is 1kb marker

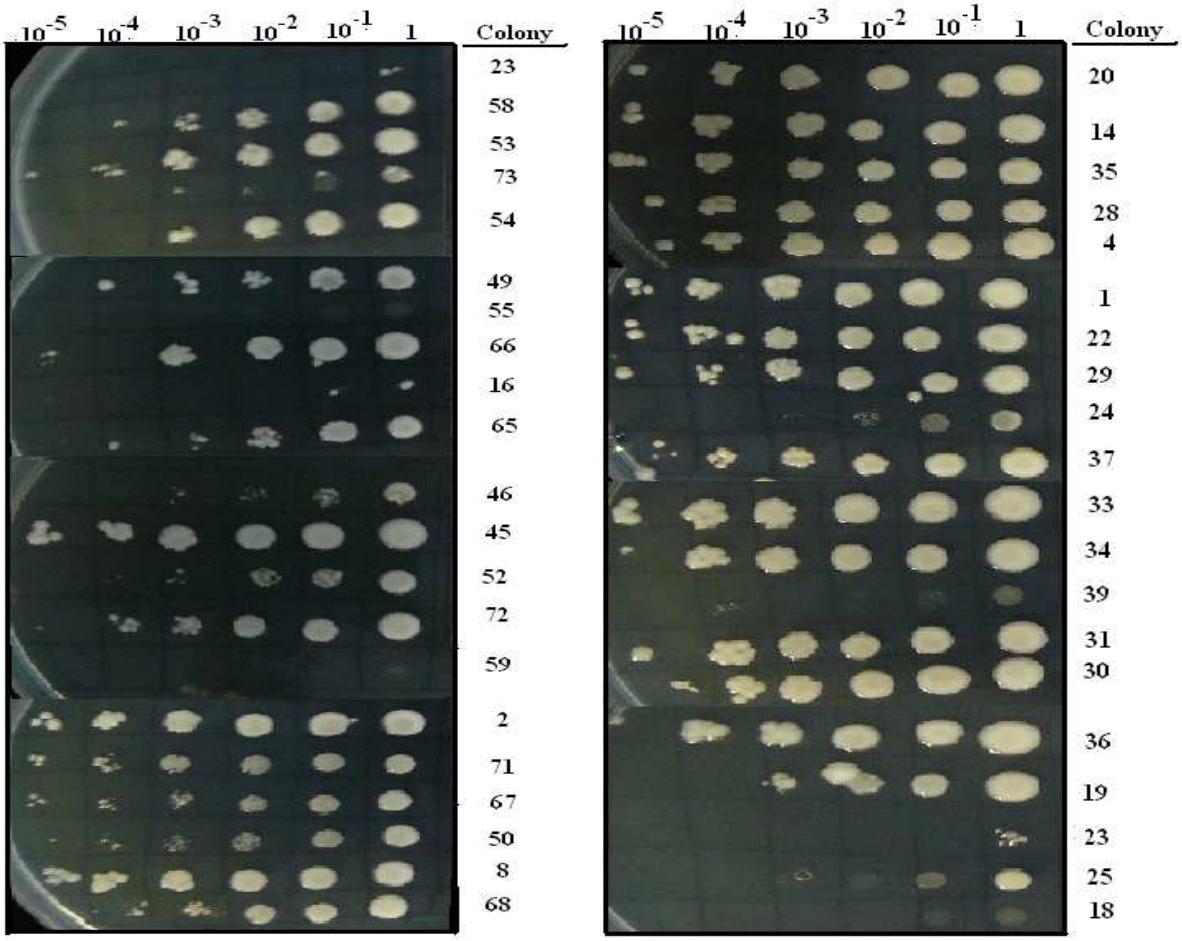

Figure 2. Dilution spot of some positive colonies from cDNA screening under different dilutions series (1 refers as undiluted and 10^{-5} as the least dilution), colonies showing growth at $\geq 10^{-3}$ are considered as true positive

