Functional characterization of *PEN1-like* genes involved in *ol-2* resistance in tomato

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Abstract

Broad spectrum resistance has been a key focus to plant breeders due to its durability potential. Different types of powdery mildew, including *Leveillula taurica*, *Oidium lycopersici* and *Oidium neolycopersici*, affect and cause huge losses in both the green house grown as well as the field-grown tomatoes. Molecular breeding focuses on susceptibility genes used by pathogens to increase pathogenicity. Silencing of susceptibility genes, confer broad spectrum resistance. *Mlo* is an example of a susceptibility gene, whose silencing results in broad spectrum resistance. Tomato *ol-2* plants are resistant lines arising from a natural mutation of tomato *Mlo* ortholog *SlMlo1*. Genetic screens for chemically induced *Arabidopsis* mutants with altered non-host interactions upon *Blumeria graminis f.sp. hordei* inoculation and resulting in increased penetration by the barley mildew led to identification of three penetration genes, *PEN1*, *PEN2* and *PEN3*. In our studies, we sought to identify the role of *PEN1* like genes in conferring broad spectrum resistance against *Oidium neolycopersici* in tomato. A blast search using AtPEN1 protein sequence helped identify two *PEN1* tomato homologs with a 74.3% and 74% sequence identity to *AtPEN1* and an 87.3% sequence identity between the two tomato *PEN1* homologs. RNAi was used to silence *PEN1* and *PEN1* homolog. Transgenic T2 tomato families had visible mycelial symptoms, increased fungal biomass with decreased expression levels of targeted genes. Microscopic analysis of transgenic susceptible plants showed germinated spores with hyphae colonies, appressorium and a feeding structure known as haustorium. There was cell wall deposition (papilla) below the germinating spores and at the neck of the feeding structures. From the results, we concluded that *PEN1* and *PEN1* homolog had an indispensable role in penetration resistance in tomato against powdery mildew *Oidium neolycopersici*.

Key words: *powdery mildew, broad spectrum resistance, papilla, penetration genes. RNAi induced silencing*

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1| Introduction and background information

1.1. Tomato fruit (*Solanum lycopersicum*)

Tomato is an important vegetable with numerous nutritional, health, and economic benefits. It is grown as a field and greenhouse perennial crop in tropical areas due to long growing seasons, while in areas with winters, characterised by frost and short daylight hours, it is grown as an annual crop. Tomato and tomato products are a source of beneficial compounds, such as lycopene, which is an antioxidant with anticancer properties (Jones *et al.* 2000).Tomatoes are considered as healthy cholesterol free foods that are low in fat and calories, and a good source of fibre as well as protein. Additionally, they are also rich in vitamins A and C, β-carotene, and potassium (Shi and Le Maguer 2000). Growers of tomato have previously faced great yield losses caused by different species of powdery mildew fungi (*Oidium* and *Leveillula*). Powdery mildew is common in several countries, affecting many important agricultural crops.

1.2. Host range of powdery mildew and geographical distribution

Worldwide, there are approximately 500 powdery mildew species that colonize and infect over 650 monocots and over 9000 dicots (Schulze-Lefert and Vogel 2000). Powdery mildew causes significant losses in agriculturally important crops, such as barley, cucumber, eggplant, pea, tobacco, and tomato (Table1). There is variation in host range among the different species of powdery mildew. Some species have a wide host range, while others have specific hosts. A unit within the powdery mildew that is distinguished by its host range is known as *forma speciales*. In the *forma speciales*, a certain powdery mildew isolate may be known to have one type of plant species as its host, for example, *Blumeria graminis* that show high specificity to grass (Trujillo *et al.* 2004). Barley, wheat and rye are infected by the powdery mildews *B. graminis f.sp.hordei, B. graminis f. sp. tritici* and *B. graminis f. sp. secale*, respectively (Schulze-Lefert and Vogel 2000) (Trujillo *et al.* 2004).

The anamorphic state of a powdery mildew first appeared on greenhouse grown tomato in southern England in 1987 (Whipps *et al.* 1998) (Fletcher *et al.* 1988). In the Netherlands, powdery mildew *O. neolycopersici* first occurred in 1988 and has spread to other European countries within 10years. *O. lycopersici* is found in Australia (Kiss *et al.* 2001). *Leveillula taurica* is another powdery mildew fungus infecting tomato. *L. taurica* can morphologically be distinguished from *O. neolycopersici* since the

mycelia of *L. taurica* grow through mesophyll layer and are visible on the abaxial side of the leaf, while *O. neolycopersici* grows mainly on the adaxial side and does not penetrate into the mesophyll layer (Lindhout *et al.* 1993) .

In *Arabidopsis*, there are four known species that establish a compatible interactions including three *Golovinomyces* spp *G. cichoracearum, G. orontii, G. cruciferarum* and *Oidium neolycopersici* which also infect tomato (Xiao *et al.* 2001). *Blumeria graminis f.sp.hordei* is a barley powdery mildew while *Erysiphe pisi* infects pea (Spanu *et al.* 2010).

Table 1: Examples of powdery mildew species and the affected host plants.

Fungus	***Host plant***	***Common plant name***
G. cichoracearum	*Arabidopsis thaliana*	*Arabidopsis*
G. orontii	*Arabidopsis thaliana*	*Arabidopsis*
G. cruciferarum	*Arabidopsis thaliana*	*Arabidopsis*
Blumeria graminis f.sp hordei	*Hordeum vulgare*	Barley
E. pisi	*Pisum sativum*	Pea
G. cichoracearum	*Cucurbita pepo*	Squash
G. orontii	*Nicotiana tabacum*	Tobacco
Oidium longipes	*Solanum melongena*	Egg plant
O. lycopersici	*Solanum lycopersicum*	Tomato
O. neolycopersici	*Solanum lycopersicum*	Tomato/*Arabidopsis*
Leveillula taurica	*Solanum lycopersicum*	Tomato

Genus names: *E; Erysiphe, G; Golovinomyces, O; Oidium*

1.3. Approaches to curb powdery mildew

Use of fungicides, like, benomyl, bupirimate, carbendazim, chlorothalonil, fenarimol, and pyrazophos has been one approach to reduce the losses caused by powdery mildew (Fletcher *et al.* 1988). A successful common greenhouse practice in Wageningen Unifarm, is the use of sulphur as a fungicide in compartments to keep away contamination by unwanted powdery mildew. The fungicide is used two times a week inform of a vapour released for at least one hour each time. However, fungicide have huge detrimental effects to the environment especially when used in open fields. For this reason, plant breeding for resistance is an appealing alternative. This method has successfully led to the

introduction of resistance genes, for example, *RPW8.1* and *RPW8.2* in *Arabidopsis* (Xiao *et al.* 2001) and *Mlo* and *Mla* in barley (Chełkowski *et al.* 2003) which confer full resistance against powdery mildew. Since cultivated tomato has limited variability, largely due to artificial selection during domestication and development of modern cultivars, tomato wild germplasm is a useful resource to improve disease resistance and other important agronomic traits (Bai and Lindhout 2007). Wild-type tomato (*Solanum* species previously *Lycopersicon*); *S.neorickii, S. lycopersicum var. cerasiforme, S. habrochaites, S. pennellii, S. cheesmaniae, S. chilense, S. peruvianum* are the known sources of resistance genes in tomato identified against powdery mildew (Lebeda *et al.* 2013). They are termed as *Ol* genes.

The use of resistance genes is quite effective but has many technical difficulties. Most resistance genes are obtained from wild-type species which have to be crossed with cultivated crops. The crosses are sometimes difficult and require more expensive techniques like embryo rescue for successful crosses. Moreover, the backcross to remove undesirable traits obtained from resistant wild-type source takes time. The specificity of resistance genes is another major problem, since only specific powdery mildew avirulence proteins are recognised by corresponding resistance genes. This specificity allows infection by powdery mildew species lacking corresponding virulence proteins. Breeders now focus on a less specific, broad-spectrum disease resistance achieved through the use of susceptibility genes (Büschges *et al.* 1997).

1.4. Breeding for disease resistance

Breeding for resistance against powdery mildew involves the use of resistance genes that are mostly derived from wild plants species. These genes have been used successfully in breeding, but with challenges in ensuring durable resistance. Presence of many transposons in powdery mildew causes genetic variations enabling the fungi to evolve and produce molecules unrecognisable by plant resistance genes (Spanu *et al.* 2010). To address this issue, breeders are now focusing on the susceptibility genes in plants that are used by pathogens to establish pathogenesis. Pathogens use these genes in order to gain entry into the host plant. Silencing of these susceptibility genes could provide a broad spectrum non-host resistance that is durable (Pavan *et al.* 2010).

1.5. Plant pathogen interaction

Plants have through evolution developed a multilayer of defences against infectious pathogens. They depend on innate immunity to defend themselves against potentially harmful pathogens, including; viruses, bacteria, and fungi (Nielsen *et al.* 2012). There are two types of defence responses in plants; pre-formed defence and inducible defence. Preformed defence, includes a physical barrier that stops pathogen entry at the site of attack. It constitutes the cell wall and toxic compounds produced at the site of infection. Inducible defence on the other hand becomes effective upon pathogen contact or entry into the plants. This defence is well described by the zigzag model (Jones and Dangl 2006). It has two layers: Pathogen Triggered Immunity (PTI) also included as a basal defence and Effector Triggered Immunity (ETI; Figure. 1).

To trigger an immune response in PTI defence, plant recognition receptors (PRR) recognise Pathogen Associated Molecular Pattern (PAMPs) which are conserved pathogen molecules and trigger an immune response. Pathogens have evolved to produce effector proteins that compromise PTI resulting to Effector triggered susceptibility (ETS). Plants have also developed resistance (R) proteins that recognise the pathogen effectors triggering an immune response known as Effector triggered immunity (ETI). Newly developed effectors by the pathogen can overcome ETI. ETI involves a gene for gene interaction and it is highly specific. It also involves an Hypersensitive Response(HR) which occurs mainly after haustoria formation.(Ellis 2006).

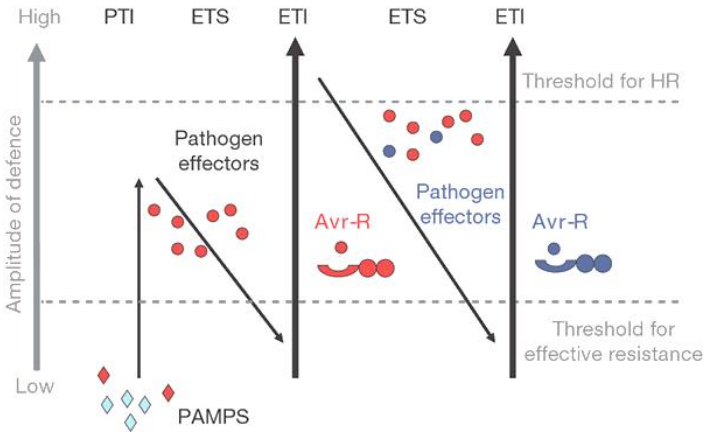


Figure 1: A scheme showing an endless battle in plant pathogen interaction. Defence starts with a pre formed defence, the second phase is PTI which is triggered by pathogen conserved molecules (PAMPs). In the third phase, pathogens have evolved to produce effectors that result in compromised resistance ETS. The plants have also evolved to produce resistance genes that recognise effectors and induce ETI defence. Pathogen can also develop unrecognisable effectors resulting in ETS. Natural selection favours new plant resistance genes that can recognize newly acquired effectors, resulting again in ETI (Jones and Dangl 2006).

1.5.1. Plant interaction with Powdery mildew (*Ascomycota*)

Powdery mildew is an obligate biotrophic fungus that relies on plant host living tissue for survival. The morphological characteristics of powdery mildew asexual stage make the fungus to be considered a member of the genus *Erysiphales* (Whipps *et al.* 1998). *O. neolycopersici* has ellipsoidal-shaped spores of approximately 15 to 30mm in size (Jones *et al.* 2000). The fungi are each specialized to infect a narrow range of plant species (Ellis 2006). Among the various economically important plants affected are tomato, pea cucumber, barley, strawberry, apples, grapes, barley and wheat. The fungi infect the stems, leaves, flowers, and fruits. Infection symptoms are visible to the naked eye in form of white mycelial colonies. Pathosystems involving *Arabidopsis*, tomato and barley have been studied (Hückelhoven 2005), (Li *et al.* 2007), (Bai *et al.* 2005) . Powdery mildew grows well in areas of high humidity and moderate temperatures. They reproduce both sexually and asexually. Genomes of *Blumeria graminis f.sp.hordei, Golovinomyces oronti* and *Erysiphe pisi* that infect barley, *Arabidopsis* and pea have been sequenced. Interestingly, the genomes contain many transposons and genome-size expansion, which could explain the increased genetic variation in powdery mildew (Spanu *et al.* 2010).

1.5.2. Life cycle and infection of powdery mildew

The life cycle of powdery mildew can be sexual (teleomorph) or asexual (anamorph) (Glawe 2008). An infection is initiated when fungal conidiospores land on the leaf surfaces of a susceptible host (Figure 2A). The conidiospore germinates, forming a germ tube that elongates to form a hypha with appressorium, penetration/infection pegs, and haustorium (Glawe 2008). The penetration peg breaches host epidermal cell walls, by physical pressure and enzymatic degradation (Ellis 2006).

Tomato powdery mildew *Oidium neolycopersici*, forms the penetration peg and haustoria directly into the epidermal cell of its host (Figure 2A (a, b, c and d)). In wheat there is a distinctive difference, since the penetration peg develops in between the cells and then goes ahead to establish feeding structures in multiple cells (Figure 2B). These structures enable the fungi to obtain nutrients from the host plant cells. In a resistant plant (Figure 2C), the spore germinates but the development of the penetration peg is stopped by papillae formation. The papillae which is composed of various chemical compounds and toxic products acts as a barrier to further infection by the powdery mildew.

