MSc thesis Plant Breeding **June 2009**
PBR-80436

Pen1 and *Pen3* are required for *ol-2* associated resistance in tomato against tomato powdery mildew.

Magali Carpentier
Supervisor: Yuling Bai

Abstract

Tomato powdery mildew *O.neolycopersici* has become a serious disease in tomato. *ol-2* is a recessive resistance gene conferring broad spectrum and prepenetration resistance of tomato to *O.neolycopersici*. ol-2 is homologous to *mlo* in barley and *Arabidopsis*. *Pen1* and *Pen3* genes are required for *mlo*-resistance in *Arabidopsis* and *Ror2* gene, homologue to *Pen1* is required for barley *mlo*-resistance. We tested using VIGS technique whether an homologue to *Pen1* and *Pen3* in tomato was also required for *ol-2*-mediated resistance against *O.neolycopersici.* Upon silencing with *Pen1, ol-2* tomatoes displayed a susceptible phenotype: big increase in host cell entry and colonization of the leaves. Moreover, papillae were not effective anymore and were penetrated by the fungus. With *Pen3* silenced, no colonization was observed, but an increase in host cell entry . This suggests that *Pen1* and *Pen3* are conserved requirements in mlo-based resistance. A chloroplast light harvesting gene (*M12E62-620)* found in a study aiming at looking for gene upregulated during infection of *ol-2* tomato with powdery mildew was also tested with the same technique. Susceptibility and microscopic increase in fungus penetration were found. This *M12E62-620* is also required for *Xanthomonas campestris* resistance suggesting a broad role for this gene.

Introduction

Tomato (*Solanum lycopersicum*) is one of the most consumed vegetable. The worldwide production is 3.98 x 10^8 ton/year (FAOSTAT data, 2007). Tomato powdery mildew (*Oidium neolycopersici* widespread in Europe, *O. lycopersici* in Australia and *Leveillula taurica* in western part of the US) is the most serious problem in tomato cultivation in greenhouses production, but nowadays also on field-grown tomatoes (1). Tomatoes infected with *O. neolycopersici* show leaf chlorosis, premature senescence, and a decrease of fruit size and quality (2). Effective fungicides have long been developed, but their use is causing serious pollution effects, and with the increase awareness of environmental issues, search for resistant cultivar is the oncoming solution (18).

- **Tomato resistance genes to *O. neolycopersici***

Several tomato genes conferring resistance to *O. neolycopersici* have already been identified, including six monogenic *Ol* genes and three quantitative trait loci (QTLs) (Table 1). On chromosome 6, three clusters can be drawn: on the long arm, one cluster contains *Ol-1* and *Ol-3* (3,9) and another closely linked cluster harbors *Ol-5* (5); on the short arm, one cluster contains the resistant genes *Ol-4* (4,9) and *Ol-6* (5). *Ol-qtl1* collocates with *Ol-1*, *Ol-3* and *Ol-5*. *Ol-qtl2* and *Ol-qtl3* are linked and collocate on chromosome 12 (8,9). *ol-2* recessive resistance gene is located on chromosome 4 (6,7,9). In summary (table1), three genetically different resistances have been identified: monogenic dominant resistance, monogenic and recessive resistance as well as polygenic resistance conferred by QTLs with additive effects. Among those resistances, three physiological mechanisms are observable when challenged with *O. neolycopersici*: the five dominant resistances involve hypersensitive response (HR), *ol-2* mediated resistance involves the formation of a papillae, and the QTL-mediated resistance involves both HR and papillae formation.

In this study, we focused on *ol-2* mediated resistance. *ol-2* allele comes from *Ol-2* gene, gene required for powdery mildew to promote disease. *ol-2* allele arises from a natural 19 base pair deletion on *Ol-2* gene, giving rise to a truncated and not functional *Ol-2* protein (13).

Table 1: Tomato resistance genes against powdery mildew and their characteristics.

gene	Genetic basis	origin	Chromosomal location	Resistance mechanism	Race specificity[a]
Ol-1	Dominant	*S .habrochaites* G.1.1560	Long arm, 6	Slow HR[b]	Race - non specific
Ol-3	Dominant	*S .habrochaites* G1.1290	Long arm, 6	Slow HR	Race - non specific
Ol-4	Dominant	*S. peruvanum* LA172	Short arm, 6	Fast HR[c]	Race specific
Ol-5	Dominant	*S. habrochaites* PI247087	Long arm, 6	Slow HR	Race - non specific
Ol-6	Dominant	Unknown origin	Short arm, 6	HR	Race specific
ol-2	Recessive	*S. lycopersicum* var *cerasiforme*	Short arm, 4	Papillae	Race non-specific
Ol-qtl1	Polygenic (Additive)	*S. neorickii* G1.1601	Long arm, 6	HR , papillae	Race non-specific
Ol-qtl2	Polygenic (Additive)	*S. neorickii* G1.1601	Short arm, 12	HR , papillae	Race non-specific
Ol-qtl3	Polygenic (Additive)	*S. neorickii* G1.1601	Short arm, 12	HR , papillae	Race non-specific