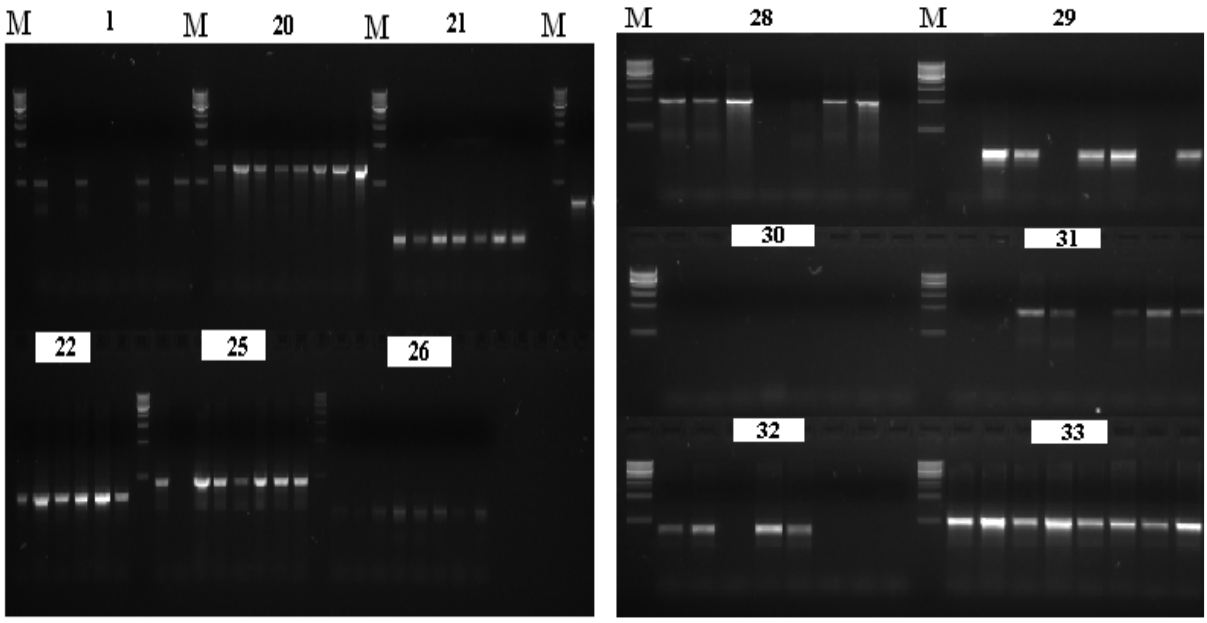

Figure 3. Colony PCR on twelve *E. coli* colonies transformed with the following plasmid numbers. Plasmid number 1,20,21,22,25,26,28,29,30,31,32 and 33 which were isolated from their respective positive yeast colony picked from the cDNA library screening. M is 1kb marker

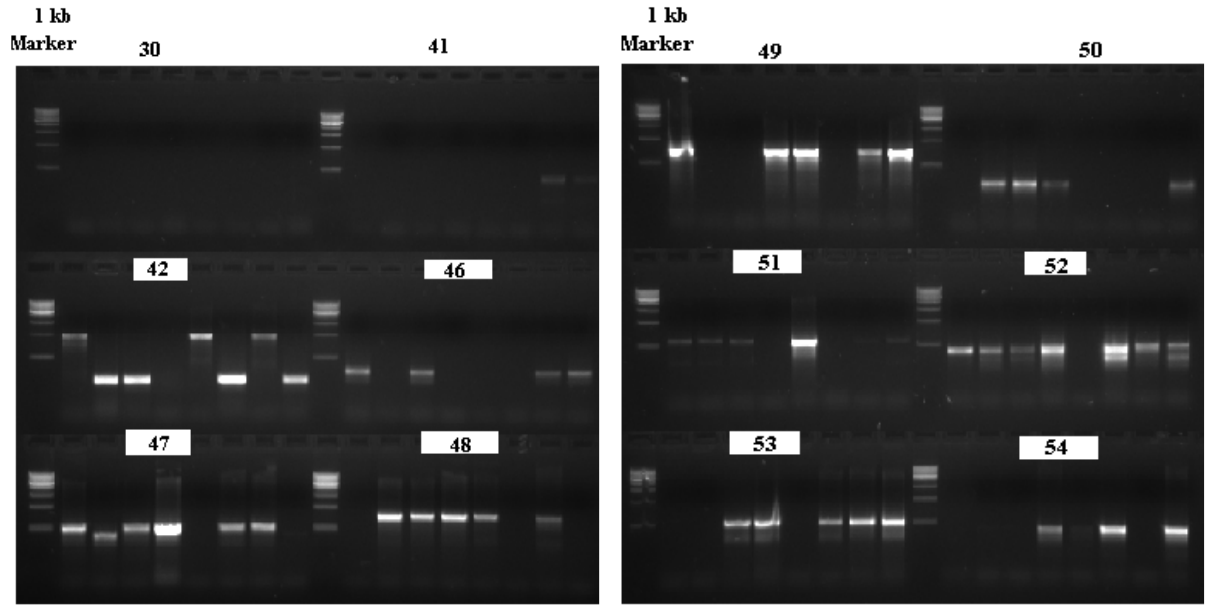

Figure 4. Colony PCR on twelve *E. coli* colonies transformed with the following plasmid numbers. Plasmid number 30,41,42,46,47,48,49,50,51,52,53 and 54 which were isolated from their respective positive yeast colony picked from the cDNA library screening. M is 1kb marker.