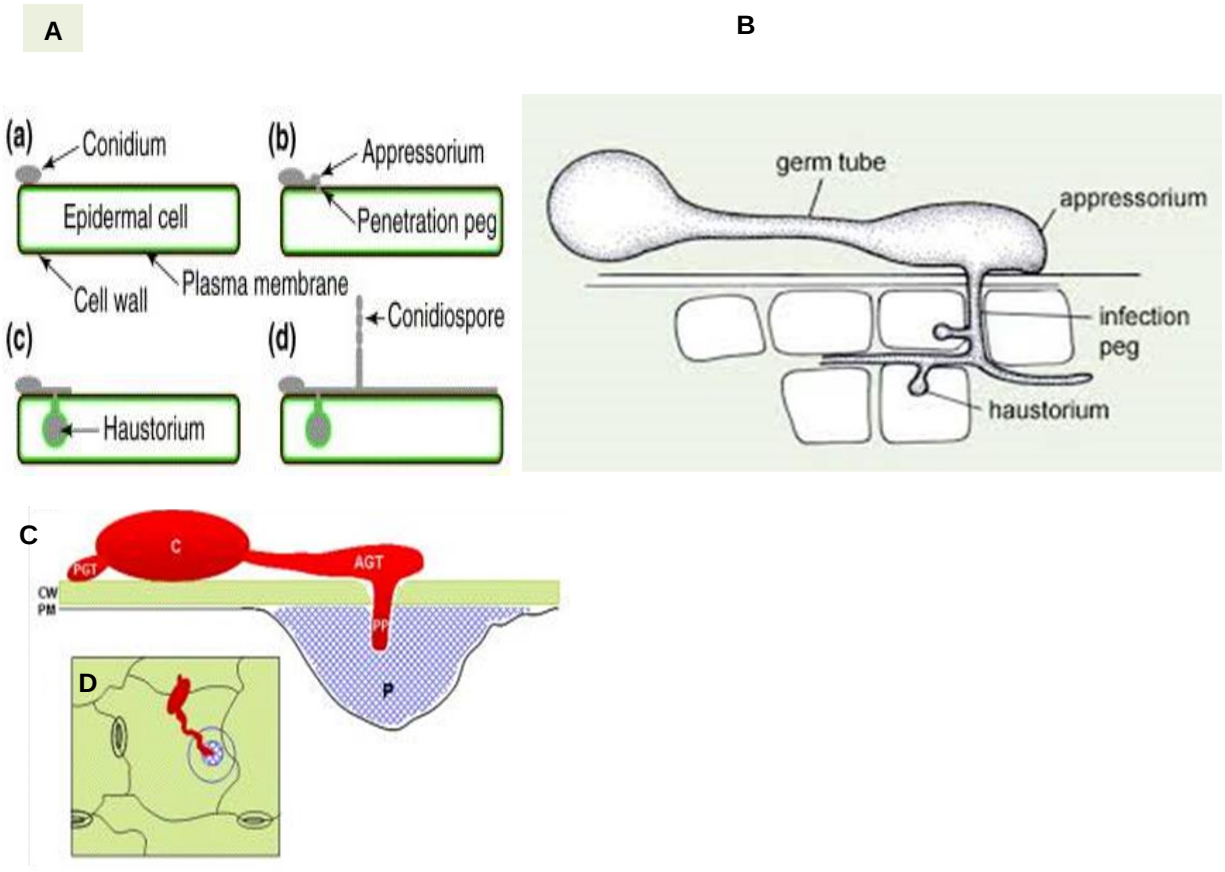


Figure 2: **A**. The structures of a powdery mildew spore after germination and successful entry in a susceptible tomato host. A single feeding structure is established into the host epidermal cell. **B**. The structures of a powdery mildew spore after germination and successful entry in a susceptible wheat host. The germinated spore establishes more than one haustoria in different host cells. **C**. A resistant plant showing unsuccessful entry of the fungi. Papilla (P) prevents further development of the penetration peg (PP) in a resistant host. (CW) Cell wall, (PM) plasma membrane, (C), conidiospore; (PGT), primary germ tube (AGT), appressorium germ tube. **D**. A top-down view of the penetration site as visualized under a light microscope. Image **A**, (Schulze-Lefert and Vogel 2000). Image **B,** courtesy of the American phytopathological society; http://www.apsnet.org/edcenter/illglossary/Article%20Images/Forms/DispForm.aspx?ID=505. Image **C** and **D,** (Underwood 2012)

1.6. Resistance genes in tomato

Powdery mildew is an obligate biotroph whose survival depends on its penetration capabilities. Wild tomato relatives contain resistance genes; *Ol-1*, *ol-2*, *Ol-3*, *Ol-4*, *Ol-5* and *Ol-6*, and three QTLs (Qualitative Trait Loci); Ol-*qtl1*, Ol-*qtl2*, and Ol-*qtl3* (Bai *et al.* 2003). *ol-2* is a recessive gene while the rest of *Ol* genes are dominant (Bai *et al.* 2005). *Ol-1*, *Ol-3*, *Ol-4*, *Ol-5*, and *Ol-6* are organized in three genetic loci on chromosome 6 of the tomato genome, while *ol-2* is found in chromosome 4 (Bai *et al.*

2005). Ol-*qtl1* is located in chromosome 6 while Ol-*qtl2* and Ol-*qtl3* are located in chromosome 12 (Bai *et al.* 2003).

The resistance mechanism of *Ol* genes is specific to certain powdery mildew races and involves mostly HR and H_2O_2 production which is strongly associated with both HR and papilla formation (Li *et al.* 2007). Accumulation of H_2O_2 in host cell occurs at an early stage during pathogen infection. H_2O_2 contributes to induced cell death, cell wall fortification and acts as a diffusible signal for induction of systemic defence response (Lamb and Dixon 1997). QTL mediated resistance is polygenic and additive, including both HR and papillae formation (Bai *et al.* 2003). The *ol-2* resistance mechanism is non-race-specific involving papilla formation (Bai *et al.* 2005; Li *et al.* 2007). Near isogenic lines carrying the *ol-2* genes have no accumulation of H_2O_2 in epidermal cells which would otherwise result to programmed cell death (Seifi *et al.* 2014). Accumulation of H_2O_2 was found only at the site of infection with cell wall apposition in *ol-2* plants. H_2O_2 involved in the cell wall fortification, is both responsible for, cross-linking of the cell wall proteins and also in serving as a substrate in cell wall apposition (Hückelhoven 2007)

Natural mutation of tomato *Mlo* gene gives rise to the *ol-2* gene. The *ol-2* gene has been isolated in a segregating population obtained by crossing *S. lycopersicum var. cerasiforme* × cv. *Super Marmande*. The initial breakthrough in breeding for resistance against powdery mildew was achieved by Ciccarese and his colleagues in 1998. They screened 132 species of *L. esculentum var. cerasiforme* and identified two resistant plants. One of the two plants upon crossing with *Marmande*, followed by selfing to F1 and F2 generations, and backcrossing, produced a resistant homozygous progeny. The F1 were susceptible suggesting that resistance was recessive. The F2 progeny segregated in a 1:3 ratio and backcrosses fitted the hypothesis of a single recessives gene *ol-2*. The backcross of F2 progeny to susceptible lines resulted in all susceptible plants, while F2 backcross to resistant plants resulted to a 1:1 segregation ratio. These proved that *ol-2* is a recessive gene characterised by low disease severity, low sporulation, and restricted mycelial growth (Ciccarese *et al.* 1998).

1.6.1. *Mlo* based resistance

Breeding approaches, which for a long time focused on resistance genes (R-genes), are slowly but progressively turning to identifying susceptibility genes (S-genes), and inactivating them for resistance (Pavan *et al.* 2010). Plant S-genes trigger a susceptible response to the pathogen and have a

negative response to plant defence (Pavan *et al.* 2010). Mutation resulting to impairment of the S-genes results in recessive resistance.

Mildew resistance locus O (*Mlo*) is a good example of an S-gene whose recessive mutation results to broad spectrum resistance in Barley. It is a member of a large gene family that encodes a class of proteins anchored on the plasma membrane by seven transmembrane domains (Figure 3) (Büschges *et al.* 1997). These polytopic proteins have no known biochemical activity (Reinstädler *et al.* 2010). Mlo protein is a negative regulator of papilla formation (Büschges *et al.* 1997). It is postulated that pathogens exploit the *Mlo* coded proteins for entry into the host (Panstruga 2005). The fungus (Figure 4) uses wild-type *Mlo* for suppression of a SNARE protein-dependent and possibly vesicle-associated defence mechanism at the cell periphery (Collins *et al.* 2003).

Mutation of *Mlo* orthologs resulting in broad spectrum resistance against powdery mildew have been discovered in Barley, *Arabidopsis*, tomato and pea (Humphry *et al.* 2006), (Bai *et al.* 2008), (Consonni *et al.* 2006), (Pavan *et al.* 2011). Development of the powdery mildew on *mlo*-resistant plants is inhibited at the prehaustorial stage through a restricted cell wall apposition (papilla formation) directly beneath the fungal appressorium. Wild-type *Mlo* gene in barley confers susceptibility to *Blumeria graminis* f.sp. *hordei.* (*Bgh*). Mutation resulting in loss of function of a protein confers broad spectrum resistance against the fungi (Humphry *et al.* 2006). This resistance constitutes papillae formation inhibiting fungal entry into the plant cell (Pavan *et al.* 2008).

Arabidopsis thaliana is used as a model plant to study the interactions between the plant and the powdery mildew. Experiments involving induced mutation and mutant screening for resistance against powdery mildew in *Arabidopsis* resulted in identification of three *mlo* resistance genes (*Atmlo2, Atmlo6,* and, *Atmlo12*). These genes confer a broad spectrum resistance with papillae formation. All the three *Mlo* genes must lose their function to confer full resistance (Consonni *et al.* 2006).

In tomato, *ol-2* gene arises from a natural mutation of tomato *Mlo* ortholog *SlMlo1* resulting in a non-functional protein (Bai *et al.* 2008). Loss of function of the gene is a result of a 19 base pair deletion in the coding region of *SlMLO1* gene, conferring full powdery mildew resistance (Bai *et al.* 2008; Bai *et al.* 2005). Previous studies have shown that *ol-2* has a broad non-host resistance which is speculated to be durable, therefore, a potential trait to control adapted pathogens (Lindhout 2002). The *mlo* resistance is independent of signal transduction pathways which activate defence mechanism, that is, salicylic acid pathway (SA), jasmonic acid pathway (JA), and ethylene pathway (ET) (Consonni *et al.*

2006). *Mlo* mediated resistance in *Arabidopsis* against *Golovinomyces* spp. is largely independent from SA, JA and ET pathways. However, in *ol-2* near isogenic lines, induced SA pathway negatively regulates ABA and JA pathways compromising *ol-2* mediated resistance (Seifi *et al.* 2014). It is assumed that induction of the ABA pathway is required for callose deposition that contributes to *ol-2* mediated resistance. JA pathway is also necessary. This is a clear indication that molecular mechanisms underlying the *mlo* mediated resistance in tomato and *Arabidopsis* are not completely the same (Seifi *et al.* 2014). It is interesting how SA, JA and ABA signalling pathways are coordinated in *ol-2*-mediated resistance associated with cell wall apposition but not with programmed cell death (Seifi *et al.* 2014).

The *mlo* mediated resistance requires proper functioning of other genes; *Ror1* and *Ror2* in barley (Hückelhoven *et al.* 2000), and three *PEN* (PENETRATION) genes, *PEN1, PEN2* and *PEN3* in *Arabidopsis* (Collins *et al.* 2003). *PEN1*, *PEN2,* and *PEN3,* inhibit and limit invasion by the powdery mildew through non host resistance in *Arabidopsis* (Lipka *et al.* 2005). These genes encode syntaxin, a glycosyl hydrolase and an ABC transporter respectively. The *PEN* genes are negatively inhibited by *Mlo* (Figure 4) (Underwood and Somerville 2008). Failure of the fungi to penetrate the cell wall is a major component of immunity of non-host plant species. It also accounts for a proportion of aborted infection attempts in susceptible plants (basal resistance). No known natural powdery mildew isolate has been shown to break *mlo*-based resistance, indicating a success in the use of S-genes in crop protection to confer durable resistance (Zheng *et al.* 2013).

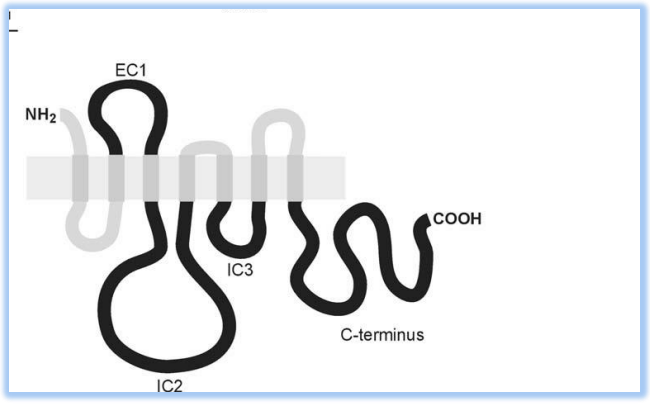


Figure 3: Diagrammatic representation of MLO proteins heptahelical topology. NH2 and COOH represent the amino and carboxyl terminal respectively. The structure depicts the loop domains and the seven transmembrane helices (longitudinal small dark grey boxes). EC1 (extracellular loop 1), IC2 and IC3; (intracellular loops 2 and 3) The large horizontal light grey box represents the plasma membrane lipid bilayer (Panstruga 2005) .

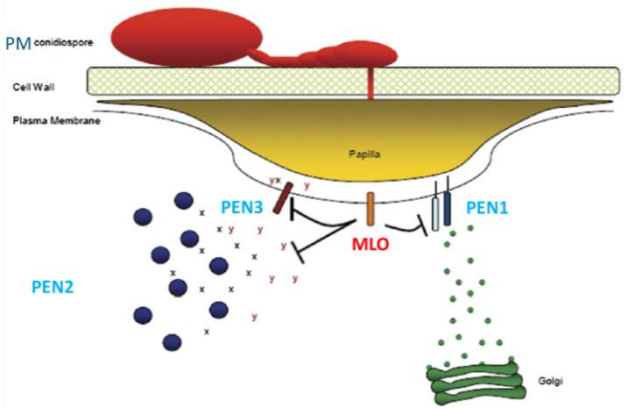


Figure 4: A germinating conidiospore trying to penetrate a resistant host. The *Mlo* gene is not active. It therefore does not act negatively on *PEN1*, *PEN2* and *PEN3*. The three genes are resistance genes that result to papilla formation preventing powdery mildew entry (Underwood and Somerville 2008).

1.6.2. Role of *PEN* genes in *mlo* based resistance

Genetic screens for chemically induced *Arabidopsis* mutants with altered non-host interactions upon *Bgh* inoculation was carried out. Results showed increased barley mildew penetration and identified *PEN1, PEN2* and *PEN3* (penetration) genes (Collins *et al.* 2003) (Assaad *et al.* 2004). Single mutants of these three genes have increased frequency of haustoria formation (Ellis 2006).

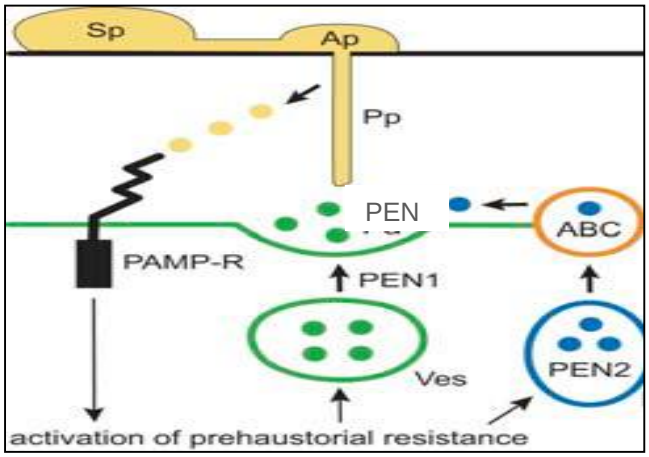


Figure 5: *PEN1*, *PEN2* and *PEN3* in prehaustorial resistance preventing further development of the penetration peg (Pp). Broad host resistance is dependent on molecules (green circles) delivered by a *PEN1*-mediated vesicle (Ves) based secretion system. It also depends on postulated toxin(s) (dark blue circles) synthesized in a *PEN2*-mediated pathway and delivered by a *PEN3*-encoded ABC transporter in the plasma membrane (PM). This results in prehaustorial resistance (Ellis 2006).

PEN1 which encodes a syntaxin containing a SNARE (for soluble N-ethylmaleimide-sensitive factor attachment protein receptor) domain is localised on the plasma membrane (Ellis 2006). This gene is a member of a large family of proteins involved in membrane fusion and secretion events (Figure 5)(Ellis

2006). Upon mutation of *PEN1*, there is evidence of cell wall penetration by powdery mildew, followed by increased hypha development, which result in a sevenfold increase in haustoria initiation (Ellis 2006). Additionally, mutants show delayed papilla formation (Figure 6)(Assaad *et al.* 2004).These observations suggest that the powdery mildew encounters an effective barriers to penetration that are defective in *pen1* mutant (Assaad *et al.* 2004). *PEN1* therefore has a highly specific role in penetration resistance.