[a] Race specificity: limited to the seven tested isolates (5).
[b] Slow HR: multi cell HR
[c] Fast HR: single cell HR

- **Plants defend themselves against powdery mildews**

In addition to tomato - *O.neolycopersici*, barley-powdery mildew (*Blumeria graminis* f.sp. *hordei: Bgh)* and *Arabidopsis*-powdery mildew *(Erysiphe cichoracearum*) pathosystems have been extensively studied for their interaction between the plant and its host powdery mildew. Several similarities amongst these pathosystems have been observed.

Three classes of barley resistance genes have been identified as representatives: *Mla*, *Mlg*, and *mlo*, for three different resistance mechanisms: *Mla* gives a slow multi cells HR taking place after fungal penetration (5). *Mlg* mediated resistance against *Bgh* triggers a single cell HR upon fungal penetration and *mlo*-based resistance in barley gives rise to a broad spectrum resistance via the formation of a papilla (17). Those three genes resemble the *Ol*-genes in tomato (Figure1).

In *Arabidopsis,* two classes of genes are known to mediate resistance against *E.cichoracearum*:

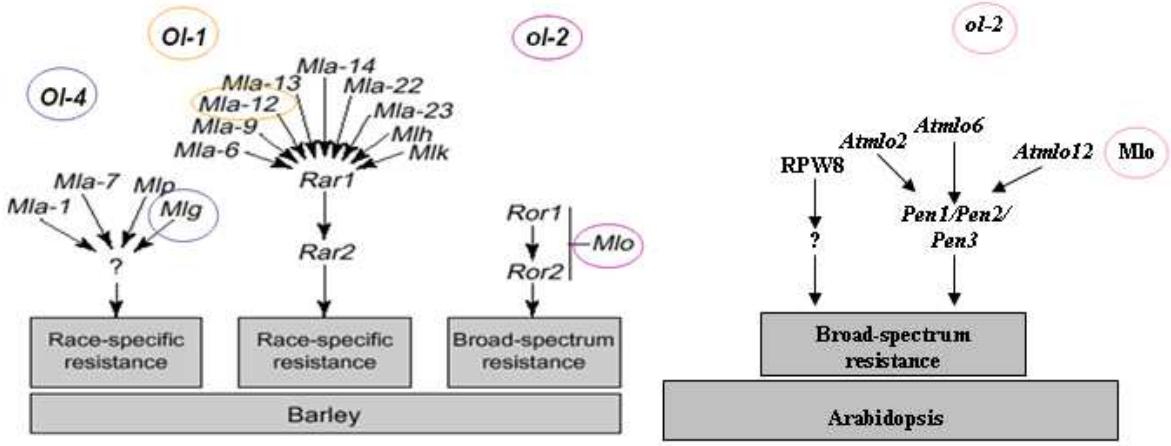
-*mlo*-resistance genes have been found in a screen for induced mutants resistant to powdery mildews: there are three *mlo* genes in *Arabidopsis* important for the resistance amongst, named *Atmlo* genes. They trigger a broad spectrum resistance via the formation of a papilla, like *mlo* in barley and *ol-2* in tomato (Table 2).

-*RPW8* gene seems not to be related to any known genes in tomato and barley, it is associated with HR in the resistance to *E.cichoracearum* in *Arabidopsis* (20).

Table 2: Genetic and mechanistic similarities between Barley, *Arabidopsis,* and tomato- powdery mildews pathosystems.

	Barley/Bgh	*Arabidopsis/E.cichoracearum*	*Tomato/O.neolycopersici*
Slow HR	*Mla12*		*Ol-1*
Fast HR	*Mlg*		*Ol-4*
Papillae formation	*mlo*	*Atmlo*	*ol-2*
HR		*RPW8*	

Figure 1: From Schulze-Lefert and Vogel, 2000 *Trends in plant science.*
Pathways leading to resistance in barley and *Arabidopsis*. There are at least two independent pathways leading to race-specific resistance in barley, and barley mutants can confer broad-spectrum resistance. In *Arabidopsis*, two categories of genes lead to broad spectrum resistance via two different mechanisms.