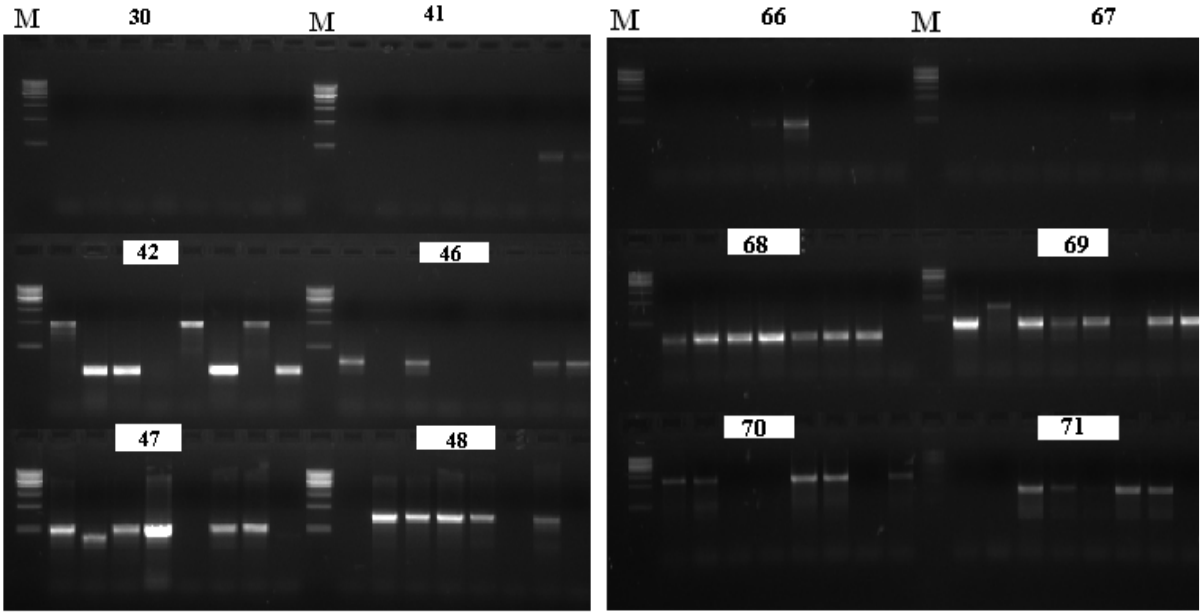

Figure 5. Colony PCR on twelve *E. coli* colonies transformed with the following plasmid numbers. Plasmid numbers 30,57,58,60,62,65,66,67,68,69,70 and 71 which were isolated from their respective positive yeast colony picked from the cDNA library screening. M is 1kb marker.

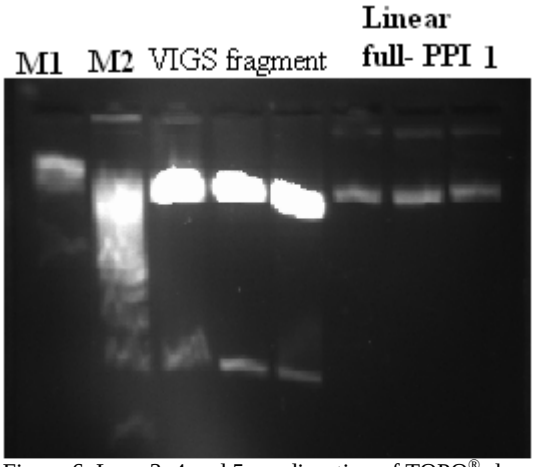

Figure 6. Lane 3, 4 and 5 are digestion of TOPO® cloned pCR4-TOPO®-VIGS*PPI*-1 vector with *EcoRI* and *BamHI*. The lower band is the VIGS fragment of *PPI*-1 and the upper band is pCR4-TOPO® vector. Lane 6, 7 and 8 are linearization of pENT-D-TOPO®-full-*PPI* plasmid with *PvuI*. M1 is 1kb and M2 is 200bp markers

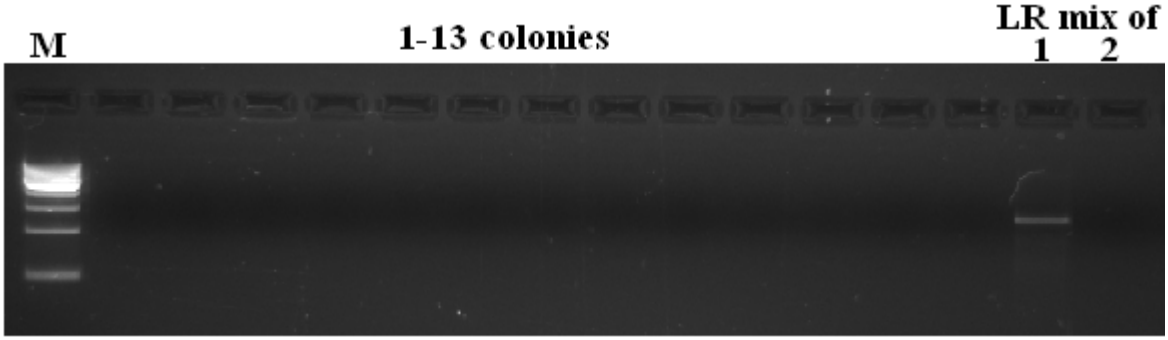

Figure 7. Colony PCR on 13 *E. coli* colonies transformed with LR Gateway® mixture of pENT-D-TOPO®-full length *PPI*-1 gene and pGWB20-10xMyc. LR mix 1 and 2 were the LR Gateway® cloning mixture of circular and linearized pENT-D-TOPO®-full length *PPI*-1 gene. M is 1 kb marker

8. Supplemental Tables

Table 1. Plasmid yield of mini-prep from section 2.3

Description	**Concentration (ng/µl)**
pMET-Cf-4(1)	164.8
pMET-peru2	156.3
pMET-Cf-2.2	163.6
pMET-AtFLS2	154.2

Table 2. Colony count of yeast transformation from section 2.4

	Number of colonies	
Description	**1st transformation**	**2nd transformation**
pMET-Cf-4(1)	376	296
pMET-peru2	16	30
pMET-Cf-2.2	448	300
pMET-AtFLS2	-	272
Untransformed JD53 yeast strain (control)	0	0

Table 3. Plasmid yield of *pMET-peru2* clones in section 2.3

Description	Concentration (ng/µl)
pMET-peru2 clone 1	270.3
pMET-peru2 clone 2	206.3
pMET-peru2 clone 3	197.6

Table 4. Plasmid yield isolated from positive yeast colonies of cDNA screening in section 2.5.1