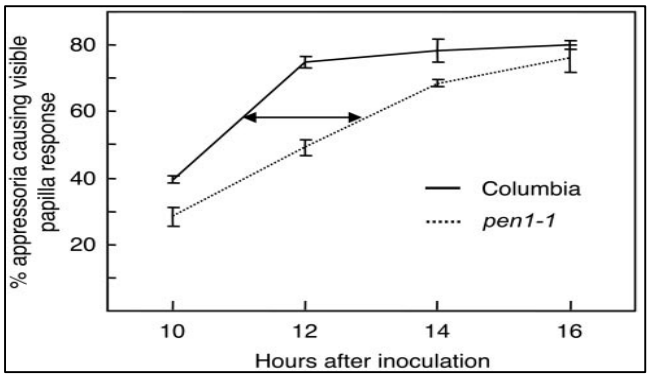


Figure 6: Papillae formation compared between *pen1* mutant and wild-type in *Arabidopsis* inoculated with *Bgh*. Straight line: wild-type; Dotted line: *pen1* mutant. Arrow highlights the delay in papilla formation (Assaad *et al.* 2004)

PEN2 and *PEN3* (Figure 5) seem to act together in a pathway distinct from *PEN1*. *PEN2*, a glycosyl hydrolase, produces glucosinolate metabolites, and *PEN3,* an ATP dependant ABC transporter, is involved in secretion of these glucosinolate metabolites to the site of fungal infection (Clay *et al.* 2009) (Bednarek *et al.* 2009). Activity of these genes drives enzymatic release and energy-dependent apoplastic secretion of toxic compounds (Lipka *et al.* 2008) The two genes may have a direct antimicrobial role. They may also be required for the deposition of callose and the encasement of powdery mildew haustoria at the site of infection, suggesting a subtle regulatory role of *PEN2* and *PEN3* in blocking infection (Dodds and Rathjen 2010).

1.7. Functional characterization of *PEN1* like genes using RNAi

Of the three *PEN* genes in mutant *Arabidopsis, PEN1* and *PEN2* have different entry control mechanisms while *PEN2* and *PEN3* work together (Lipka *et al.* 2005). The *PEN* genes defence response suffices to completely prevent non-adapted fungal entry. Gene expression studies carried out in tomato identified several genes that were upregulated in resistant plants against powdery mildew using cDNA-AFLP (Amplified Fragment Length Polymorphism) (Li *et al.* 2006). Functional

information on some of the Transcript Derived Fragments (TDFs) obtained upon blasting their sequence against the NCBI non redundant database, showed that these TDFs were derived from genes involved in direct defence responses, photosynthesis, or signal transduction (Li *et al.* 2006).

Identifying the function of specific candidate genes involved in *mlo* based non-host defence response could be key to understand this durable resistance mechanism. *PEN1*, is an example of a candidate gene whose silencing in *ol-2* background show increased susceptibility to powdery mildew with 80% colony in tomato (Carpentier 2009).

Various tools including VIGS (Virus Induced Gene Silencing) and RNAi could be used to study the functionality of candidate genes. VIGS is a transient transformation assay that exploits a homology based defence mechanism triggered by an infecting virus. The aim of the virus at this point is to take over the host machinery by targeting host endogenous genes for silencing. Transcript of the gene to be silenced is cloned in TRV-RNA 2 (Tobacco Rattle Virus)-RNA 2.

RNAi (RNA interference) is described as PTGS (Post transcriptional gene silencing) which results from the degradation of mRNAs (Mourrain *et al.* 2000). RNAi involves the formation of double stranded RNA which initiates silencing of the corresponding endogenous gene. Transformations with double stranded RNA corresponding to a certain target endogenous gene enables the silencing of the gene for functional characterization.

1.8. Problem statement

Powdery mildew causes huge losses in both the green house and field grown tomatoes. Various strategies have been used to try and curb the menace of these ferocious fungi. The most common approach is the use of fungicides, which among other disadvantages cause serious environmental pollution. Dominant resistance genes have also been used in breeding strategies with remarkable resistance. These approaches, however, have several disadvantages, including lack of durability, the resistance can be overcome by pathogen evolution of unrecognised effector (Jones and Dangl 2006) . Moreover, resistance genes confer a race specific resistance, in that, they only recognise specific avirulence genes. Plant breeders are focusing on the use of broad spectrum resistance that is less specific, not easily broken, and appears to be more durable as compared with race specific resistance. Broad non-host resistance involves the arrested entry of fungi through cell wall apposition at the entry site. The resistance is as a result of silencing the genes utilised by pathogens to establish pathogenesis. The genes are known as susceptibility (S) genes an example being *Mlo*. Mutation of

Mlo orthologs resulting in broad spectrum resistance against powdery mildew have been discovered in Barley, *Arabidopsis*, tomato and pea (Bai *et al.* 2008; Consonni *et al.* 2006; Humphry *et al.* 2006; Pavan *et al.* 2011). *SlMlo1* is a susceptibility gene in tomato used by the fungi to increase its entry into the host (Panstruga 2005) (Bai *et al.* 2005) (Bai *et al.* 2008). Loss of *SlMlo1* gene function resulted in an *ol-2* recessive gene conferring broad spectrum resistance.

Initial evidence for the existence of a plant-controlled process terminating fungal entry at the cell wall level was observed in non-host interactions between *Arabidopsis* and the barley powdery mildew, (Bgh) (Consonni *et al.* 2006). Three *Arabidopsis pen* genes mutant loci that permit, at high frequency, the entry of non-adapted fungi were identified (Collins *et al.* 2003).

Oidium neolycopersici can grow on other species apart from tomato including *Arabidopsis (Xiao et al. 2001).* An experiment was carried out by (Zheng 2012) to identify the defence pathway for *mlo* based resistance to *Oidium neolycopersici*. In his results, he demonstrated the importance of *PEN*-dependant and *PEN*–independent pre-penetrative defences in *mlo* based resistance. The silencing of *PEN1, PEN2 and PEN3* in *Atmlo2* background showed a dramatic increased penetration of *Oidium neolycopersici* in the double mutants *Atmlo2/pen1, Atmlo2/pen1* and *Atmlo2/pen1*. From these results, we can speculate the active pre-penetrative role of *PEN* genes

A crucial step is to therefore identify the underlying mechanism in *mlo* based resistance in tomato against *Oidium neolycopersici*. Understanding the role of *PEN* genes in conferring broad spectrum resistance in *Arabidopsis* against *Oidium neolycopersici* by (Zheng 2012) acts as a lee way to further investigate the crucial role of these genes in broad spectrum resistance in tomato. This is part of a new breeding strategy using susceptibility genes, that could be an effective way to ensure the tomato plants have durable broad spectrum powdery mildew resistance (Pavan *et al.* 2010). Once the pre-penetrative defence mechanism is well understood, breeding for broad spectrum resistance will be an effective approach to curbing the losses caused by powdery mildew with effective and durable resistance.

1.9. Aim

With the hypothesis that using RNAi to silence *PEN1*; a gene involved in the resistance against powdery mildew in *ol-2* tomato line, increases susceptibility of resistant tomato plants, we sought to investigate the involvement of *PEN1* like genes in the defence mechanism against powdery mildew. We focused on: (1) Investigating the effectiveness of RNAi of *PEN1* and *PEN1* homolog. (2) Finding

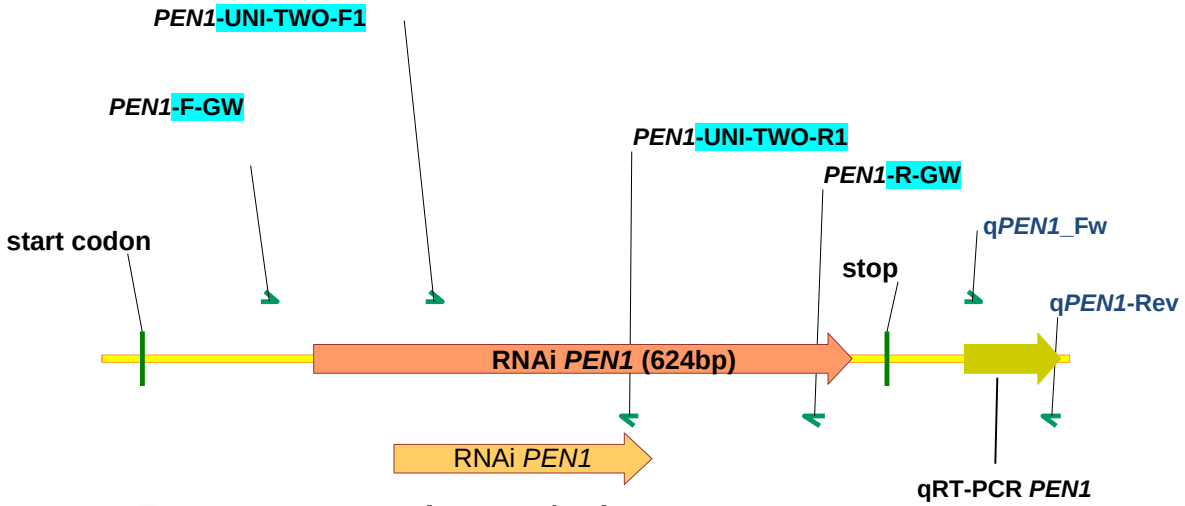
out if there is increased susceptibility from the silencing with increased fungal growth. (3) Identifying the fungal structures formed in transgenic plants with compromised immunity.

2| Materials and methods

Previous studies to determine the role of penetration genes in conferring full penetration resistance against *Oidium neolycopersici* in *Arabidopsis* were carried out by (Zheng 2012). In his results, mutants of *PEN1* gene had increased penetration of *Oidium neolycopersici* in *Arabidopsis.* We therefore wanted to investigate the role of the *AtPEN1* homologs in tomato against *Oidium neolycopersici*. AtPEN1 protein sequence was blasted in the Sol Genomic Network (SGN) database to identify how many homologs were present in tomato. The homologs were then used to build a phylogenetic tree to identify the true tomato homologs.

2.1. Constructs

The constructs used in these study, were initially made by Yan Zhe. The gene regions amplified are shown in figure 7. For *PEN1,* two primer pairs were designed: the first pair primer (*PEN1*-UNI-TWO-F1 and *PEN1*-UNI-TWO-R1) amplified a region of 259 base pairs named *PEN1*_short. The second pair (primer *PEN1*-F-GW and primer *PEN1*-R-GW) amplified a region of 624 base pairs termed as *PEN1*_long. For *PEN1* homolog, primers named SGN-U584182-F2 and SGN-U584182-R2 amplified a region of 250 base pairs. The primers *qPEN1-Fw* and *qPEN1-Rev* were used in real time PCR for expression analysis of *PEN1.* There were two pairs of primers used for expression analysis of *PEN1* homolog (q*PEN1*-hom Fw1 and q*PEN1*-hom Rev1: q*PEN1*-hom Fw2 and q*PEN1*-hom Rev2) (Figure 7). The PCR products of the genes of interest were cloned into the silencing vector pHellsgate 8 (Figure 8) using the gateway recombination technology.



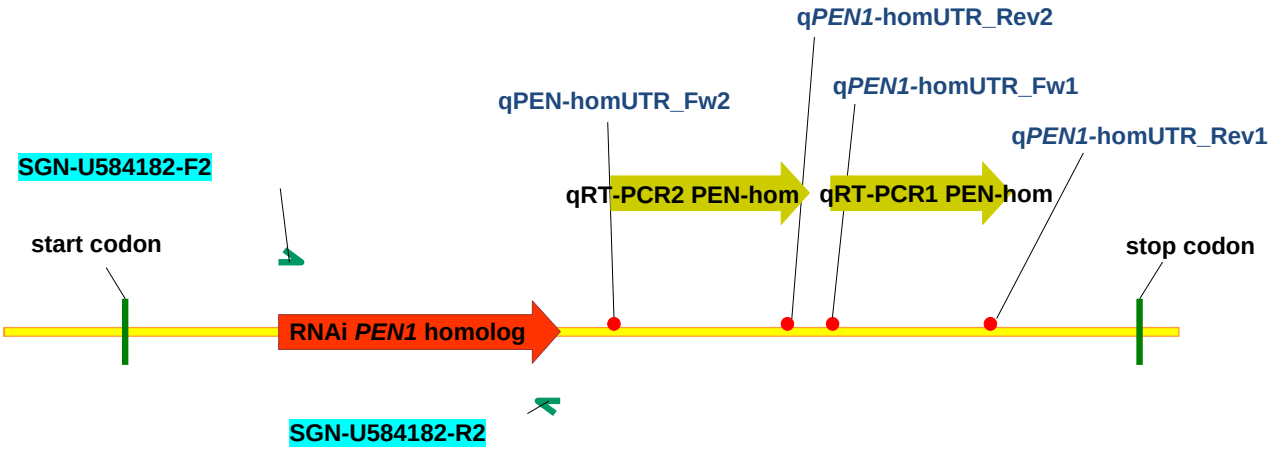


Figure 7: Insertions used for RNAi and the primer pairs used to amplify them. For *PEN1* gene two constructs were made, named short and long, of 259bp and 624bp respectively. For *PEN1* homolog one construct was made with an insertion of 250bp. Primer pairs highlighted in blue were used for amplification of the genes. Primer pair in blue writing were used in real time PCR for expression analysis.

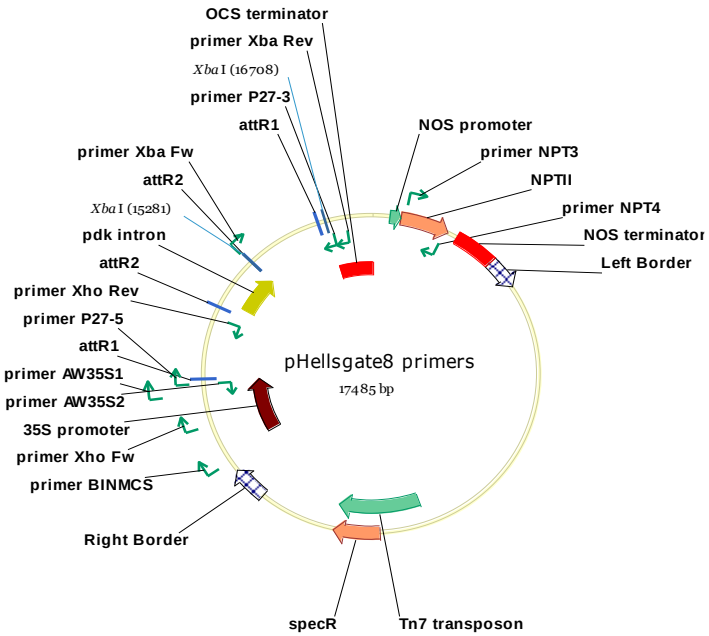


Figure 8: The pHellsgate vector scheme showing different primers used to amplify specific regions. The part between the right and left border is integrated into the plant genome.

2.2. Plant material and fungal material

Initially, in the first experiment, we used 15 transgenic T2 families in this study. The families included 4 families containing *PEN1* homolog construct, 9 families containing *PEN1*_short and 2 families with *PEN1*_long (Table 2). The background genotype of the transgenic plants analysed, was from the resistant tomato line *ol-2* which carries a 19 base pair deletion in the *SlMlo* gene. The genotypes used as controls were, *S .lycopersicum* cv Money maker (MM) and the *ol-2* line (PV103110), developed from a cross between *S. lycopersicum* cv Super Marmande, and *S. lycopersicum* var cerasiforme. Plants were grown at 20±2 ºC, 16h daytime, and 70±5% relative humidity.

A second experiment was carried out but the plants were not used since they suffered from an unknown stress hindering visual scoring and leaf material collection for molecular and histological analysis. A third and last experiment was carried out and the plants were visually scored and leaf material collected for molecular and histological analysis.