- ***mlo* mutant in barley, *Arabidopsis* and tomato (*mildew resistance locus o*)**

One of the wild-type *Mlo* function is to promote mildew fungus penetration into the plant (40). *Mlo* family is a small size gene family in *Arabidopsis* and barley (42).
Loss of function of a single *Mlo* gene in barley confers broad spectrum resistance to powdery mildews with formation of a papillae (44). The mutation that gives rise to the resistance is a tandem repeat array inserted upstream the wild type gene leading to aberrant transcripts and non functionality of the MLO protein (41).
In *Arabidopsis*, mutations in three *Mlo* (*Atmlo* 2, 6 and 12*)* co-orthologues are required for a full resistance to powdery mildew with formation of a papillae (43; 14).
In tomato, the *ol-2* allele arised from a 19 base pair natural deletion on Ol-2 leading to a genetic frame shift, and to an unfonctional *ol-2* gene, *ol-2* recessive gene giving rise to broad spectrum resistance against powdery mildew with the formation of a papillae (45).

- **Pathway genes involved in the resistance against powdery mildew**

Some aspects of the molecular mechanisms underlying the resistance have already been found: the resistance pathway in *ol-2* resistant cultivars seems to be independent of the defense pathways mediated by ethylene, JA and SA. On the other hand, it seems to be dependant on the lipoxygenase pathway. *Ol-4* mediated resistance involves ethylene and lipoxygenase pathway, whereas it is independent from JA and SA. *Ol-1* mediated resistance on the contrary seems to depend upon SA and ethylene pathways. Though, in the study of Achuo et al (2004), it had been demonstrated that activation of the SA dependent defense pathway did not induce resistance against *O. neolycopersici* in S-MM (12).
In *Arabidopsis* and barley, the involvement of known pathways in resistance against powdery mildew have been studied as well. Pathways monitored by the resistance gene *RPW8* (small, basic proteins with putative N-terminal trans membrane domain and a coiled coil domain) leading to the resistance of *Arabidopsis* to powdery mildew via a HR response seem to be associated with SA accumulation and SA-dependent feedback regulation of the expression of *RPW8* (20).
In a study of the pathosystem barley-powdery mildew by Hein et al (2004) it had been demonstrated that *Mla*-mediated HR and *mlo*-mediated papilla formation in mildew-challenged barley were associated with H_2O_2 but not with SA. Some key genes are specific, they are used to monitor each defense pathway (Table 3).

Table 3: Key genes used in tomato to monitor known defense pathways: JA,SA and ethylene

gene	Pathway monitored in tomato	publication
PR-1	JA, SA, ethylene	21
Beta 1,3 glucanase	JA, SA	22
Coronatine insensitive1	JA	20
ETR-1	ethylene	
NIF1	SA	23
LoxD	lipoxygenase	24

- **Candidate genes involved in resistance of tomato against powdery mildew**

- Few genes have been proven to be involved in the resistance pathways in Barley and *Arabidopsis*: *Pen1*, *Pen2* and *Pen3*, essential for *Atmlo* mediated resistance in *A. thaliana* (14), *ror1* and *ror2*, essential for *mlo* mediated resistance in Barley (22), *rar1* and *rar2* for *Mla* resistance in barley (21; 23). *Pen1* and *Ror2* genes are homologues in function and in sequence (29).

- In tomato, several resistance genes have been identified, but genes in the defense pathway are not known. Based on a gene expression study, Chengwei Li (2006) identified several candidate genes which were upregulated for tomato resistance against powdery mildew.

His study examined DE-TDFs (Differentially Expressed Transcript Derived Fragments) of challenged or unchallenged tomato plants (with all kind of genotypes: S-MM, NIL-Ol-1, F3-ol-2, NIL-Ol-4, Ol-QTL- see Materials and Methods for description) via cDNA-AFLP (Amplified Fragment Length Polymorphism). Amongst the 141 DE-TDFs differently regulated in the four genotypes tested in the study of Li et al (2007) (S-MM, NIL-Ol-1, F3-ol-2, NIL-Ol-4), 50 TDFs were sequenced and blasted against the TIGR database (The Institute for Genomic Research). Twenty six TDFs showed functional information, such as known direct defense responses, or signal transduction and photosynthesis. Twenty four TDFs on the other hand matched with unknown proteins or did not match at all (9). In another similar study of Li et al, three genotypes were tested (S-MM, NIL-*Ol-1*, and *Ol*-QTL), and that time, amongst the 230 sequenced DE-TDFs, 120 matched with known proteins, and 110 with unknown ones, or had no hits found in the database (11). A simplistic classification of the DE-TDFs consists of four classes. There are redundancy amongst those classes and the differential expressions observed into each class are not always homogeneous in timing or in level (Table 4). For a more detailed table, see the work of Chengwei Li (Thesis 2006).