Colony number	**Plasmid concentration (ng/µl)**	**Colony number**	**Plasmid concentration (ng/µl)**	**Colony number**	**Plasmid concentration (ng/µl)**	**Colony number**	**Plasmid concentration (ng/µl)**
1	62	19	129.7	38	181.8	58	32.7
2	76.7	20	50.4	40	114.6	60	29.5
3	54.8	21	96.9	41	77.9	62	102.1
4	74.3	22	130.4	42	34.1	65	103.3
5	45.7	25	59.5	43	3.6	66	91.4
6	80.2	26	52.8	44	27.6	67	110.7
7	79.3	28	71.5	46	149.2	68	118.4
8	110.9	29	39.5	47	151.7	69	57.8
9	135.1	30	24.8	48	90.8	70	111.4
10	147.7	31	36.8	49	112.1	71	63.8
11	95.6	32	73.0	50	95.4	72	137.6
12	39.2	33	28.0	51	30.8	73	135.8
13	57.8	34	31.5	52	141.8	74	128.5
14	61.5	35	68.3	53	129.1	75	310.4
15	63.5	36	86.7	54	295.8		
17	70.8	37	124.7	57	73.2		

* Plasmid isolated from colony 43 was very less (3.6 ng/µl), then discarded from the experiment.

Table 5. Plasmid yield isolated from positive *E. coli* colonies of colony PCR in section 2.5.2

Colony number	**Plasmid concentration (ng/µl)**	**Colony number**	**Plasmid concentration (ng/µl)**	**Colony number**	**Plasmid concentration (ng/µl)**	**Colony number**	**Plasmid concentration (ng/µl)**
2 (7)	180.1	17 (2)	153.3	37 (1)	145.2	60 (2)	139.8
3 (1)	199.5	17 (5)	139.5	37 (8)	139.1	62 (4)	187.8
4 (5)	161.8	19 (1)	134.8	38 (7)	133.8	65 (3)	169.2
5 (8)	155.9	1 (3)	104.9	40 (2)	136	65 (5)	96.8
11 (1)	173.1	20 (6)	132.9	41 (7)	132.4	66 (5)	108.9
11 (2)	166.7	21 (7)	141.6	42 (1)	151.4	67 (6)	115.5
11 (3)	140.4	22 (5)	126.8	42 (6)	114.9	68 (4)	162.3
6 (8)	150.1	25 (8)	115.8	46 (3)	133.5	69 (2)	174.6
7 (4)	171.4	26 (4)	123.5	47 (2)	130.4	69 (7)	149.6
8 (4)	106.7	28 (3)	135.8	47 (6)	113.8	70 (5)	183.8
9 (2)	135.5	29 (6)	119	48 (2)	139.9	71 (3)	193.6
10 (5)	137.3	31 (7)	148.9	49 (4)	172.3	72 (1)	147.3
12 (1)	144.3	32 (4)	135.9	50 (2)	133.9	73 (4)	92.5
12 (2)	151.4	33 (2)	146.4	51 (5)	157.6	74 (3)	116.5
12 (8)	139.7	34 (1)	153.6	52 (4)	164.5	75 (2)	139.6
13 (2)	116.2	35 (6)	114.6	53 (3)	139.3	Gus-1	164.8
13 (6)	108.3	35 (7)	143.5	54 (6)	131.9	Gus-2	128.9
14 (2)	124.3	36 (3)	115.6	57 (5)	113.7		
15 (4)	143.6	36 (6)	139.5	58 (4)	190.1		

Table 6. Plasmid concentration of the LR Gateway® cloned *pZmL-Avr4* clones in section 2.8.1

Description	Concentration (ng/µl)
pZmL-Avr4 clone 1	86.4
pZmL-Avr4 clone 2	140.8
pZmL-Avr4 clone 3	85.1

Table 7. HR score of Cf-4 and wild *N. benthamiana* plants infiltrated with (A) *Agrobacterium* expressing Avr4, (B) supernatant of *pZmL-Avr4* containing yeast culture, (C) supernatant of pZmL-dest. containing yeast culture, (D) liquid DO-L media. Four leaves from each plants and each treatment infiltrated on each leaf (++ strong HR, + mild HR and – no HR)

		plant	**Leaf 1**				**Leaf 2**				**Leaf 3**				**Leaf 4**			
			A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Day 1	*N. benthamiana:Cf-4* plants	1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
		2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		4	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	Wild *N. benthamiana* plants	1	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-
		2	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
		3	-	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-
		4	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-
Day 2	*N. benthamiana:Cf-4* plants	1	++	-	-	-	++	+	-	-	+	-	-	-	++	-	-	-
		2	++	+	-	-	+	-	-	-	+	-	-	-	++	-	-	-
		3	+	-	-	-	++	-	-	-	++	-	-	-	++	-	+	-
		4	+	-	-	-	++	-	-	-	++	-	-	-	++	-	-	-
	Wild *N. benthamiana* plants	1	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-
		2	-	++	++	-	-	+	+	-	-	+	+	-	-	+	+	-
		3	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-
		4	-	+	+	-	-	+	+	-	-	+	++	-	-	-	++	-
Day 3	*N. benthamiana:Cf-4* plants	1	++	-	-	-	++	+	-	-	++	-	-	-	++	-	-	-
		2	++	+	-	-	++	-	-	-	++	-	-	-	++	-	-	-
		3	+	-	-	-	+	-	-	-	++	-	-	-	++	-	+	-
		4	++	-	-	-	++	-	-	-	++	-	-	-	++	-	-	-
	Wild *N. benthamiana* plants	1	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-
		2	-	++	++	-	-	+	+	-	-	+	+	-	-	+	+	-
		3	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-
		4	-	+	+	-	-	+	+	-	-	+	++	-	-	-	++	-