O. neolycopersici was used to study the disease incidence. The powdery mildew was maintained on susceptible Money maker (MM) tomato plants in a greenhouse compartment at 21 ºC with 75% ±2 relative humidity.

Table 2: List of the 15 transgenic T2 families containing different constructs that were used in the analysis in experiment 1.

no. families	TV number	Gene	no. of plants
1	TV123342	homolog *PEN1*	20
2	TV123343	homolog *PEN1*	20
3	TV123356	homolog *PEN1*	20
4	TV123357	homolog *PEN1*	20
1	TV123344	*PEN1* (short)	20
2	TV123345	*PEN1* (short)	20
3	TV123346	*PEN1* (short)	20
4	TV123347	*PEN1* (short)	20
5	TV123348	*PEN1* (short)	20
6	TV123349	*PEN1* (short)	20
7	TV123350	*PEN1* (short)	20
8	TV123351	*PEN1* (short)	18
9	TV123352	*PEN1* (short)	20
1	TV123353	*PEN1*(long)	20
2	TV123354	*PEN1*(long)	20

2.3. Disease assay

Leaves of highly infected MM were collected and gently washed in water to release the spores. The suspension was sprayed on one month old (*ol-2)* transgenic tomato plants, maintaining the inoculum concentration at approximately 2.5×10^4 spores per millilitre for normal disease assay. Plants to be used for histological analysis were sprayed with a higher dosage of 2.5×10^5 per millilitre. Plants were visually scored at a regular interval using a scale of 0-3. Scoring was done on leaf number three and four of each tomato plant.

We used a scale of 0-3 where 0 was scored for resistant plants with hardly any formation of mycelial colonies and 3 for susceptible plants with leaves infested with >30% leaf area covered with mycelial colonies.

2.4. DNA, RNA isolation and RT-PCR

Leaf material was harvested in liquid nitrogen and grinded to a fine powder. DNA isolation was done using the DNeasy kit including the RNAse treatment (Qiagen, Germany). The DNA quality was checked using NanoDrop ND-1000, UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA).

The DNA was then used in normal PCR for *NPTII* scoring and for confirmation of the inserted fragment, using primer pairs listed in table 3. *NPTII* (neomycin phosphotransferase II) gene is used for plant selection in *Agrobacterium tumefaciens* mediated transformation. The pHellsgate vector contains the *NPTII* gene that makes transgenic plants resistant to the antibiotic kanamycin. Primer pair designed to amplify the *NPTII* gene were therefore used IN PCR to aid in the selection of transgenic plants from the non-transgenic ones in T2 families segregating for the transgene inserted. To further confirm the presence of the transgene, obtained using the *NPTII* marker, a PCR using primer pair targeting the 35S promoter was also performed. To confirm insertion of the target gene, different primers pairs were used; Xba-Fw and P27-3-Rev, Xho-Fw and 35S2-Rev, 35S1-Fw and PDK-Flip-Rev, Xho-Fw and PDK-Rev, and, Xho-Fw and PDK-flip-Rev. (Table 3).

The amplification program was typically made of three steps: 94ºC (5 minutes) denaturation; then the cycle repeated 35-40 times made of three parts: denaturation at 94 ºC (30 seconds), annealing at 55 ºC (30 seconds) and extension at 72ºC for typically (1 minute per Kb), and final extension at 72 º C for 5 minutes) . The PCR product was then run in agarose gel electrophoresis using TBE buffer at

110Voltage for 30-40 minutes. The loading dye Ds-red was used to enable visualization of the bands under UV light. Some of the PCR products were purified and sent for sequencing to confirm the presence of the insert in transgenic plants. The PCR product was selected from DNA that showed a nice single clear band observed from the gel electrophoresis and purification was done using a PCR purification kit. A sequencing tube was prepared according to the indications of GATC sequencing service.

Table3: Primers used in PCR to amplify different gene regions.

PCR primers		**Purpose of using the primers to amplify certain gene regions**
35S-1	GCTCCTACAAATGCCATCA	To identify transgenic plants
35S-2	GATAGTGGGATTGTGCGTCA	
NPTII_Fw	TCGGCTATGACTGGGCACAAC	
NPTII_Rev	AAGAAGGCGATAGAAGGCGA	
Xho_Fw	TGCTGACCCACAGATGGTTA	To confirm the insertion of the gene of interest
Xho_Rev	CGGCACTACCCGAAGTATGT	
Xba_Fw	TGGGTTCGAAATCGATAAGC	
Xba_Rev	TTAGGTTTGACCGGTTCTGC	
P27-3	GAGCTACACATGCTCAGG	
P27-5	GGGATGACGCACAATCC	
PDK_flip_rev	ACAGTTGGGAAATTGGGTTCGA	
PDK_rev	5'-ATTTCCTTACCAAGCTGGGGT-3'	

Some of the DNA was used to carry out real time PCR to quantify the fungal biomass. This was done using SYBR green dye on Bio-Rad iCycleriQ machine. The PCR reaction mixture was prepared by mixing 5µl SYBR green dye, 3.4µl water, 0.3 of each primer (forward and reverse) and 1µl of DNA to make at total volume of 10µl. The transcript level was then calculated with reference to tomato housekeeping genes Ef 1α. The primers used for *Oidium* quantification include On_Fw (cgccaaagacctaaccaaaa) and On_Rev (agccaagagatccgttgttg) designed based on the internal transcribed spacer (ITS). The primer pair Ef1α_Fw (GGAACTTGAGAAGGAGCCTAAG) and Ef1α_Rev (CAACACCAACAGCAACAGTCT) were used to detect and quantify tomato DNA (Table 4). RNA was extracted using kit Mag Max 96 total RNA isolation kit (Ambion). The RNA was used for cDNA synthesis using superscript reverse transcriptase kit iScript (Biorad). The cDNA obtained was then used in real time PCR to quantify the expression levels of the target genes in comparison to the

reference gene Ef 1α. The primers used for expression analysis include q*PEN1*_Fw and q*PEN1*_Rev *PEN1*, and, q*PEN1*_homUTR_Fw and q*PEN1*_homUTR_Rev (Figure 7, Table 4).

Elongation factors were used as the reference to normalize the DNA proportion of plants using the 2-.ΔΔCt method (Livak and Schmittgen 2001). This method is used to calculate relative changes in gene expression, for example, lowered gene expression resulting from silencing.

To calculate the relative expression,

$$\Delta\Delta Ct = \big(Ct(gene\ of\ interest) - Ct(reference)\big)_{time\ x} - \big(Ct(gene\ of\ interest) - Ct(reference)\big)_{time\ 0}$$

Amount of the target = 2-.ΔΔCt

Table 4: Primers used in real time PCR to quantify the amount of *Oidium*.

qpcr primers		**Purpose of primer**
q*PEN1*_Fw	CGAGATGCTTTGTGCATCAG	For expression analysis of *PEN1.*
q*PEN1*_Rev	CAGTCTCCTTCAGCTCCATTTC	
q*PEN1*_homUTR_Fw	tggtttagttgttgatggacctc	For expression analysis of *PEN1* homolog.
q*PEN1*_homUTR_Rev	acccccatccaacttacttctc	
On_Fw	cgccaaagacctaaccaaaa	For expression analysis of *Oidium* biomass.
On_Rev	agccaagagatccgttgttg	
Ef1α_Fw	GGAACTTGAGAAGGAGCCTAAG	Primers of housekeeping genes used to normalise the possible variation in the experiment.
Ef1α_Rev	CAACACCAACAGCAACAGTCT	

2.5. Histological analysis

To study the fungal structures formed by powdery mildew germinating spores, one month old transgenic tomato plants, belonging to three T2 families (TV123349, TV123354 andTV123343) (Table 5) from the third experiments were inoculated with a high dosage of *Oidium* 2.5×10^5 per millilitre. Plants from the first experiment could not be used in histological analysis since we had inoculated a low *Oidium* dosage and could hardly see anything under the microscope.

Plants used for histological analysis were prior to inoculation scored for the presence/absence of the *NPTII* to identify transgenic plants to be used for histological analysis. This was done by picking a very small leaf part of approximately 1cm and isolating DNA. Two methods, Tris method and CTAB were separately used to compare DNA quality. Using *NPTII* primers for PCR, transgenic plants were

identified. To confirm our results, primers of the 35s promoter were also used to run a second PCR. The results obtained, enabled the selection of two plants (indicated in red arrow) in table 5 per genotype to be used in the analysis. The selected transgenic plants were positive for NPTII and 35S in all the PCRs (Table 5).

Table 5: The families used for histological analysis in experiment three. Two DNA isolation methods were used (Tris and CTAB). PCR with primers of NPTII and 35S was carried out to identify transgenic plants. The visual scoring was done for the rest of the plants. (√) presence of the gene, (-) absence of the gene, (←) plants selected for histological analysis at 48hpi.

Genotypes	Gene inserted	*Tris*		*CTAB*			
		NPTII	**35S**	**NPTII**	**35S**	**visual scoring 12 days after inoculation**	**Selected plants**
349-1	*PEN1* _short	√	-	√	-	0.5	
349-2		√	√	√	-	1	
349-3		√	√	√	-	2	
349-4		√	-	√	√	0.75	
349-5		√	-	√	-	1	
349-6		√	-	√	-	0.5	
349-7		√	√	√	√	1.5	←
349-8		√	√	√	√	2.5	←
349-9		-	-	√	√	2	
349-10		-	-	-	√	2	
343-1	*PEN1* homolog	√	√	√	√	0.5	
343-2		√	√	√	√	0.75	
343-3		-	-	√	-	0.5	
343-4		√	√	√	√	0.5	
343-5		√	√	√	-	0.5	
343-6		√		√	-	0.5	
343-7		√	√	√	√	0.5	←
343-8		√	√	√	√	0.75	←
343-9		√		√	-	0.5	
343-10		√	√	√	-	0.5	
354-1	*PEN1*_long	√	√	√	√	0.5-0.75	
354-2		√	√	√	-	1.5	
354-3		√	√	√	-	1.5	
354-4		√	√	√	√	2	←
354-5		√		√	-	0.5	
354-6		√	√	√	√	1.5	
354-7		√	√	√	√	1.5	←
354-8		√	√	√	-	0.5	
354-9		-	-	√	√	1	
354-10		√	√	√	√	0.75	←

There was a variation in the transgenic plants obtained from the use of *NPTII* and 35S primers. We can conclude that the use of 35S primers is more reliable unlike the *NPTII* primers since there is a clear distinction between transgenic and non-transgenic plants while *NPTII* shows almost all the plants are transgenic leading to the possibility of false positives. The DNA isolated by two different methods (Tris and CTAB) have different PCR results. We however cannot conclude if the different isolation methods have an effect on quality of the DNA resulting to different PCR results when using different primers. Leaf samples of 1×3 cm in size from the third and fourth leaf of the selected plants were taken at two time points (24hpi and 48hpi). The leaf fragments were fixed in acetic acid and ethanol in a ratio of 1:3 for bleaching, followed by stained with 0.03% Trypan blue dye in lactophenol and ethanol in a ratio of 1:3. During the staining, the leaves were incubated in the Trypan blue solution for 4-5 minutes at 90ºC. The stained leaves were then immersed in chrolohydrate for 24 hours for decolourization.

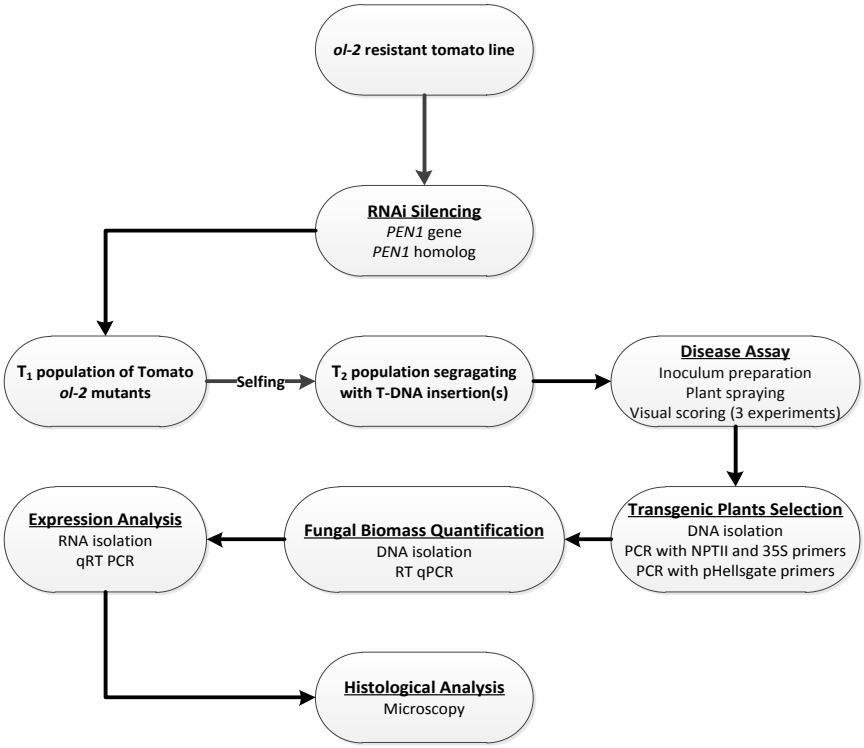


Figure 9: A schematic overview of the experimental approach used in studying candidate genes in *ol-2* resistant tomato. RNAi is used to silence the gene of interest for functional analysis. A T1 population is established and selfed to obtain a T2 population. Inoculum is prepared and spared on transgenic plants and visual scoring is done. Leaf material is then harvested for DNA and RNA isolation. The DNA is used to run PCRs for identification of transgenic plants as well as real time PCR for biomass quantification. The RNA was used for real time PCR to quantify the expression levels of the target genes. Histological analysis and microscopy are done to identify the fungal structures established by the germinating conidiospores as well as the identification of papilla formation by the plant

The leaf samples were then used to make slides. Two slides per plant were made by fixing the stained leaf in glycerol for only 48hpi. The slides were then observed under a conventional phase contrast microscope. The magnification of ×10 and ×40 was used. In total, we observed four slides per family. The scores made were on infection units (IU), hyphae number, and formation of primary appressorium, papilla and haustorium, formation of secondary appressorium, papilla and haustorium as well as occurrence of HR. The rest of the transgenic plants from which the transgenic leaf material for histological analysis were obtained were scored for disease for a period of three weeks before they were disposed. This was to help compare disease index to structures of germinated spores observed under the microscope. The overall work plan of this study is summarized in Figure 9.

3| Results

3.1. Identification of *AtPEN1* homologs in tomato

To identify how many homologs of *PEN1* were present in tomato, *Arabidopsis* At*PEN1* protein sequence At3g11820 was obtained from The *Arabidopsis* Information Resource (TAIR). Using this protein to blast query the Sol Genomic Network (SGN) database, with $10e^{-16}$ e-value threshold yielding ten hits. However, a phylogenetic analysis revealed only two of the ten tomato proteins clustered on the same node as *AtPEN1*, thus true homologs termed as, *PEN1* (Solyc10g081850*)* and *PEN1* homolog (Solycg006950) (Figure 10). The homology of the two homologs was 87.3%. The similarity of *PEN1* and *PEN1* homolog to At*PEN1* was 74.3%.and 74.0 respectively (Figure 11)

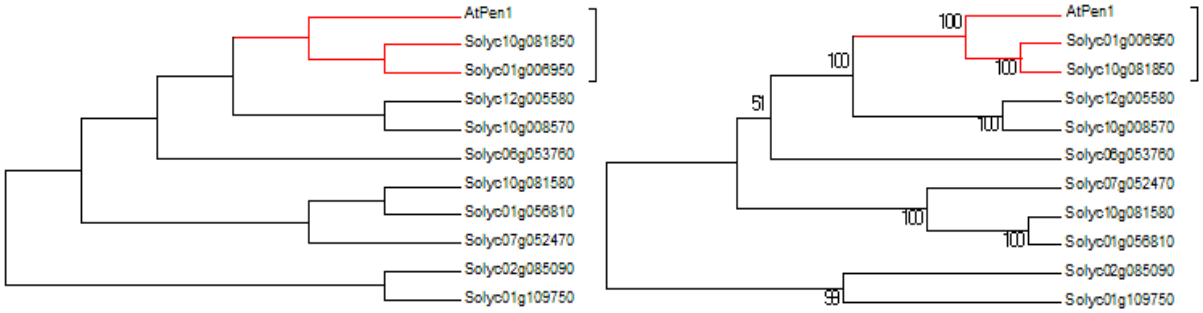


Figure 10: **A.** A thousand-fold bootstrapped consensus neighbour joining phylogeny of *PEN1* homologs from *Arabidopsis* (At) and tomato (Solyc). **B.** Bootstrap confidence values are shown on the branches. The clade representing *AtPEN1* and its putative tomato homologs, Solyc10g081850 and Solyc01g006950, is highlighted in red.