Table 4: Different expression classes of the DE-TDFs

Class	Description of the expression pattern
I	Increase in all genotypes tested
II	Increase in all resistant genotypes tested
III	Increase in a specific resistant genotype only
IV	Increase in S-MM only
V	Increase in S-MM and one resistant genotype

- **Aim of the study**

The aim of the study is to identify genes involved in the defense mechanism to fight against *O.neolycopersici*. We shall focus on (1) some known proteins involved in resistance pathways in the *Arabidopsis* and Barley-powdery mildew pathosystems, and on (2) an unknown one, chosen amongst those described in Chengwei Li thesis (Table 5).

Those interesting genes will be silenced via VIGS (Virus Induced Gene Silencing) in the *ol-2* genotype. *ol-2* is chosen because the resistance phenotype is complete and clear.

Table 5: Tested genes on the genotype showing the clearest pattern of resistance

genes	**expression pattern/putative function**	**genotype**	**class**
Pen1 *Pen 3*			
M12E62-620[b]	Upregulated in *ol-2* genotype/Chloroplast light-harvesting complex II protein Lhcbm6[a]	*Ol-2*, S-MM	III=induced only in one genotype

[a]Putative function of *M12E62-620* has been found by comparative blast search on genbank and TIGR databases.

[b]TDFs are called from the cDNA-AFLP size pattern.

- **Expected results**

Silencing of *Pen1* and *Pen3* genes in *ol-2* carrying genotype should lead to susceptibility, likely observable at the microscopic level, with a higher penetration of the pathogen into the cells, or even at the plant level, with visible symptom spots onto the leaves. Indeed similar study in Barley and *Arabidopsis* lead to this observation.

M12E62-620 is upregulated in challenged *ol-2* plants. Its silencing should lead to susceptibility.

- **Technique used**

The technique used for gene silencing is the VIGS described by Lu et al (10). VIGS is a transient transformation assay technique which exploits the homology-based-defense mechanism (RNAi), which is triggered by incoming viruses, to target individual endogenous genes for silencing. Approximately 100 base pairs of the transcript of

the gene to be silenced are cloned into Tobacco Rattle Virus (TRV)- RNA 2 (TRV-RNA 2). This construct is transferred into *Agrobacterium. tumefasciens* which culture is agro infiltrated in the desired tomato genotypes together with an *A. tumefasciens* culture harboring TRV-RNA1 construct. TRV makes the construct spread through the whole plant. A Phytoene Desaturase Protein (PDS) construct control is done to observe the white silencing pattern, as well as an empty vector negative control, to observe the effect of TRV alone in the plant. Expression of the silenced genes will be monitored along the experiment.

Materials and methods

Plant materials:
Two tomato genotypes were used in this study. *S. lycopersicum* cv. Moneymaker (S-MM) was used for the compatible interaction. F3-ol-2 (a F3 line of *S.lycopersicum* cv. Marmande x *S.lycopersicum* var. cerasiforme carrying homozygously the recessive *ol-2* gene (5)) was used as the resistant line.
Plants were grown at 20 ± 2°C, 16 h daytime, and 70 ± 5% relative humidity.

Constructs:
Fragment of the chosen TDF was ligated into TRV2 plasmid (described by Liu et al, 2002) amplified in *Escherichia coli*, and transformed in *A. tumefasciens* (15). *Pen1* and *Pen3* constructs were done by Zheng Zheng. See table 5 for the primers used, forward then reverse sequences are listed. Primers were designed based on sequence analysis using the cDNA-AFLP set sequences, and TIGR (plant transcript assemblies program) and NCBI blastN with the expressed sequence tag database. Each primer combination allowed an amplification of approximately 150bp length. Primers were designed using DNASTAR software (v6.0; Laser Gene, Madison, WI, USA) For a more detailed protocol see appendix.

Table 6: Primers used for the VIGS experiment.

gene	primers
Pen1[a]	TCTGCATAATGCTAAAAATGTCAAA; TTTCTTGAACTTTTCTGGTGTTTTC
Pen 3[a]	CCTTCTTCTCCTTC; AACATTTTTGTGCTA
M12E62-620	GAACGCGAACTTGAGGTGAT; AGATGGCCAAGATGCTTTGT

[a]constructs done by Zheng Zheng

Sequencing of the TDF cloned:
VIGS constructing workPurified PCR products obtained with the multiple cloning site primer set were sent to Base clear to be sequenced. The result was compared with the expected sequence from Chengwei Li work.

Agrobacterium mediated virus infection:
Cultures of *A.tumefasciens* containing the construct TRV2-TDFs and TRV1, and a control *A.tumefasciens* culture containing an empty TRV2 construct were grown and adjusted to an OD_{600} of 1.0. Equal volumes of *A. tumefasciens* cultures were infiltrated to the desired tomato plants, on the lower side of both cotyledons using a 1 ml-syringe lacking a needle.