Table 8. HR score of *N. benthamiana:Cf-4* plants infiltrated with (A) *Agrobacterium* expressing Avr4, (B) Apoplastic fluid containing Avr4, (C) *Agrobacterium* expressing autoactive Rx and (D) *Agrobacterium* expressing BAX. Four leaves from each plants and each treatment infiltrated on each leaf (++ strong HR, + mild HR, C collapsed cell and – no HR); Score taken after 3 days of infiltration

Silenced construct (gene)	plant	**Leaf 1**				**Leaf 2**				**Leaf 3**				**Leaf 4**			
		A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
PPI-1	1	C	-	++	+	C	-	++	+	C	-	++	+	C	-	++	+
	2	-	-	++	+	+	-	++	+	C	C	++	C	-	-	++	-
	3	+	-	+	-	C	-	++	+	C	-	++	C	C	C	++	+
Cf-4	1	C	C	++	++	C	-	++	+	C	-	++	+	-	-	++	++
	2	-	-	++	++	C	-	++	++	-	-	++	++	+	-	++	C
	3	C	-	++	C	C	-	++	+	C	-	++	+	-	-	C	-
SGT1	1	-	-	-	-	-	C	-	-	-	C	-	-	-	-	-	-
	2	C	+	-	C	-	-	-	-	-	-	C	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SOL2735	1	-	-	++	++	C	-	++	+	-	-	++	++	+	-	++	C
	2	C	-	++	C	-	-	++	++	C	-	++	++	-	-	++	++
SOL2771	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GUS	1	++	-	++	+	+	-	++	+	++	-	++	-	+	-	++	++
	2	+	-	++	++	+	-	++	+	+	C	++	-	+	-	++	++

Table 9. Tomato Cf-4 interactors from Split- Ubiquitin yeast two-hybrid screening which were not in a correct reading frame based on the NuI N-terminal ubiquitin moiety. [(1)] Corresponding SGN-unigene based on SGN blast, [(2)] Predicted protein by NCBI protein BLAST including the GenBank accession number [(3)] predicted domains based on SMART domain blast

Cf-4 Interactor	SGN-unigene [(1)]	Predicted protein by BLAST [(2)]	Predicted domains [(3)]
4.5	SGN-U590039	unknown [Zea mays], ACR34970.1	Low complexity region
5.8	No hit	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase (Salmo salar), BT059958.1	Low complexity region
7.4	SGN-U579843	chloroplast chlorophyll a-b binding protein (*Nicotiana tabacum*), AY554168.1	Pfam:Chloroa_b-binding
8.4	SGN-U579085	peptide transporter, (Ricinus communis,) XM_002520381.1	Pfam:Sugar_tr, Pfam:MFS_1 and Pfam:PTR2
11.1	SGN-U572479	Solanum lycopersicum cDNA, clone: LEFL1001CA06, HTC in leaf AK246939.1	Signal peptide
12.1	SGN-U581066	26S ribosomal (*Nicotiana tabacum*), AF479172.1	None
12.8	SGN-U566099	phosphoglycerate mutase-like protein (PGM) (Glycine max), AY004241.1	PGAM domain
13.6	No hit	40S ribosomal protein S7 (Salmo salar), BT059944.1	None
14.2	SGN-U568670	proteasome-like protein alpha subunit (*Solanum tuberosum)*, DQ228349.1	Proteasome_A_N domain, Pfam:proteasome
17.2	SGN-U564007	putative acyl-CoA synthetase (*Capsicum annuum*), AF354454.1	Pfam:AMP-bindinig
17.5	SGN-U580976	4-coumarate:coenzyme A ligase (4CL1) (*Nicotiana tabacum)*, U50845.1	Pfam:AMP-bindinig
19.1	SGN-U575965	protease inhibitor/seed storage/lipid transfer protein family protein (*Tamarix hispida)*, ACM78616.1	Signal peptide, AAI and low complexity
21.7	No hit	DNA sequence from clone LE_HBa-27B24 (S .lycopersicum) CU914528.4	None
22.5	SGN-U568650	phosphoinositide binding (*Arabidopsis thaliana)*, NP_564781.1	2 RING domains and low complexity
25.8	SGN-U580697	putative ubiquitin extension protein (*Solanum tuberosum)*, EU294350.1	UBQ, low complexity and Pfam:Ribosomal_S27
26.4	SGN-U565102	protein phosphatase 2C,(Nicotiana tabacum), AB110954.1	PP2Cc and PP2C_SIG
29.6	SGN-U581488	ribosomal protein S29 (*Jatropha curcas)*, ADB02896.1	Pfam;Ribosomal_S14
31.7	SGN-U580235	adenylyl-sulfate reductase (Lycopersicon esculentum), AY568717.1	Low complexity, Pfam:PAPS_reduct and Pfam:Thioredoxin
32.4	SGN-U581090	10kDa polypeptide precursor of photosystem II, (*L. esculentum*), X95987.1	Pfam:PsbR
33.2	SGN-U578242	ubiquitin conjugating enzyme E2, (*L. esculentum)*, X73419.1	UBCc domain
35.6	SGN-U586460	small heat shock protein Hsp23.5 (*Triticum aestivum*), AAD03604.1	Low complexity region and Pfam:HSP20
35.7	SGN-U577641	dihydrolipoamide dehydrogenase precursor (lpd2) (*Solanum tuberosum)*, AF295339.1	Pfam:Thi4, Pfam:HI0933_like, Pfam:DAO, Pfam:FAD_binding_2, Pfam:GIDA, Pfam:pyr_redox_2 and Pfam:pyr_redox, Pfam:pyr_redox_dim