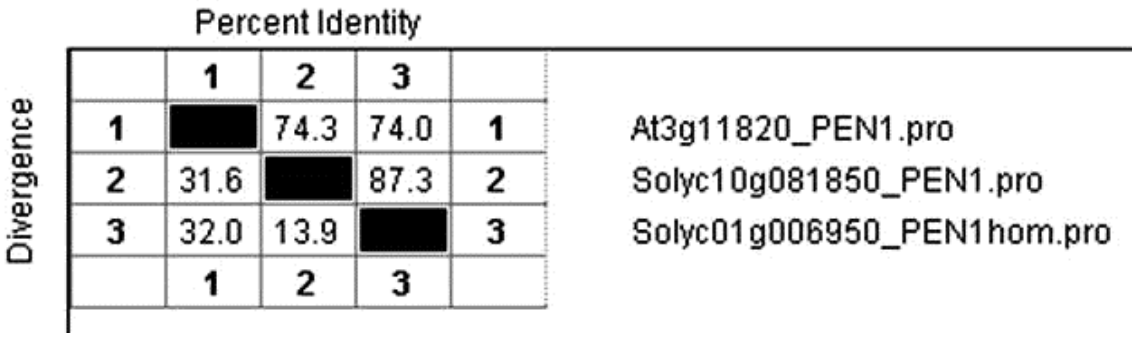


Figure 11: Alignment analysis showing the percentage identity of *Arabidopsis* PEN1 protein and tomato *PEN1* and *PEN1* homolog

3.2. Silencing *PEN1* and *PEN1* homolog confers susceptibility to resistant *ol-2*

3.2.1. Disease assay

Visual scoring was done for three experiments. During the first experiment, we observed a restoration of susceptibility in the 15 transgenic T2 families from visual scoring (Table 6). The individual plant scoring for disease was done at 9 and 14 days after inoculation and the average visual scoring was calculated as well as a T-test for disease significance in transgenic plants (appendix i). The varying disease incidence in transgenic plants compared to controls was an indication of a segregating T2 population. To further correlate the observed phenotype with the indication of presence/absence of the transgene (which is segregating in T2 families), we decided to focus on four families with a significantly high average scoring for disease (Table 7).

Table 6: Average visual scoring for disease in 15 transgenic families with different constructs in experiment 1.

no. families	TV number	Gene	no. of plants	average visual scoring	t test
1	TV123342	homolog *PEN1*	20	0.83	0.04
2	TV123343	homolog *PEN1*	20	**0.95**	**0.02**
3	TV123356	homolog *PEN1*	20	0.80	0.20
4	TV123357	homolog *PEN1*	20	0.83	0.15
1	TV123344	*PEN1*_short	20	0.84	0.14
2	TV123345	*PEN1* _short	20	0.48	0.09
3	TV123346	*PEN1* _short	20	0.53	0.31
4	TV123347	*PEN1* _short	20	**1.11**	**0.01**
5	TV123348	*PEN1* _short	20	0.89	0.04
6	TV123349	*PEN1* _short	20	**1.49**	**0.00**
7	TV123350	*PEN1* _short	20	0.69	0.52
8	TV123351	*PEN1* _short	18	0.76	0.28
9	TV123352	*PEN1* _short	20	0.59	0.67
1	TV123353	*PEN1*_long	20	0.54	0.30
2	TV123354	*PEN1*_long	20	**0.91**	**0.04**

We also took some photos (Figure 12). The pictures show susceptible transgenic plants with visible white mycelial growth on leaf surface. There is a difference in mycelial growth on leaves of transgenic plants, wild-type resistant *ol-2* and susceptible MM controls. Silencing the *PEN1* and *PEN1* homolog restores susceptibility in transgenic *ol-2* plants (Table 6; Figure 12). This is due to increased penetration of the powdery mildew as a result of a compromised entry barrier. The non-transgenic

plants and the wild-type *ol-2* plants have little or no visible disease. In the *ol-2* wild-type, we noticed that the little mycelial colonies formed were more spread and did not form defined circular colonies as seen in transgenic plants. The average visual disease scoring was the highest for families silenced with *PEN1* _short (347 and 349) at 1.11 and 1.49 respectively. The families silenced for *PEN1*_long (354) and *PEN1* homolog (343) had a slightly lower disease incidence with a value of 0.95 and 0.91 respectively. However the visual scoring for the four families did not have significant differences among the four transgenic families. We carried out two more disease assays with the four families (experiment 2 and 3) which showed similar results (data from experiment 2 not included as plants seemed to experience some environmental (nutrients and humidity stress)). Visual scoring for experiment 1 and 3 (appendix i).

Table 7: The results of disease assay for four families with highest visual scoring.

Family	**Gene silenced**		**Average visual scoring**
TV123343	*PEN1* homolog	0.5-1.5	0.95
TV123347	*PEN1*_short	0.5-1.5	1.11
TV123349	*PEN1*_short	0.5-2.0	1.49
TV123354	*PEN1*_long	0.5-1.5	0.91

3.2.2. *Oidium* biomass quantification and expression analysis

DNA and RNA was isolated from the four families 343,347, 349 and 354. The DNA was used in PCR using NPTII and 35S primers to identify transgenic plants in the four families (appendix ii). PCRs with primers of pHELLs gate were done to confirm insertion of the T-DNA (Table 3). To quantify fungal biomass, real time PCR was done using DNA. In the quantification of the fungal biomass, we used *Oidium* primers to quantify the fungal biomass quantity. The *Oidium* quantities were significantly high in transgenic plants as compared to wild-type *ol-2* tomato plants and non-transgenic controls. RNA was used in real time PCR to verify the expression level of the target gene and also the expression of the homolog to check for cross silencing. The data was correlated with the *Oidium* biomass results, grouping together transgenic plants and non-transgenic plants (Figure 13-16).

3.2.2.1. *PEN1*_short silencing

The transgenic plants silenced for *PEN1*_short had high biomass levels of the *Oidium* (Figure 13 and 14) as compared to non-transgenic plants. In 347 family, the *Oidium* quantity level is 4.15 folds higher as compared to non-transgenic plants. In the 349, the *Oidium* level are quantified as 2.56 folds higher

compared to the non-transgenic plants. The RNAi silencing of *PEN1*_short is effective, the expression levels are significantly lower in the two transgenic families (347 and 349) compared to controls (non-transgenic plants and *ol-2* plants). The expression level is 0.69 for 347 family and 0.45 in 349 family as compared to controls with an expression value of 1. The expression of *PEN1* is variable in the two families, presumably due to different silencing effect in the transgenic plants. In the same families, the expression level of *PEN1* homolog is the same in both transgenic and non-transgenic plants.

The family 347 has 15 transgenic and 4 non-transgenic plants (appendix ii Table 3). According to the chi-square statistic (x^2=2.4). The predetermined alpha level of significance (0.5), and the degrees of freedom (df =1) see appendix ii Table 4. Since the x^2 statistic (2.4) is less than the critical value for 0.05 probability level (3.841) we can accept the null hypothesis that the observed values of our segregating population are the same as the theoretical distribution of a 3:1 ratio for a single gene segregation. The *Oidium* biomass was 4.15 folds higher compared to non-transgenic plants. The expression level was low at 0.69 compared to 1 in non-transgenic plants. The visual disease scoring of this family was 1.11 which confirmed the high level of *Oidium* as well as a low expression level of *PEN1*, explaining the increased susceptibility resulting from silencing of the *PEN1* gene.

The family 349 had 13 transgenic and 6 non-transgenic plants (appendix ii Table 3). The chi-square statistic (x^2=0.467). The predetermined alpha level of significance (0.5), and the degrees of freedom (df =1) see appendix ii Table 4. Since the x^2 statistic (0.467) is less than the critical value for 0.05 probability level (3.841) we can accept the null hypothesis that the observed values of our segregating population are the same as the theoretical distribution of a 3:1 ratio for a single gene segregation. The family had the high visual scoring of 1.49. The *Oidium* biomass was however slightly lower at 2.56 folds, compared to 347 family that had a greater *Oidium* biomass of 4.15. The difference could be as a result of variation in *Oidium* biomass among transgenic plants in 349 family. However, silencing level was more in the 349 family at 0.45 compared to 0.69 in 347 family. This could explain the high visual scoring in the 349 family as compared to 347 family.

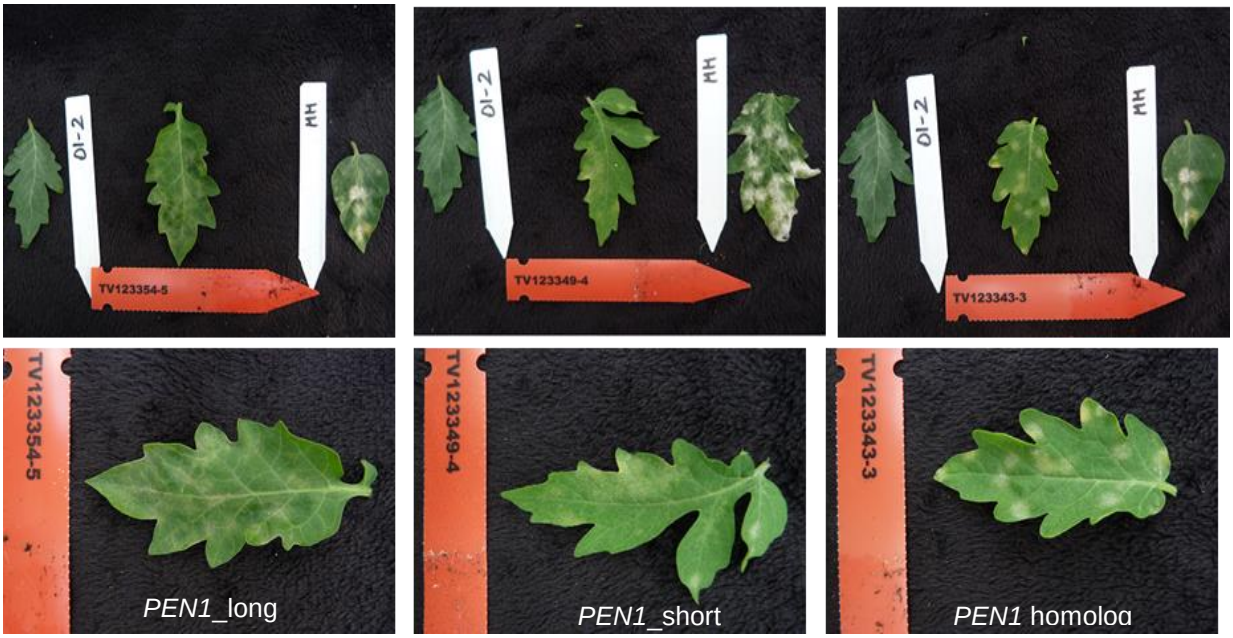


Figure 12: Visual scoring for formation of white mycelial growth in transgenic plants compared with controls in experiment 1. *ol-2* ((resistant) white label) transgenic plants ((susceptible) red label) and Money maker MM ((susceptible) white label).

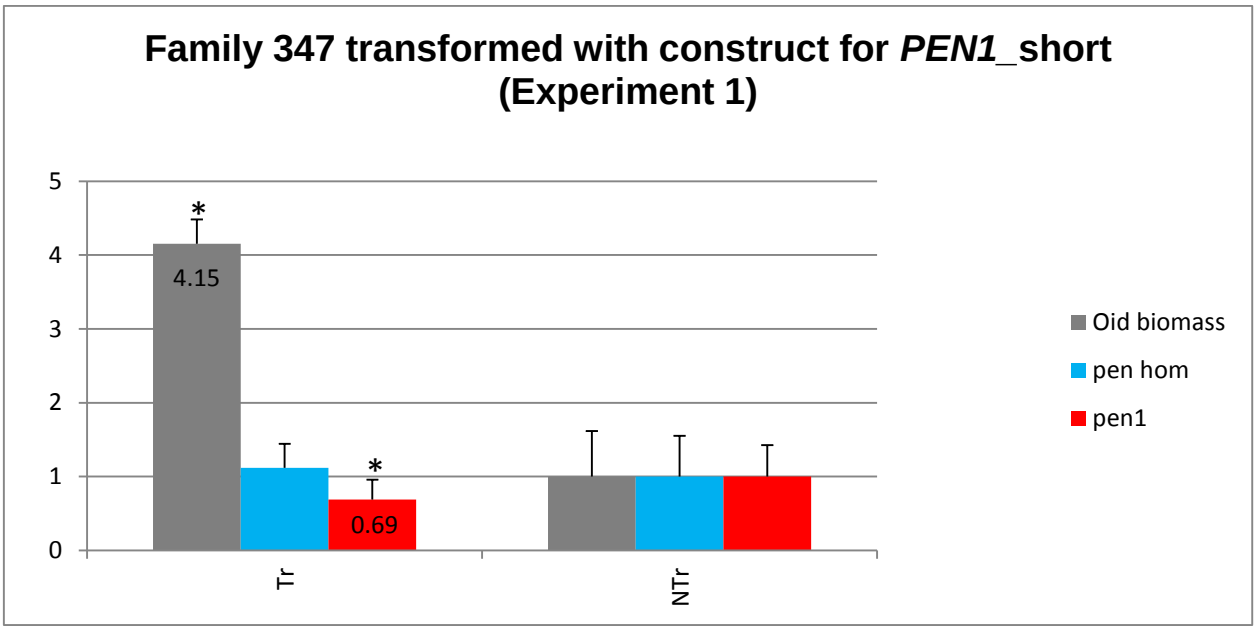


Figure 13: *PEN1*_short transgenic family 347. The *Oidium* biomass is high (4.15) and the *PEN1* expression level is low (0.69) due to RNAi silencing. Non-transgenic plants have low *Oidium* biomass (1) and the *PEN1* expression level is higher (1). The expression level of *PEN1* homolog is the same in both transgenic and non-transgenic plants. Transgenic plant (Tr), n=15 and non-transgenic plant (NTr), n=9 (4Ntr +5 *ol-2*). Asterix (*) represent significant differences in fungal biomass and silencing level in transgenic plants compared to controls with ($p<0.05$)