Fungal material:
The pathogenic fungus *O. neolycopersici*, which originated from infected commercial tomato plants (Lindhout et al. 1994a), was maintained on S-MM plants in a greenhouse compartment at 20 ± 3ºC with 70 ± 15% relative humidity. A disease test was performed by spraying two-week-old tomato plants (first PDS silenced symptoms) with a suspension of 4×10^5 conidia per ml for the first experiment and of 6×10^5 conidia per ml for the second experiment . The inoculum was prepared by washing conidial spores from freshly sporulating leaves of heavily infected S-MM plants in tap water and was used immediately (8).

Disease test:
Plants were scored every two days using a scale of three points. Plants scored 1 or more were classified as susceptible. Leaves number one and two were not scored, because of the silencing effect less effective on leaves one and two.

Microscopic slide preparation:
Leaf fragment of 1 x 3 cm in size were fixed in acetic acid- ethanol (1:3), and stained with 0.03% trypan blue in lactophenol-ethanol (1:2) as described in Huang et al study, 1998 (32) to study fungal structure and penetration. Callose deposition or papillae formation were observed with aniline blue, in 0.07 M K_2HPO_4 for UV light observation (32).

Three leaf fragments were taken on the third and fourth leaves from each randomly chosen plants as replicates. Approximately 100 infection units were scored amongst twelve different slides for each silenced genotype. Empty vector plants were also scored as a control. An infection unit was defined as a germinated spore which produced at least a first appresorium. Presence of a first haustorium, of secondary appressoria, of secondary haustoria, the number of hyphae, the presence or not of a necrotic cell at the point of penetration as well as the presence of conidia were recorded.

Total RNA isolation:
To monitor chosen transcript accumulations in experiment No1, total RNAs were extracted using the Trizol reagent (Invitrogen Life Technologies Co., Carlsbad, CA, U.S.A.) and were purified with a NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany).
In experiment No2, total RNA were extracted using MagMAX™-96 Total RNA Isolation Kit (Ambion Inc., Austin, TX).
cDNAs were synthesized with oligo(dT)18 primers using the SuperScript III RTS first-strand cDNA synthesis kit (Invitrogen) (45).

RT-PCR:
Primers for the RT-PCR were selected using primer select program of DNASTAR (v6.0; Laser Gene, Madison, WI, USA) (Table5). Real-time PCR was done using SYBR green PCR kits (Brilliant SYBR Green QPCR core reagent kit; Stratagene, La Jolla, CA,U.S.A.) on a iQ5 Multicolor real-time PCR detection system (Bio-Red, Munched, Germany). Relative transcript levels were calculated using the ΔΔct detection methods (17).

Experiment no 1

Three constructs were agroinfiltrated in *ol-2* genotype tomato plant, S-MM was used as a control. Control plants (PDS, water and empty vector) were made for *ol-2* genotype and S-MM.

F3-ol-2:
10 plants each construct (*Pen1*, *Pen3*, M12E62-620)
3 PDS plants
3 empty vector plants
3 water plants

S-MM:
5 plants each construct (3 vectors= 15 plants)
3 PDS plants
3 empty vector plants
3 water plants

Experiment no 2

Two constructs were agroinfiltrated in *ol-2* genotype tomato plants, S-MM was used as a control. Control plants (PDS, water and empty vector) were made for *F3-ol-2* and S-MM.

F3-ol-2:
35 plants *Pen1* construct
15 plants *M12E62-620* construct
5 PDS plants
35 empty vector plants
10 water plants

S-MM:
35 plants *Pen1* construct
15 plants *M12E62-620* construct
5 PDS plants
35 empty vector plants
10 water plants

Sampling was done at 4, 8, 24 and 48 hpi.

Results

- **Experiment No 1**

Sequencing:
TRV2- *M12E62-620* construct is verified, this construct should silenced chloroplast light-harvesting complex II protein Lhcbm6 gene*:* GACTGGACGGACCCATGGAGGCCTTCTAGAAATTCACTAGTGATTGAACG
CGAACTTGAGGTGATCCACTGCAGATGGGCTATGCCTGGTGCTCTTGGAT
GTGTCTCCCTGAGCTCTTGGCCCGTAATGGTGTCAAGTTTGGTGAGGCTG
TCTGGTTCAAGGCTGGATCTCAAATCTTCAGTGAGGGTGGACTTGACTAC
TTGGGCAACCCAAGCTTGGTCCATGCACAAAGCATCTTGGCCATCTAATC
GAATTCGGTAACCTTACTCACAGAATC, putative chloroplast light-harvesting complex II protein Lhcbm6. (TIGR database).