36.6	SGN-U579171	N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase THT1-4 (THT1-4) pseudogene (*Lycopersicon esculentum*), AY081908.1	Pfam:Acetyltransf_1
37.1	SGN-U588828	PREDICTED: hypothetical protein (Vitis vinifera), XP_002283422.1	Pfam:UPF0041
38.7	SGN-U580869	RuBisCo small (*Capsicum annuum)*, AF065615.1	Low complexity region and Pfam:RuBisCo_small
40.2	SGN-U582179	TatD DNase domain-containing deoxyribonuclease, (*Ricinus communis*), XM_002524024.1	Pfam:TatD_DNase and Low complexity region
41.7	SGN-U564368	hypothetical protein (*Nicotiana benthamiana)*, BAF02556.1	None
42.1	SGN-U577195	Rab GDP dissociation inhibitor (GDI) (*Nicotiana benthamiana),* FJ755907.1	Pfam:GDI
42.6	SGN-U294014	imp biosynthesis protein (*Oryza sativa* Indica Group), ABR26072.1	Low complexity
46.3	SGN-U570812	glucose-methanol-choline (gmc) oxidoreductase, (*Ricinus communis),* XM_002509648.1	Signal peptide, Pfam:GMC_oxred_N, Pfam:DAO and Pfam:GMC_oxred_C
47.2	SGN-U578562	cevi-1 gene, (*Lycopersicon esculentum)*, Y19023.1	Signal peptide and Pfam_peroxidase
49.4	SGN-U584857	unnamed protein product [Vitis vinifera], CBI28100.3	Low complexity region
50.2	SGN-U591178	cDNA, clone: LEFL1027AB10, HTC in leaf, (*Solanum lycopersicum)*, AK247065.1	None
51.5	SGN-U564931	RecName: Full=Pathogen-related protein *(Hordeum vulgare)*, P16273.2	None
52.4	No KKV site		
57.5	SGN-U579575	ribosomal protein PETRP-like (*Solanum tuberosum*), DQ235197.1	Pfam:Ribosomal_L22
69.7	SGN-U572726	DNAJ heat shock N-terminal domain-containing protein (*Arabidopsis thaliana)*, NP_188036.1	DnaJ domain and 2 low complexity region
71.3	SGN-U582565	cell death associated protein (*Solanum tuberosum*), AB200918.1	Pfam:Abhydrolase_3
73.4	SGN-U580694	ribosomal protein L12-1a (*N. tabacum*), X62368.1	Low complexity region and Pfam:Ribosomal_L12

9. Appendixes

Appendix A

Yeast Medium

YEPD medium (1L)

20 g Difco peptone
10 g Yeast extract
40ml Glucose 50% (or 20g of glucose) to 1L water

Dropout Medium (1L)

	-H	-T	-HT	-HTU	-U
glucose (g)	20	20	20	20	20
Yeast Nitrogen Base (g)	6.7	6.7	6.7	6.7	6.7
Dropout mix (selective AA / g)	-H / 1,92	-T / 1,92	-HTUL / 1,4	-HTUL / 1,4	-U / 1,92
Ura (mg)	-	-	50	-	-
His (mg)	-	-	-	-	-
Trp (mg)	-	-	-	-	-
Lys (mg)	-	-	-	-	-
Leu (mg)	-	-	380	380	-
Agar (g)	15	15	15	15	15
Water	to 1L	to 1L	to 1L	to 1L	to 1L

Absolute Minimal Medium (1L)

	-H	-T	-HT	-HTU	-U	HU
glucose (g)	20	20	20	20	20	20
Yeast Nitrogen Base (g)	6.7	6.7	6.7	6.7	6.7	6.7
Ura (mg)	50	50	50	-	-	-
His (mg)	-	76	-	-	76	-
Trp (mg)	76	-	-	-	76	76
Lys (mg)	76	76	76	76	76	76
Leu (mg)	380	380	380	380	380	380
Agar (g)	15	15	15	15	15	15
Water	to 1L	to 1L	to 1L	to 1L	to 1L	1L

FOA-containing Minimal Medium (1L)
Dissolve 1g of FOA in 1oml of DMSO (or water) by vortexing, incubating at 37°C for 15-20min can help

L- MEDIUM for *E. coli* (1L)

Glucose (g)	1
Trypton (g)	10
Yeast extract (g)	5
Nacl (g)	5
Agar (g)	15
Water	to 1L

Appendix B

Fast Yeast Transformation

- 10 ml o/n culture of yeast strain in selective dropout media or full media (YEPD) if it is not carrying any plasmid
- centrifuge 5min at 3000rpm
- resuspend pellet in 10ml H_2O
- centrifuge 5min at 3000rpm
- resuspend pellet in 1ml LiAc 100mM freshly prepared from a 1M LiAc solution
- transfer to Eppi tube and centrifuge 15sec at 13000rpm
- resuspend in 0,5ml LiAc 100mM
- use 50µl for each transformation (better to use 2ml Eppi tube)
- spin briefly at 11000rpm (removing the supernatant is not necessary: avoid to lose a lot of yeast cells)
- add DNA into the pellet (from 100ng up to 1µg)
- **while vortexing** add 300µl of the PEG solution
- incubate at 30° for 30min
- switch to 42° for 60min (but it can vary according to the yeast strain)
- centrifuge 1min at 13000rpm
- resuspend pellet in 300/400µl H_2O
- plate 100 µl on dropout media (or minimal media if the bait is methionine-sensitive)