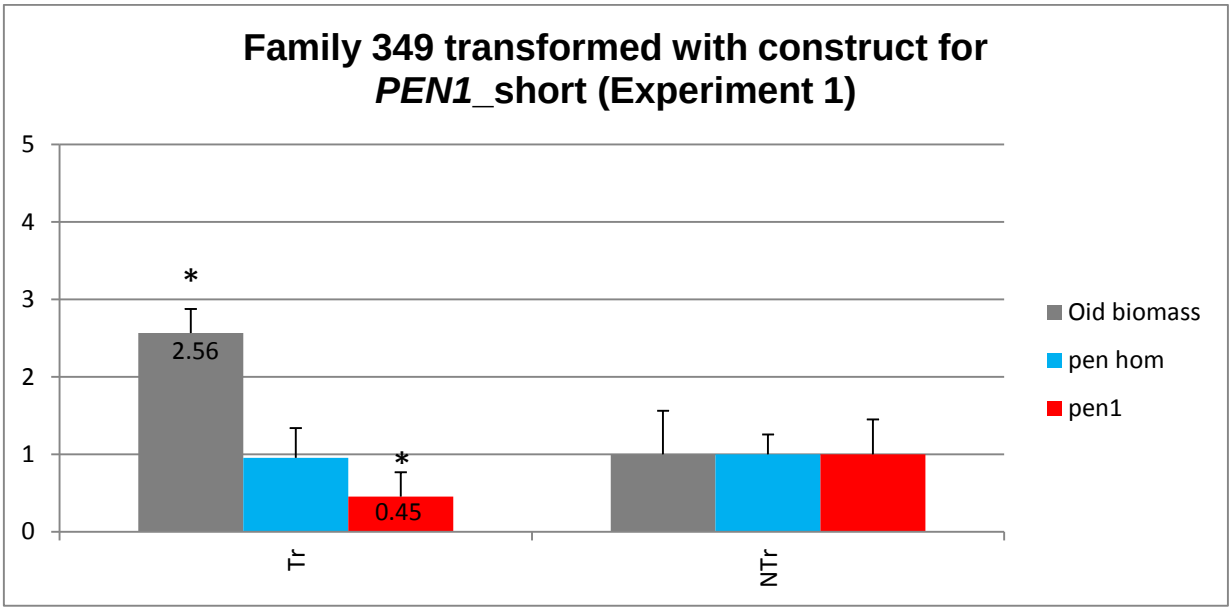


Figure 14: *PEN1*_short transgenic family 349. The *Oidium* biomass is high (2.56) and the *PEN1* expression level is low (0.45) due to RNAi silencing. Non-transgenic plants have low *Oidium* biomass (1) and the *PEN1* expression level is higher (1). The expression level of *PEN1* homolog is the same in both transgenic and non-transgenic plants. Transgenic plant (Tr), n=13 and non-transgenic plant (NTr), n=10 (6Ntr +4 *ol-2*). Asterix (*) represent significant differences in fungal biomass and silencing level in transgenic plants compared to controls with ($p<0.05$)

3.2.2.2. *PEN1*_long silencing

The 354 family, silenced with *PEN1*_long, (Figure 15) had 17 transgenic and 3 non-transgenic plants (appendix ii Table 3). The chi-square statistic (x^2=1.067). The predetermined alpha level of significance (0.5), and the degrees of freedom (df =1) see appendix ii Table 4. Since the x^2 statistic (1.067) is less than the critical value for 0.05 probability level (3.841) we can accept the null hypothesis that the observed values of our segregating population are the same as the theoretical distribution of a 3:1 ratio for a single gene segregation. T The segregation is 5:1 which is not compatible to the 3:1 ratio for a single gene segregation. This family, silenced for *PEN1*_long had 3.64 times higher *Oidium* compared to control. The silencing of *PEN1* gene was significantly lower at 0.42 in this family compared to controls. The visual scoring for 354 family was 0.91 which corresponds to the *Oidium* biomass and effective silencing. This family had a lower disease incidence compared to the transgenic plants 347 and 349 silenced with *PEN1*_ short construct.

From these results we can conclude that silencing of *PEN1* gene with *PEN1*_short was more effective than the silencing with *PEN1*_long construct as seen from the visual scoring. In 347 and 349 families, the expression level of *PEN1* homolog is the same in both transgenic and non-transgenic plants. Both

*PEN1*_long and *PEN1*_short seems to be only effective in silencing *PEN1* and not its homolog. The *PEN1*_short construct was initially made with the aim of silencing both *PEN1* and *PEN1* homolog. The results are contradictory to the intended use of this construct which silences only *PEN1* and not *PEN1* homolog.

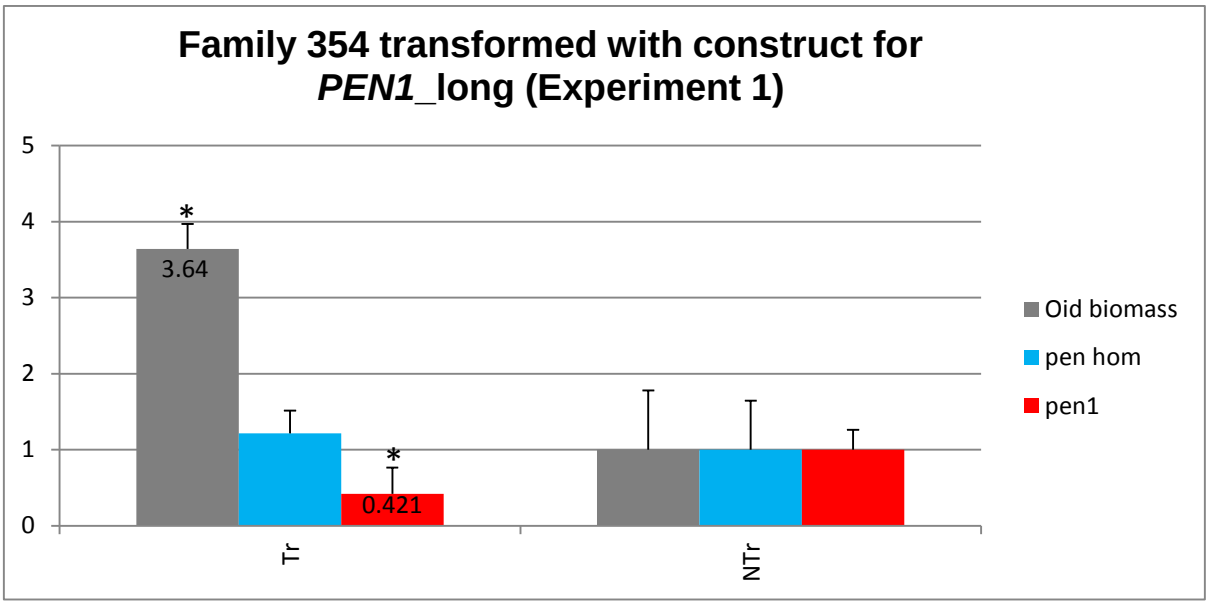


Figure 15: The family 354 contains the *PEN1*_long construct. The *Oidium* biomass is high (3.64) and the *PEN1* expression level is low (0.421) due to RNAi silencing. Non-transgenic plants have low *Oidium* biomass (1) and the *PEN1* expression level is higher (1). The expression level of *PEN1* homolog is the same in both transgenic and non-transgenic plants. Transgenic plant (Tr), n=17 and non-transgenic plant (NTr), n=7 (3Ntr +4 *ol-2*). Asterix (*) represent significant differences in fungal biomass and silencing level in transgenic plants compared to controls with ($p<0.05$)

3.2.2.3. *PEN1* homolog silencing

The family 343 (Figure 16) silenced with *PEN1* homolog, had 12 transgenic and 8 non-transgenic plants (appendix ii Table 3). The chi-square statistic ($x^2=2.4$). The predetermined alpha level of significance (0.5), and the degrees of freedom (df =1) see appendix ii Table 4. Since the x^2 statistic (02.4) is less than the critical value for 0.05 probability level (3.841) we can accept the null hypothesis that the observed values of our segregating population are the same as the theoretical distribution of a 3:1 ratio for a single gene segregation. The transgenic family silenced for *PEN1* homolog family had 2.21 folds increase in *Oidium* biomass compared to the controls. This family has a slightly lower *Oidium* biomass compared to the transgenic plants silenced for *PEN1*. Interestingly, this family has the lowest expression of *PEN1* homolog at 0.23 compared to controls with a value of 1.The visual scoring is 0.95 which is lower compared with the families silenced for *PEN1*_short but slightly higher than the *PEN1*_long. The *Oidium* biomass is also lower compared to the 347 and 349 silenced with

*PEN1*_short. In the 343 family, the expression level of *PEN1* is the same in both transgenic and non-transgenic plants. *PEN1* homolog construct used for the silencing is only effective for silencing *PEN1* homolog gene and not *PEN1* gene. We conclude although only a few families were analysed, *PEN1*_short was the most effective construct as seen in the visual scoring, and *Oidium* biomass.

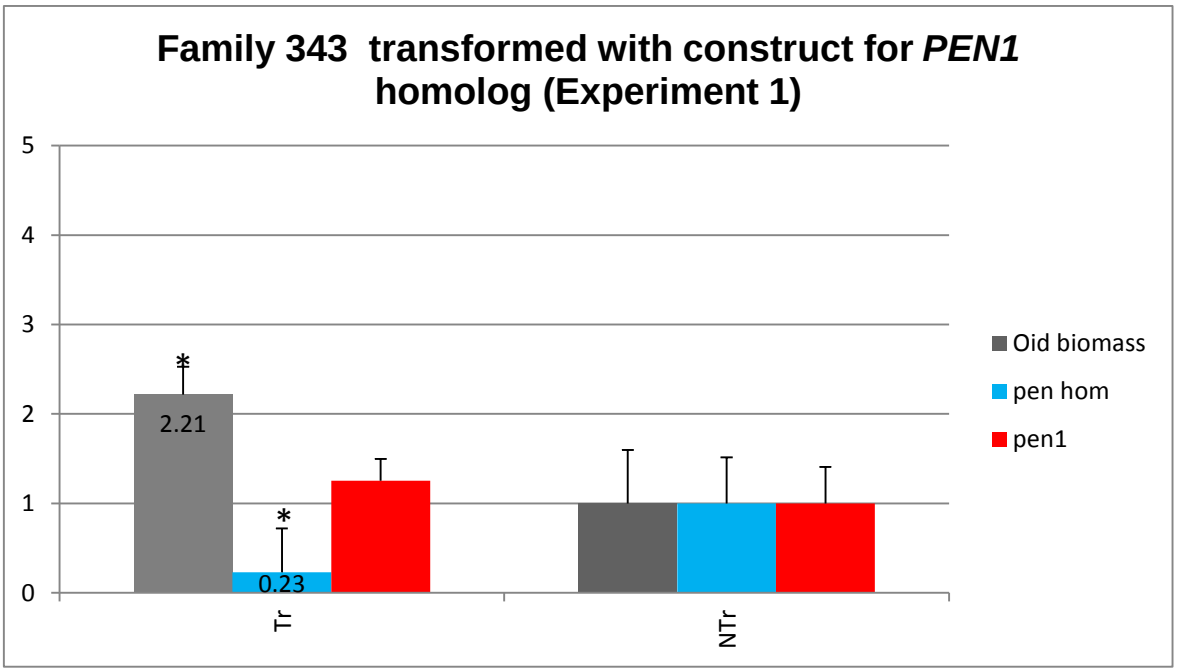


Figure 16: *PEN1* homolog transgenic family 343. The *Oidium* biomass is high (2.21) and the *PEN1* expression level is low (0.235) due to RNAi silencing. Non-transgenic plants have low *Oidium* biomass (1) and the *PEN1* expression level is higher (1). The expression level of *PEN1* is the same in both transgenic and non-transgenic plants. Transgenic plant (Tr), n=12 and non-transgenic plant (NTr), n=12 (8ntr +4 *ol-2*). Asterix (*) represent significant differences in fungal biomass and silencing level in transgenic plants compared to controls with ($p<0.05$)

3.3. Microscopy

The histological analysis identifies the type of structures established by the germinated spore in susceptible host. The experiment was done using the transgenic families 349, 354 and 343 silenced with *PEN1*_short, *PEN1*_long and *PEN1* homolog respectively. For each genotype, we selected two plants and made two slides per plant. In total we observed four slides per genotype including controls *ol-2* and MM at 48hpi. The slides were scored for infection units (IU), hyphae number, and formation of primary appressorium, papilla and haustorium, formation of secondary appressorium, papilla and haustorium as well as occurrence of HR (Figure 17) (Figure1; Appendix iii)

The family silenced with the *PEN1*_short, 349, has the highest spore penetration rate. The colonies formed on this family were the largest at 2.3 hyphae per infection unit (Table 8) (Figure2; Appendix iii).

This corresponds to the highest visual scoring in the 349 family. The spores formed had primary and secondary penetration structure (Figure 19). Spores that germinate on *PEN1*_long and *PEN1* homolog silenced families had both 1.2 hyphae per infection unit (Figure2; Appendix iii) which is lower compared to the transgenic 349 (*PEN1*_short). In the transgenic family 354 silenced with the *PEN1*_long construct, we initially used two transgenic plants 354-10 and 354-7 at 48hpi. However due to large differences in the hyphae number between the two transgenic plants, we added another plant 354-4 at 24hpi which was comparable to the 354-10 in hyphae number. This results can however not be conclusive since the different time points (48hpi and 24hpi) have different spore germination stages.

From these results we can conclude from hyphae number that, the average colony formation is high in plants silenced with *PEN1*_short compared to wild-type *ol-2* as also seen in visual scoring. Money maker is highly susceptible, this is confirmed from our results. The spores germinating on this genotype have the largest colony forming spores with an average of 2.8 hyphae per infection unit. The wild-type *ol-2* interestingly allows germination and colony formation with an average of 1.9 hyphae per IU. This is higher than in transgenic families silenced with *PEN1*-long and *PEN1* homolog. However, the colonies do not develop further due to cell wall apposition. We confirmed microscopically that mutants of *PEN1 and PEN1* homolog have an increased susceptibility evident from the various structures formed by germinating spores, such as appressorium, hyphae and haustorium as well as an ineffective papilla that allows penetration of *Oidium neolycopersici* (Table 8).