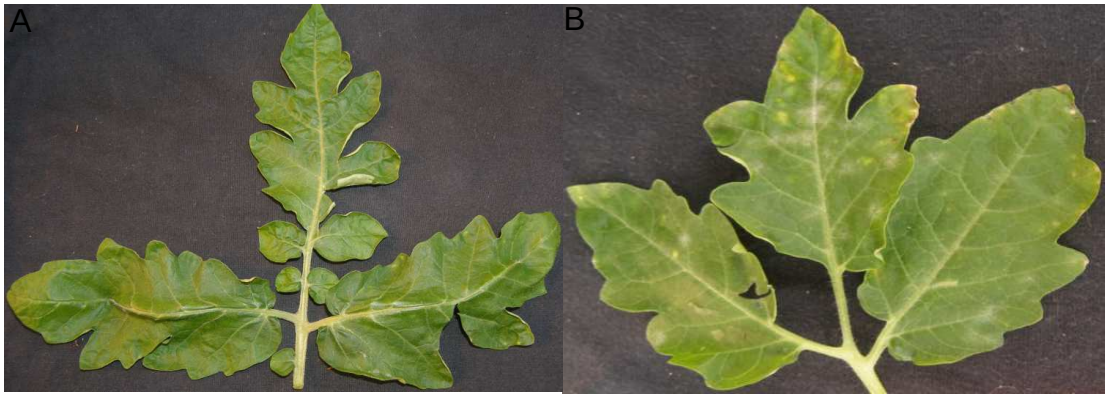
Disease test:
Seven days post inoculation, p*en1*-silenced S-MM plants showed first disease symptoms. *Pen3* and *M12E62-620* silenced S-MM showed first symptoms together with water control S-MM plants, i.e. eleven days after inoculation.
Fifteen days post inoculation, ev *ol-2*, *M12E62-620* and *Pen3* silenced *ol-2* plants were completely cleaned of any symptoms, whereas six out of ten *Pen1- ol-2*-silenced plants showed fungal colonies on leaves three and four (Table 7, Figure 2).

Table 7: Disease test on S-MM and *ol-2* silenced plants

Gene silenced	S-MM	Ol-2
Control plants[a]	++ [c]	-[b]
Pen1	+++ [c]	+[c]
Pen3	++	-
M12E62-620	++	-

[a] control plants, water and empty vector plants.
[b] -, no symptoms
[c] +, symptoms appeared at 15 dpi; ++, symptoms appeared at 11 dpi; +++, symptoms appeared at 7 dpi.

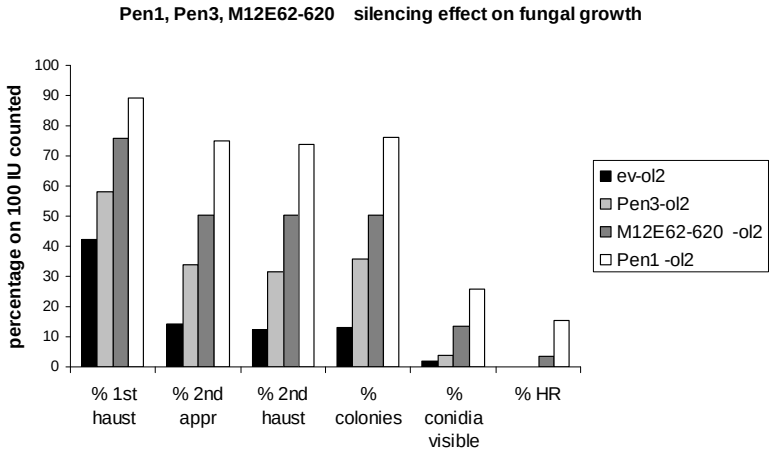
Figure 2:Leaves of **A** ev and **B** *Pen1*- silenced plant infected with powdery mildew at 15 dpi. *Pen3* and *M12E62-620* are not shown, they display no visible symptoms.



Microscopic slides observation:
- Infected ev-*ol-2*- plants were used as reference. Ev plants showed the same histology as *ol-2* genotype plants in Bai et al study, 2005 (5): no necrotic cells, but callose deposition and less than half infection units gave a first haustorium (Figure 3).

- *Pen1*-silenced plants show a high fungal penetration rate at the microscopic level (90%), *pen3*-silenced plants are only slightly more infected than ev control plants, whereas *M12E62-620* displays intermediate microscopic observations between *Pen1* and *Pen3* silenced plants (Figure 3; Table 8).

Figure 3: Fungal growth on *pen1*, *pen3*, and *M12E62-ol-2*- silenced plant 15 dpi. Ev plants are used as control for fungal development. haust: haustorium; appr: appresorium.



- **Pen1**

Nearly all germinated spores scored for *Pen1* silenced plants had formed a first haustorium, and 80% show colony formation, with the formation of long hyphae and secondary infection structures. 15% of the penetration points gives necrotic cells.