PEG Solution (1ml)

50% PEG 3350	680 µl
1M LiAc	100 µl
2mg/ml carrier DNA	140 µl
H_2O	80 µl

Dilute 1:5 carrier DNA (10 mg/ml stock Roche)
Incubate at 95°C for 5 min
Add to the PEG solution just before use

Appendix C

Yeast Transformation (Library Scale)

- 10 ml o/n culture of yeast strain in selective dropout media or full media (YEPD) if it is not carrying any plasmid
- determine OD_{600} of the o/n culture (1 OD_{600}= 2,5 * 10^7 cells/ml)
- inoculate 50ml of a main culture in YEPD with 2,5 *10^8 cells (=5*10^6 cells/ml)
- grow for 4-5 hours at 30°C shaking up to a density of 2 * 10^7 cells/ml
- centrifuge 5min at 3000rpm
- resuspend pellet in 25ml H_2O
- centrifuge 5min at 3000rpm
- resuspend pellet in 1ml LiAc 100mM freshly prepared from a 1M LiAc solution
- transfer to Eppi tube and centrifuge 15sec at 13000rpm
- resuspend in 0,5ml LiAc 100mM
- use 50µl for each transformation (better to use 2ml Eppi tube)
- spin briefly at 11000rpm (removing the supernatant is not necessary)
- add DNA to the pellet (from 100ng up to 1µg)
- **while vortexing** add 300µl of the PEG solution
- incubate at 30° for 30min
- switch to 42° for 60min (but it can vary according to the yeast strain)
- centrifuge 1min at 13000rpm
- resuspend pellet in 1ml H_2O (200ul)
- plate 10 µl (2ul) on dropout media to determine the transformation efficiency
- plate all the rest on a selective minimal media

PEG Solution (1ml)

50% PEG 3350	680 µl
1M LiAc	100 µl
2mg/ml carrier DNA	140 µl
H_2O	80 µl

Dilute 1:5 carrier DNA (10 mg/ml stock)
Boil for 5 min
Add to the PEG solution just before using

Appendix D

Heat shock transformation *E. coli*

- *E. coli* DH5α competent cell from -80°C. thawed and directly put on ice
- 39 µl *E. coli* + …. µl (+/- 50 ng) appropriate plasmid
- (Or for ligation 75 µl *E. coli* + ... µl ligation mix)
- Put on ice for 30 min
- Heat shock: put tube for 90 sec at 42°C
- Put tube on ice for 1 min
- Add per tube 1 ml of L-medium, mix by pipetting
- Incubate for 1 hour at 37°C
- Plate on appropriate selection medium

Electroshock Transformation *E. coli*

- *E. coli* DH5α competent cell from -80°C. thawed and directly put on ice
- Add 2-3 µl of ligation mix or LR Gateway® mix to cells
- Electroshock 2.4kV 200ohm
- Add 300 ul L media
- Incubate 1 hour 37C
- Plate on selection media

Gateway® LR cloning (own protocol)

4 ul pENT/pDONR
1 ul pDEST
0.5 ul TE pH8
0.3 ul LR Mix

2-4hours 25C, + 0.5 µl protinase K and 15 min 37C

Ligation

- ….ng of plasmid
- 1 µl of T4 ligase buffer
- 1 µl of T4 ligase
- … ng of fragment
- Add MilliQ till 10 µl
- Ligate at 14-16°C o/n

(Put tube in a red-cap, close well with parafilm, add weights and sink in water bath 14°C)

***Agrobacterium* transformation**

- *Agrobacterium* tumefaciens C584 + pCH32 electrocompetent cells in -80 freezer
- Thaw aliquot from the -80 freezer
- Add 40 µl of cells to a tube and add 2-4 µl plasmid
- Transfer mixture to a electroporation cuvette placed on ice
- Give a pulse at 2.4 kv at 400Ω, time constant should be around 9 ms
- Add directly after the pulse 1 ml L-medium
- Transfer suspension to 1.5 ml tubes and incubate at 28°C for 1.5-2 h
- Plate 200 µl of the suspension on L+kan+tet plates
- Incubate plates at 28°C for 2-3 days

Appendix E

Protocol: Plasmid DNA Purification Using the Qiaprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 μg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods.

Note: All protocol steps should be carried out at room temperature.

1. Resuspend pelleted bacterial cells in 250 μl Buffer P1 and transfer to a microcentrifuge tube.
Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. Add 250 μl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.
Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.
If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. Add 350 μl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy.
If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
A compact white pellet will form.

5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

6. Centrifuge for 30–60 s. Discard the flow-through.

7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.
This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5□™ do not require this additional wash step.