Table 8: Histological analysis of spore formation in mutants of *PEN1* and *PEN1* homolog at 48hpi (Experiment 3). Infection unit (IU), Primary (P), Secondary (S), appressorium (app), Average (Ave)

Genotype	**Silencing construct**	Slide number	IU	Hyphae no	Ave. hyphae	P. app	papilla	haustoria	S. app	Papilla	S haustoria
349-7	*PEN1*_short	1	31	2.4	2.3	100%	100%	84%	58%	42%	40%
		2	21	2.6							
349-8		1	14	2.4							
		2	7	1.8							
343-7	*PEN1*_homolog	1	24	1.2	1.2	100%	97%	63%	15%	9%	8%
		2	30	1.3							
343-8		1	25	1.2							
		2	13	1.4							
354-4	*PEN1*_long	1	28	1.3	1.2	99%	95%	26%	14%	11%	0%
		2	16	1.2							
354-10		1	21	1.1							
		2	12	1.3							
354-7		1	21	2.1							
		2	16	2.3							
MM		1	>30	2.8	2.8	100%	94%	89%	80%	98%	59%
		2	>30	2.9							
MM		1	>30	2.6							
		2	25	2.9							
OI-2		1	8	1.5	1.9	95%	100%	14%	0%	0%	0%
		2	11	2.1							
OI-2		1	13	1.8							
		2	9	2.4							

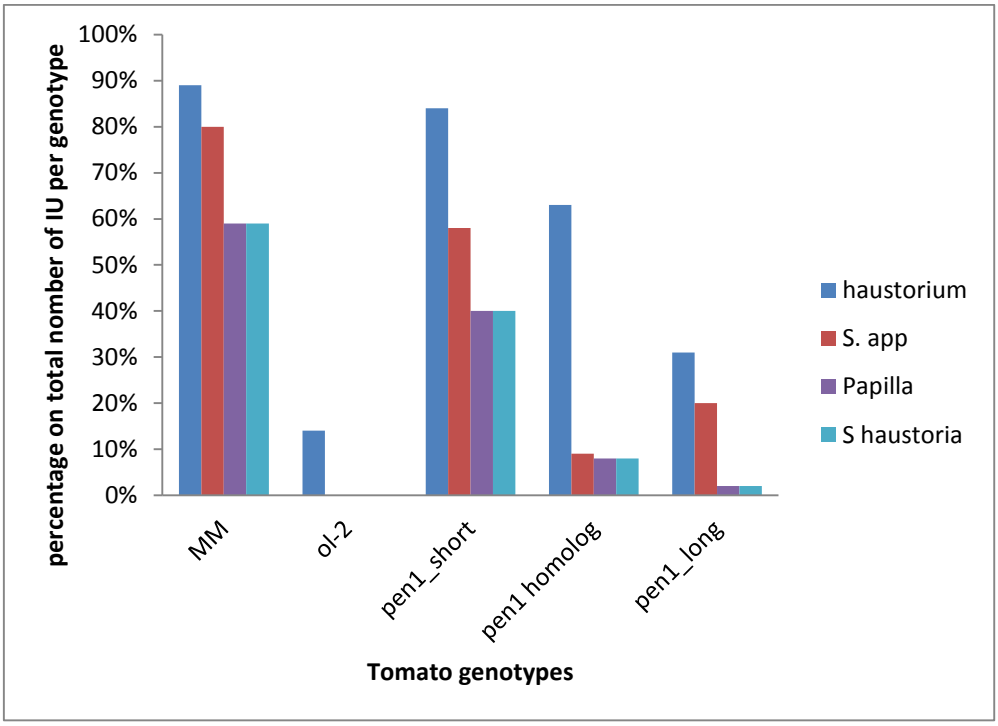


Figure 17: Different structure formed by germinating *Oidium neolycopersici* spores in different tomato mutants, wild-type *ol-2* and MM (experiment 3).

From the observations under the microscope, we were able to distinguish the different structures formed by *Oidium neolycopersici* on the surface as well as the inside of the epidermal cells of the host (Figure 18). The Appressorium is hammer shaped as observed from the germinated spore. The haustorium formed had a circular shape and looks intact in an epidermal cell with no HR. The papilla formed beneath the appressorium and in the haustorium is circular shaped.

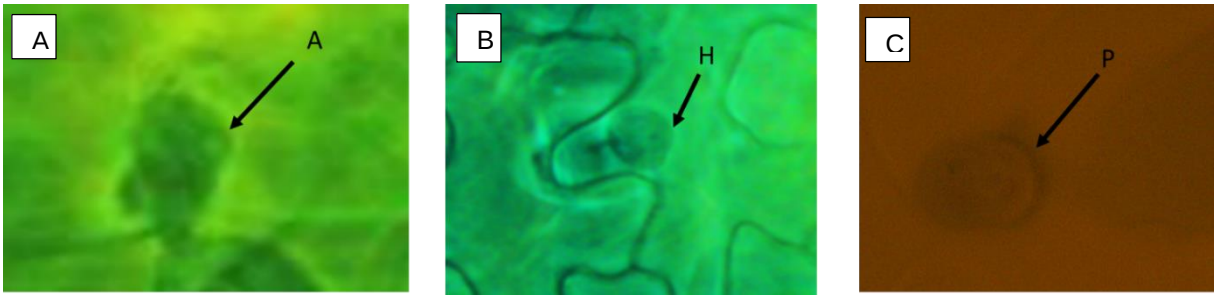


Figure 18: Appressorium, haustorium and papillae in the interaction of tomato and *Oidium neolycopersici.* **A,** An appressorium (indicated as A) with a hammer shape. The hammer like shape exerts a physical force on the host cell wall to aid in penetration. **B,** a normal circular shaped haustorium (indicated as H) in an epidermal cell with no HR. **C,** Papillae (indicated as P) formation beneath the appressorium (experiment 3).

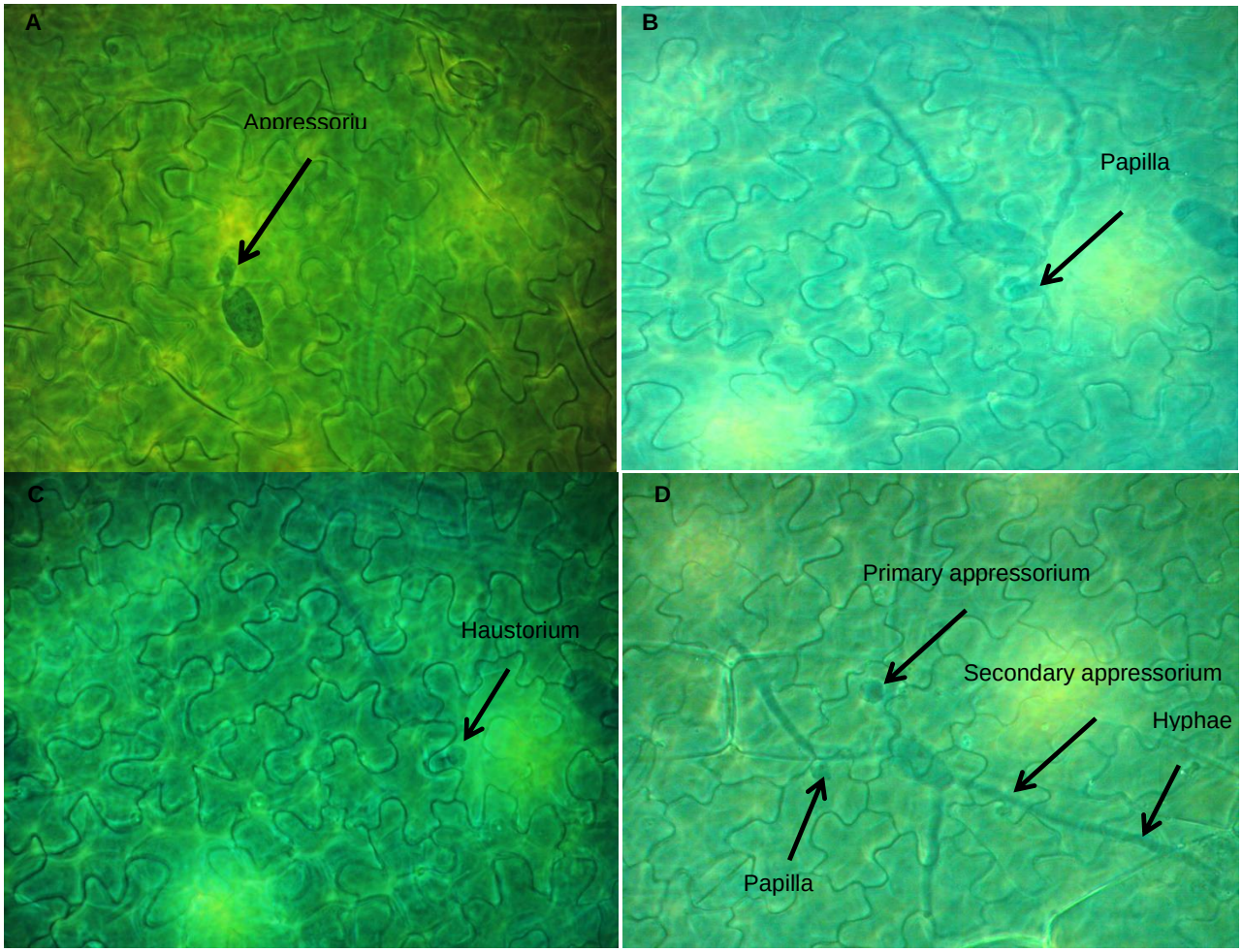


Figure 19: Microscopy images. A. An appressorium formed by a germinated spore. B. Cell wall deposition forming a papilla. C. Spores that breaks the penetration barrier forming a feeding structure known as the haustorium. D. Germinated spore forming a large colony with primary and secondary structure (experiment 3)**.**

4| Discussion and conclusion

In the study of *mlo* based resistance in *Arabidopsis*, three penetration (*PEN1, PEN2* and *PEN3*) genes were originally discovered based on their essential requirement in effective extracellular defences against the non-adapted powdery mildews *Blumeria graminis f. sp. hordei* and *Erysiphe pisi* (Collins *et al.* 2003) (Lipka *et al.* 2005). Studied done by (Zheng 2012) on role of *PEN* genes in *mlo* based resistance against *Oidium neolycopersici* in *Arabidopsis,* demonstrated the importance of the *PEN*-dependent and *PEN*–independent pre-penetrative defence mechanism required in *mlo* resistance.

In this study, we sought to investigate whether silencing of tomato *PEN1* homologs conferred susceptibility in *ol-2* resistant tomato plants. *ol-2* is a recessive gene. The *ol-2* mutants is characterised by low disease severity, low sporulation and restricted mycelial growth of *Oidium neolycopersici* (Ciccarese *et al.* 1998). For this, disease assays were performed using transgenic T2 tomato families, obtained with three RNAi constructs (*PEN1*_short, *PEN1*_long and *PEN1* homolog), which silenced *PEN1* and *PEN1* homolog in *ol-2* background.

From the disease assay, the transgenic plants have restored susceptibility, with fungal growth on the third and fourth leaf (Appendix i; Table 1; Table 2) compared to the wild-type (Carpentier 2009). The symptoms in wild-type *ol-2* were minimal ranging from 0-0.5. The disease index range between 0.5-2 in T2 plants. The mycelial colonies formed in *ol-2* were small and spread unlike the intact round colonies formed in transgenic susceptible plants. The silenced genes leads to compromised basal defence since the physical barrier is compromised and cell apposition though present is not tight enough to inhibit fungal entry, as observed by fungi penetration despite the presence of papilla (Figure 18 B and C) and formation of visible white mycelial colonies. This confirms that a membrane syntaxin protein *PEN1* is involved in secretion of components for the pathogen-induced cell wall appositions that inhibits fungal penetration into the host cells (Ellis 2006) (Zhang *et al.* 2007).

In molecular analysis, the real time PCR showed increased *Oidium* biomass in transgenic plants as compared to wild-type control. This proves that there is increased fungal entry as a result of defective pre penetration resistance. *PEN1* encodes a plasma membrane-anchored syntaxin with a SNARE domain with a key role in vesicle-associated membrane fusion and secretion processes that include exocytosis and endocytosis (Collins *et al.* 2003). Silencing *PEN1* could therefore have a negative effect on secretion of important defence material in the cell surface thus increasing fungal penetration. All the three RNAi constructs; *PEN1*_short, *PEN1* homolog and, *PEN1_long* resulted in significantly

high fungal biomass as compared to wild-type *ol-2* plants. This concludes that indeed the silencing of *PEN1* and *PEN1* homolog has restored susceptibility in *ol-2* plants.

The expression levels of the silenced genes was significantly lower compared to controls. This is a good indication that the constructs used for RNAi were effective in silencing *PEN1* and *PEN1* homolog. The *PEN1*_short construct was initially targeted to silence both *PEN1* and *PEN1* homolog, however the construct only silenced *PEN1*. This could be due to the fact that it had higher similarities to *PEN1* and not to *PEN1* homolog. Interestingly, we tested the functionality of the *PEN1* and the *PEN1* homolog constructs to see if they could silence both the target gene and its homolog. However, the constructs could only silence the target gene and not it homolog, which is due to the low identity at nucleotide level of *PEN1* and *PEN1* homolog with only 87% sequence identity.

The results are similar to those of an experiment carried out in barley to investigate the *ROR2* gene against (*Bgh*) and *PEN1* in *Arabidopsis*. *ROR2* and *PEN1* are functionally homologous syntaxin family members that confer broad non-host resistance conserved between monocotyledons and dicotyledons (Collins *et al.* 2003). Upon silencing *ROR2* and *PEN1* in barley and *Arabidopsis*, there was increased *Bgh* penetration (Collins *et al.* 2003). The experiment was a good model to study both host and non-host resistance involving the syntaxin proteins *PEN1* and *ROR2*. The results also overlap to studies carried out by ((Lipka *et al.* 2005). According to their experiments, the penetration rate of *Bgh* and *E. pisi* in *Arabidopsis* increased seven folds in *PEN1* mutants as compared to wild-type. However both studies (Collins *et al.* 2003) and (Lipka *et al.* 2005) show presence of HR that stops further infection.

We on the other hand see no HR in the interaction between transgenic *ol-2* tomato plants with *O. neolycopersici*. In *ol-2* mediated resistance, JA pathway is active and is a regulator of programmed cell death (Reinbothe *et al.* 2009). Biochemical characteristics of *ol-2* mediated resistance have shown that, H_2O_2 which is known contributes to induces cell death and cell wall fortification only accumulates at the site of infection and not in the epidermal cells (Lamb and Dixon 1997) (Seifi *et al.* 2014). These two features of *ol-2* mediated resistance could therefore explain why we see no HR in our transgenic *ol-2* plants. In *Arabidopsis* interaction with *O. neolycopersici,* SA up-regulation might be an important feature of *Atmlo2* resistance hence the reason for presence of HR (Seifi *et al.* 2014)

Previous work done on *Arabidopsis* interaction with tomato *Oidium neolycopersici* (Zheng 2012) show that, double mutant *Atmlo2/PEN1* had a decreased fungal penetration with respect to *Atmlo2* with insignificant disease symptom which contradicts our findings of increased fungal penetration in *PEN1*

silenced *ol-2* plants. We hypothesise that interaction between (*Arabidopsis* and *Oidium*) and (tomato and *Oidium*) could result in different pathways leading to *PEN1* penetration resistance. Host interactions between tomato and *Oidium neolycopersici* could have an indispensable role of the *PEN1* gene that confers resistance in tomato, while in *Arabidopsis*, there exists a *PEN* independent defence mechanism that restricts penetration of *Oidium neolycopersici* (Zheng 2012).

On structure development from germinating spores, hyphae germinated from one end and the short germ tube terminated in an appressorium (Figure 18 and 19). The hyphae then continued to develop both from the appressorium and from further germ tubes arising from the spore (Whipps *et al.* 1998) Studies by (Jones *et al.* 2000), described deposits of extracellular material beneath the *O. neolycopersici* germ tubes, the hyphae, around the margins of the appressorium and surrounding the haustorium, however, not beneath ungerminated spores This results overlap with our findings where we saw the cell wall depositions around the appressorium and also surrounding the neck of the haustorium. There was also no cell wall deposition where the spore had not germinated. In transgenic plants especially in highly susceptible *PEN1*_short transgenic 349 family , there was formation of a secondary germ tube (the colony forming hypha) arising from another tip of the spore and forming a small opposite or spread, lobed-shaped (secondary) appressorium and further development of a secondary haustoria (Jones *et al.* 2000). Some spores have a third and fourth germ tubes (colony forming hyphae) emerging from the remaining poles of the spore (MieslerovÁ *et al.* 2004).There is germination in the wild-type *ol-2* , an indication that spore germination is present in resistant wild-type accessions. This is a clear indication that the initiation of broad host resistance only becomes effective after spore germination (MieslerovÁ *et al.* 2004). The *ol-2* have an effective papilla that stops further development of fungal structures. At 48 hpi the colony forming hyphae of *O. neolycopersici* were greatly elongated on highly susceptible accessions (MieslerovÁ *et al.* 2004). We also find differences both in the length of germ tubes and colony forming hyphae and in the number of hyphae of *O. neolycopersici* between resistant and susceptible host lines.

From our findings, we conclude that *PEN1* and *PEN1* homolog gene is essential to for resistance in *ol-2* tomato against *Oidium neolycopersici.* Silencing of the genes restores susceptibility in transgenic *ol-2* tomato.