- **Pen3**

Pen3 silenced plants gave no necrotic cell. Only 10% more IU than in ev plants formed a first haustorium, but 50% more formed secondary haustoria, colonies and conidia.

- **M12E62-620**

M12E62-620 silenced plants display results intermediate between *Pen1* and *Pen3* silenced plants. Nearly 80% of the infection units scored displayed a first haustorium. Half of them formed colonies, out of which 33% are large colonies (colonies with more than ten secondary appressoria).

- **Papillae shape**

Pen1, *Pen3* and *M12E62-620* silenced plants are not distinguishable by the shape and the size of the papillae. Slightly bigger papillae are observable for *Pen1*-silenced plants, but the variation between the plants is too big to obtain any significant results about it (Figure 4, 5).

Figure 4: Haustoria, necrotic cells, conidia and papillae in the interaction of tomato and *Oidium neolycopersici*
A. conidia in *Pen1* silenced plant. x 100 **B**. secondary haustoria in *M12E62-620*-ol2 silenced plants.x 40 **C.** hypersensitive response in *Pen1* silenced plant. x 40 **D.** haustorium in *Pen3* silenced plant. x 100 **E.** appressorium in a control (ev) plant. x 100

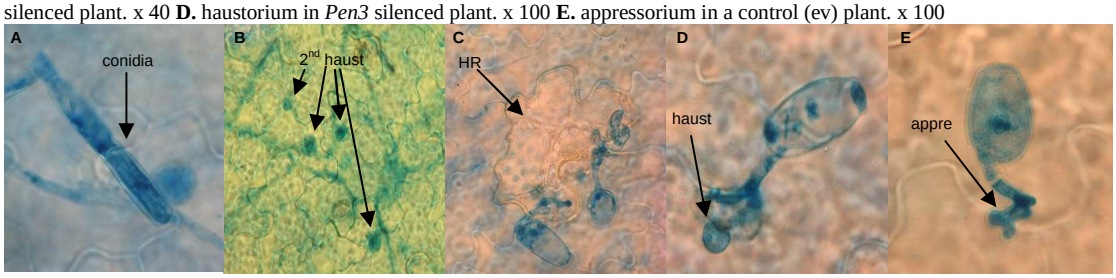
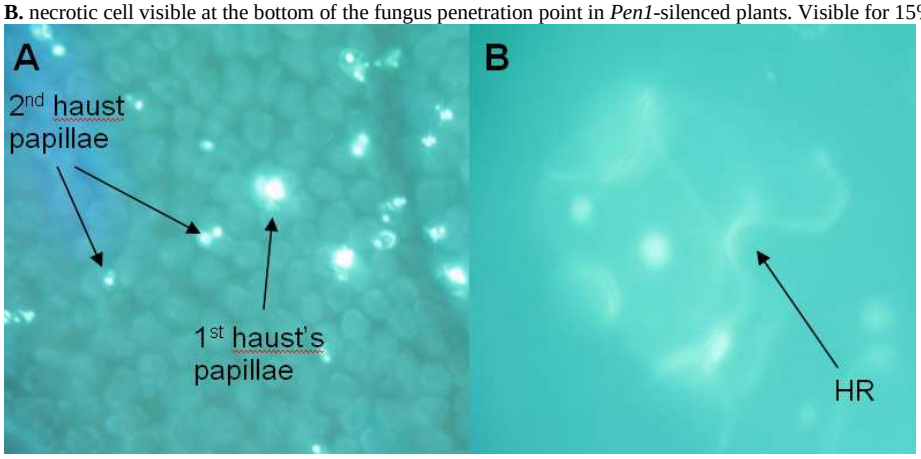


Table 8: Development of *O.neolycopersici* on silenced *ol-2* plants[a]

Genotype	Primary haustorium (%)	Secondary appressorium (%)	Secondary haustorium (%)	Colonies[b] (%)	visible conidia (%)	necrotic cells (%)	big colonies[c] (%)
Ev-ol2	42	14	12	13	2	0	7
Pen3-ol2	58	34	32	36	4	0	17
M12E62-620-ol2	76	50	50	50	14	4	35
Pen1-ol2	90	75	74	76	26	15	46

[a] values represents percent of infection units scored of 100 IUs.
[b] colonies is defined as more than one hyphae visible
[c] big colonies is defined as colonies with more than ten secondary appressoria.

Figure 5: Necrotic cells and papillae in *Pen1*-silenced tomato plant staining: aniline blue. x 40. **A**. First appressorium leads to a large papilla, secondary appressoria give smaller papillae aligned along the hyphae. *Pen3*, ev, and *M12E62-620*-ol2 silenced plants give the same results. **B.** necrotic cell visible at the bottom of the fungus penetration point in *Pen1*-silenced plants. Visible for 15% of the infection units scored.