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

Appendix F

Zymoprep™ II -Yeast Plasmid Miniprep: Protocol for Liquid Culture

1. Aliquot 0.1-1.5 ml of the yeast cells into 1.5 ml microfuge tubes and spin down the cells at 600 x g for 2 minutes.

Note: For multiple sample process, add 15 ul **Zymolyase™** for each ml of **Solution 1** to make a **Solution 1-enzyme mixture**. Use 200µl of this mixture to resuspend the pellet for each sample. Generally fresh culture gives better plasmid recovery than old culture. Add more Zymolyase™ for old culture to ensure efficient lysis. Ideally cells should be harvested in early log phase (OD600: 0.2-0.6). If cells are harvested from stationary phase cultures or old culture is used, add more Zymolyase™ to ensure efficient lysis.

2. Add 200 ul **Solution 1** to each pellet.

3. Add 3 ul of **Zymolyase**™ to each tube. Resuspend the pellet by flicking with finger or mild vortexing.

4. Incubate at 37°C for 15-60 minutes (15 minutes is the minimal incubation time. Longer incubation is optional, but is suggested for stationary phase or older cells).

5. Add 200 ul **Solution 2** to each tube. Mix well.

6. Add 400 ul **Solution 3** to each tube. Mix well.

7. Centrifuge at maximum speed for 3 minutes.

8. Transfer the supernatant to the **Zymo-Spin-I** column.

9. Spin the **Zymo-Spin I** column for 30 seconds.

10. Discard the flow-through in the collection tube. Make sure the liquid does not touch the bottom part of the column.

11. Add 550 ul of **Wash Buffer** (ethanol added) onto the column with the collection tube and spin for 1-2 minutes. Discard the wash buffer. Place column into a new 1.5 ml microfuge tube (not provided).

12. Add 10 ul of water or TE and spin for 30 seconds-1 minute to elute plasmid off the column into a new 1.5 ml microfuge tube. For plasmids larger than > 15 Kb, incubate the column and elution buffer (water or TE) for 5 minutes before centrifugation to increase plasmid yields.

Appendix G

Illustra GFX PCR DNA and Gel Band Purification Kit

Protocol for purification of DNA from TAE and TBE Agarose gels

1. Sample Capture

a. Weigh a DNase-free 1.5 ml microcentrifuge tube and record the weight.
b. Using a clean scalpel, long wavelength (365 nm) ultraviolet light and minimal exposure time cut out an agarose band containing the sample of interest. Place agarose gel band into a DNase-free 1.5 ml microcentrifuge tube (user supplied).
c. Weigh microcentrifuge tube plus agarose band and calculate the weight of the agarose slice.
Note: The sample may now be stored at -20°C for up to 1 week.
d. Add 10 µl **Capture buffer type 3** for each 10 mg of gel slice, for example, add 300 µl Capture buffer type 3 to each 300 mg gel slice.
Note: If the gel slice weighs less that 300 mg, add 300 µl Capture buffer type 3. DO NOT add less than 300 µl Capture buffer type 3 per sample.
Note: To save time when purifying multiple samples of gel bands (each weighing less than 500 mg), add 500 µl Capture buffer type 3 to each gel slice. DNA recovery will be unaffected providing the volume of Capture buffer type 3 is in excess of the weight of each gel slice.
e. Mix by inversion and incubate at 60°C for 15–30 minutes until the agarose is completely dissolved. Mix by inversion every 3 minutes.
Note: If sample contains DNA > 5 kb, do not vortex, this may cause shearing of DNA.
f. Once the agarose has completely dissolved check that the **Capture buffer type 3**-sample mix is yellow or pale orange in color.
Note: If the color of binding mixture is dark pink or red, add a small volume (~ 10 µl) of 3 M sodium acetate pH 5.0 and mix. Ensure that the binding mixture turns a yellow or pale orange color before loading onto GFX MicroSpin column. Refer to section 4.1 for more information.
g. For each purification to be performed, place one GFX MicroSpin column into one Collection tube.

2. Sample Binding

a. Centrifuge **Capture buffer type 3**- sample mix briefly to collect the liquid at the bottom of the tube.
b. Transfer up to 800 µl **Capture buffer type 3**- sample mix onto the assembled GFX MicroSpin column and Collection tube.
Note: the cap of the Collection tube can be used to cap the GFX MicroSpin column. If the cap is not required cut if off.
c. Incubate at room temperature for 1 minute.
d. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.
e. Discard the flow through by emptying the Collection tube. Place the GFX MicroSpin column back inside the Collection tube.
f. Repeat Sample Binding steps b. to e. as necessary until all sample is loaded.

3. Wash and Dry

a. Add 500 µl **Wash buffer type 1** to the GFX MicroSpin column.
b. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.
Note: If purity is paramount (e.g., if the sample is to be used in a blunt-ended ligation), repeat Wash and Dry step a and perform step b twice. After the first spin, discard flow through, place the GFX MicroSpin column back inside the Collection tube and centrifuge at 16 000 × g for an additional 30 seconds. This extra wash step may reduce yield by 4%.
c. Discard the Collection tube and transfer the GFX MicroSpin column to a fresh DNase-free 1.5 ml microcentrifuge tube (supplied by user).

4. Elution

a. Add 10–50 µl **Elution buffer type 4** *OR* **type 6** to the center of the membrane in the assembled GFX MicroSpin column and sample Collection tube.
b. Incubate the assembled GFX MicroSpin column and sample Collection tube at room T^0 for 1 minute.
c. Spin the assembled column and sample Collection tube at 16 000 × g for 1 minute to recover the purified DNA.
d. Proceed to downstream application. Store the purified DNA at -20°C. 30 seconds