Recommendation for further research would be on histochemical analysis on the constituents of the defective papilla formed in susceptible transgenic plants compared to the one formed in resistant *ol-2*

plants. Studied of wild-type barley papilla have shown presence of many constituents including, Fe^{3+}, cell wall cross linked phenolics H_2O_2, cell wall cross linked proteins and phenolic conjugates, p-coumaroyl-hydroxyagmatine (Liu *et al.* 2007) (von Röpenack *et al.* 1998) (Thordal-Christensen *et al.* 1997). Callose is a major component in papilla, whose deposition by callose synthase, POWDERY MILDEW RESISTANT4 (PRM4) has been identified in *Arabidopsis* (Böhlenius *et al.* 2010). Studies have shown that plants lacking callose have a decreased penetration resistance. Further research on the expression of *PRM4* gene is recommend to correlate callose production as well as deposition at site of infection with effective pre penetration barriers to PM.

In (Zheng 2012), *PEN2* and *PEN3* roles were investigated on their role in pre-penetration resistance against *Oidium neolycopersici* in *Arabidopsis*. Double mutants, *Atmlo2/pen2* and *Atmlo2/pen3*, had an increased *Oidium neolycopersici* penetration rate in *Arabidopsis*. Interestingly, a protein blast search using AtPEN2 protein sequence against the tomato genome shows result of no true *PEN2* homolog since the best hit, Solyc01g074030, shares only 47% sequence identity. However, a similar search with AtPEN3 protein sequence as a query identifies Solyc03g120980, which shares a 72% sequence identity and could be the only true homolog. It would therefore be interesting to identify if for sure there is *PEN2* and *PEN3* in tomato as well as their role in *mlo* based resistance against *Oidium neolycopersici.*

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Appendices

Appendix i

Two different visual scoring (Experiment 1 and 3) for individual plants after inoculation with *Oidium neolycopersici.* (Table 1 and Table 2).

Table 1: Visual scoring for individual plants scored in experiment 1 containing 20 transgenic plants per family. R28_15_2 (code representing *ol-2* background)**.**

***ol-2* background**	**TV number**	**9 days after inoculation**	**2 weeks after inoculation**	**average visual scoring**	**t test**
R28_15_2	TV123343-1	clean	1.5	0.95	0.0152
	TV123343-2	normal leaf	1		
	TV123343-3		1.5		
	TV123343-4		1		
	TV123343-5		0.75		
	TV123343-6		1.5		
	TV123343-7		0.75		
	TV123343-8		0.75		
	TV123343-9		1		
	TV123343-10		1		
	TV123343-11		0.75		
	TV123343-12		0.75		
	TV123343-13		1		
	TV123343-14		0.75		
	TV123343-15		1		
	TV123343-16		0.5		
	TV123343-17		0.75		
	TV123343-18		1		
	TV123343-19		1		
	TV123343-20		0.75		
R28_16_4	TV123347-1	clean	1	1.1125	0.0053
	TV123347-2	normal leaf	1		
	TV123347-3	greyish myc	1		
	TV123347-4		1.5		
	TV123347-5		1.5		
	TV123347-6		1.5		
	TV123347-7		0.75		
	TV123347-8		1.5		
	TV123347-9		0.75		
	TV123347-10		0.75		
	TV123347-11		0.5		
	TV123347-12		0.5		
	TV123347-13		1.5		

	TV123347-14		1.5		
	TV123347-15		1.5		
	TV123347-16		0.5		
	TV123347-17		1.5		
	TV123347-18	clean	1		
	TV123347-19	S-HR	1		
	TV123347-20	S-HR	1.5		
R28_16_7	TV123349-1	clean	1.5	1.4875	0.0002
	TV123349-2	normal leaf	1.5		
	TV123349-3		2		
	TV123349-4		1.5		
	TV123349-5		2		
	TV123349-6		1.5		
	TV123349-7		2		
	TV123349-8		2		
	TV123349-9		2		
	TV123349-10		2		
	TV123349-11		1.5		
	TV123349-12		0.75		
	TV123349-13		0.75		
	TV123349-14		2		
	TV123349-15		1		
	TV123349-16		0.75		
	TV123349-17		1		
	TV123349-18		0.5		
	TV123349-19		1.5		
	TV123349-20		2		
R28_11	TV123354-1	different leaf	1	0.9125	0.0368
	TV123354-2	lighter and	0.5		
	TV123354-3		1		
	TV123354-4		1		
	TV123354-5	smaller/branc	1		
	TV123354-6		0.75		
	TV123354-7		1.5		
	TV123354-8	smaller/greyis	1.5		
	TV123354-9		0.75		
	TV123354-10	normal leaf	0.75		
	TV123354-11		0.75		
	TV123354-12		0.75		
	TV123354-13	like 2	0.5		
	TV123354-14		0.75		
	TV123354-15		1		
	TV123354-16	greyish myc	0.75		
	TV123354-17		0.75		
	TV123354-18	greyish myc	1		
	TV123354-19		0.75		
	TV123354-20		1.5		

Table 2: Visual disease scoring for individual plants scored in experiment 3 with 15 transgenic plants per family.

Construct	Genotypes	11 days after inoculation	18 days after inoculation	Average visual scoring
***PEN1* homolog**	343-1	0.75	1	**0.933**
	343-2	0.75-1	1	
	343-3	0.75-1	1	
	343-4	1	1.5	
	343-5	0.75-1	1.5	
	343-6	0.5	1	
	343-7	0.5	1	
	343-8	0.5	0.5	
	343-9	0.5	1	
	343-10	1.5	1.5	
	343-11	0.5	0.5	
	343-12	0	0.5	
	343-13	0.75	0.75	
	343-14	0.5	0.5	
	343-15	0	0.75	
***PEN1*_short**	347-1	0.75	1.5	**1.052**
	347-2	0.5	0.5	
	347-3	0.5	0.75	
	347-4	0	0	
	347-5	0.5	1	
	347-6	0.5	1	
	347-7	0.5-0.75	1	
	347-8	1.5	1.5	
	347-9	1.5	1.5	
	347-10	0.75	1	
	347-11	0.5	0.5	
	347-12	0	0.75	
	347-13	0.5	0.5	
	347-14	0.5	0.5	
	347-15	1	1.5	
***PEN1*_short**	349-1	0.75	1	**1.283**
	349-2	2	2	
	349-3	1.5	2	
	349-4	1-1.5	1.5	
	349-5	1.5	2	
	349-6	0.5	0.5	
	349-7	1	1	
	349-8	2	2	
	349-9	2	2	
	349-10	2	2	
	349-11	0.5	0.5	
	349-12	0	0.5	
	349-13	0.5	1	
	349-14	0	0.75	

	349-15	0.5	0.5	
***PEN1*_long**	354-1	0	0	**0.916**
	354-2	2	2	
	354-3	1	1	
	354-4	0.5	0.5	
	354-5	0	0.5	
	354-6	0.75-1	1.5	
	354-7	0.5	1	
	354-8	1.5	1.5	
	354-9	0.75	0.75	
	354-10	0	0	
	354-11	0.5	0.75	
	354-12	0.75	0.75	
	354-13	0	0.5	
	354-14	1.5	1	
	354-15	1.5	2	

Appendix ii

Table 3: The results from PCR amplification of NPTII, 35S and pHELLs gate primers .Tr, transgenic, Ntr, Non-transgenic plants.

Genotype	NPTII	35S	Xba_F and P27_R	Segregation ratio (Tr: Ntr)
TV123343-1	-	−	-	12:8 X^2=2.4 (<3.84) Compatible with 3:1 ratio for a single gene segregation.
TV123343-2	+	+	+	
TV123343-3	+	+	+	
TV123343-4	+	+	+	
TV123343-5	−	−	-	
TV123343-6	+	+	+	
TV123343-7	+	+	+	
TV123343-8	−	−	-	
TV123343-9	+	+	+	
TV123343-10	−	−	-	
TV123343-11	+	+	+	
TV123343-12	−	+	-	
TV123343-13	+	+	+	
TV123343-14	+	+	+	
TV123343-15	−	−	-	
TV123343-16	+	−	-	
TV123343-17	−	−	-	
TV123343-18	+	+	+	
TV123343-19	+	+	+	
TV123343-20	−	−	-	
TV123347-1	+	+	+	15:4 X^2=0.2 (<3.84) Compatible with 3:1 ratio for a single gene segregation. segregation
TV123347-2	+	+	+	
TV123347-4	+	+	+	
TV123347-5	+	+	+	
TV123347-6	+	−	-	
TV123347-7	−	+	-	
TV123347-8	−	+	-	
TV123347-9	+	+	+	

TV123347-10	+	+	+	
TV123347-11	−	−	-	
TV123347-12	+	−	-	
TV123347-13	+	+	-	
TV123347-14	+	+	+	
TV123347-15	+	+	+	
TV123347-16	−	−	-	
TV123347-17	−	+	-	
TV123347-18	+	+	+	
TV123347-19	−	+	-	
TV123347-20	−	+	-	
TV123349-1	+	+	+	13:6 X^2=0.467 (<3.84) Compatible with the 3:1 ratio for a single gene segregation.
TV123349-2	+	+	+	
TV123349-3	+	+	+	
TV123349-4	−	−	-	
TV123349-5	+	+	-	
TV123349-6	+	−	+	
TV123349-7	+	−	+	
TV123349-8	+	+	+	
TV123349-9	+	+	-	
TV123349-10	+	+	-	
TV123349-11	+	+	+	
TV123349-12	−	−	-	
TV123349-13	−	−	-	
TV123349-15	−	−	-	
TV123349-16	−	−	-	
TV123349-17	+	+	+	
TV123349-18	−	−	-	
TV123349-19	+	+	+	
TV123349-20	+	+	-	

TV123354-1	+	−	-	17:3 X^2=1.067 (<3.84) Compatible with 3:1 ratio for a single gene segregation.
TV123354-2	+	+	-	
TV123354-3	+	+	-	
TV123354-4	+	+	-	
TV123354-5	+	−	-	
TV123354-6	+	+	-	
TV123354-7	+	+	+	
TV123354-8	+	+	+	
TV123354-9	+	−	+	
TV123354-10	−	−	-	
TV123354-11	−	+	-	
TV123354-12	+	+	+	
TV123354-13	+	+	+	
TV123354-14	+	+	-	
TV123354-15	+	−	-	
TV123354-16	+	+	+	
TV123354-17	−	−	-	
TV123354-18	+	−	+	
TV123354-19	+	+	+	
TV123354-20	+	−	+	

Table 4. Chi Square distribution table.

Probability level (alpha)

Df	0.5	0.10	0.05	0.02	0.01	0.001
1	0.455	2.706	3.841	5.412	6.635	10.827
2	1.386	4.605	5.991	7.824	9.210	13.815
3	2.366	6.251	7.815	9.837	11.345	16.268
4	3.357	7.779	9.488	11.668	13.277	18.465
5	4.351	9.236	11.070	13.388	15.086	20.517

Appendix iii

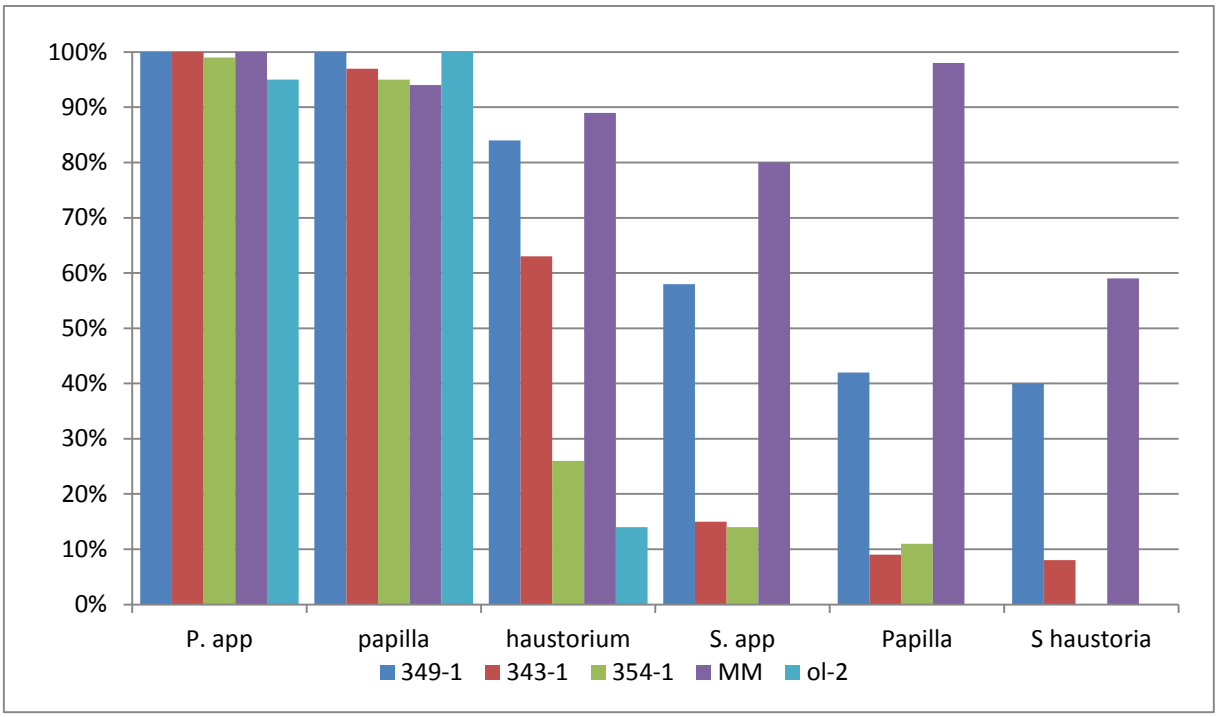

Figure 1: The graphical representation of structures formed by germinating spores in different genotypes.

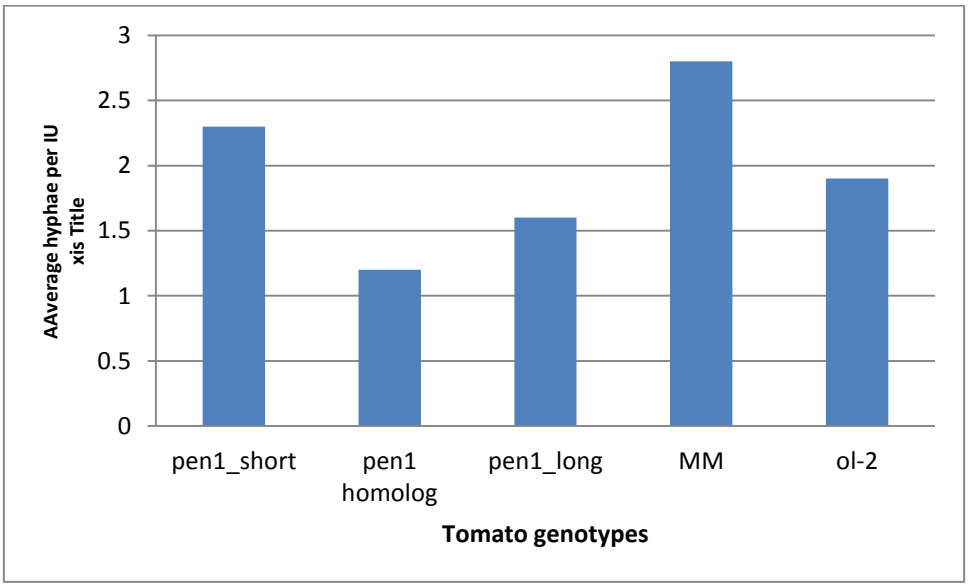

Figure 2: Colony size (average hyphae per IU) in different tomato mutant, wild-type *ol-2* and MM.