- **Experiment No2**

Disease test

A disease index was attributed to each plants every two days, following the DI scale of three points. S-MM plants showed symptoms after 6 days of inoculation (Table 9).

Table 9: Disease test on S-MM, EV-*ol2*, *Pen1-ol2* and *M12E62-620-ol2*

	Genotype			
dpi	*S-MM*	*EV-ol2*	*Pen1-ol-2*	*M12E62-620-ol2*
6	55/55 (3) [a]	0/20	0/25	0/10
8	55/55 (3)	0/20	0/25	0/10
10	55/55 (3)	0/20	0/25	0/10
12	55/55 (3)	0/20	0/25	0/10
14	55/55 (3)	0/20	4/25 (1)	0/10
16	55/55 (3)	2/20 (1)	7/25 (1)	0/10
18	55/55 (3)	2/20 (1)	7/25 (1)	2/10 (1)

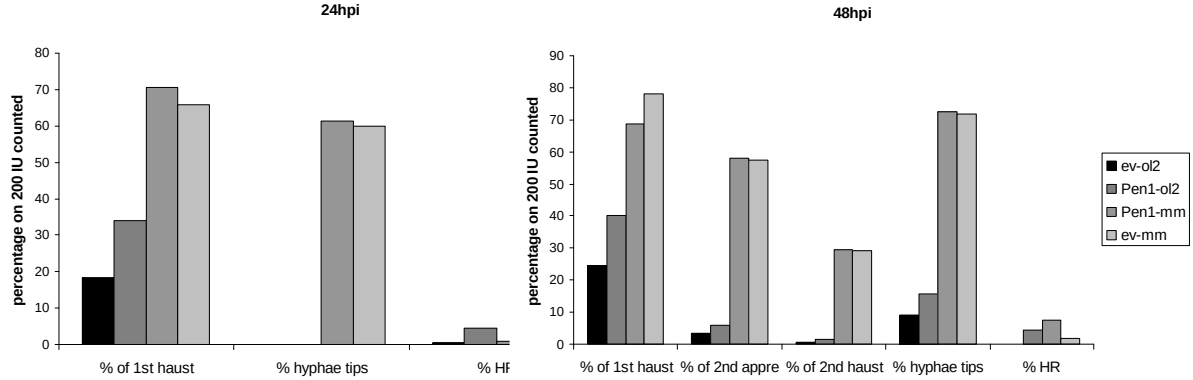
[a] (3) indicates the DI.

At 14 dpi, *Pen1-ol2* was more susceptible than its control genotype, *ev-ol2*. Eighteen dpi, *M12E62-620-ol2* also displayed a higher susceptibility than *ev-ol2*.
No differences were observed between *Pen1*-MM, *M12E62-620*-MM, and EV-MM plants. Therefore the result of those plants were put together. In the first experiment, a clear difference was observed between Pen1-MM plants and the others. The explanation can be that the amount of inoculum sprayed for the first and the second experiment was not the same, higher in the second experiment.

Microscopic slides observation:

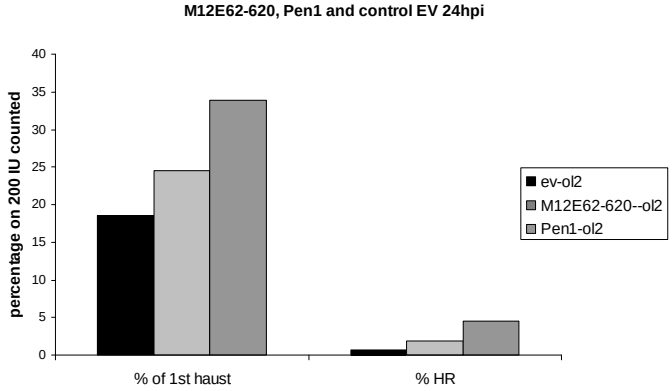
Two leaf fragments were taken from third and fourth leaves of five replicates for each time points (See appendix for the detailed layout).
Tryptan blue slides from leaves taken at 4 hpi and at 8 hpi were analyzed, but the results are not presented here. At 4hpi, no haustorium was detected on any genotype. At 8 hpi, few haustoria were seen on EV-*ol-2* and *Pen1-ol2* genotype and on S-MM.

Figure 6: Fungal growth on *pen1 -ol-2-* silenced plant, and *pen1 -S-M 24 and 48 hpi.* Ev plants are used as control for fungal development . haust: haustorium; appr: appresorium



From 24 hpi, *Pen1-ol2* plants showed a higher percentage of first haustorium at the site of attempted penetration than control ev-ol2 plants (Figure 6).

Figure 7 : Fungal growth on *pen1 -ol-2* and *M12E62-620-ol2.* Ev plants are used as control for fungal development haust: haustorium.



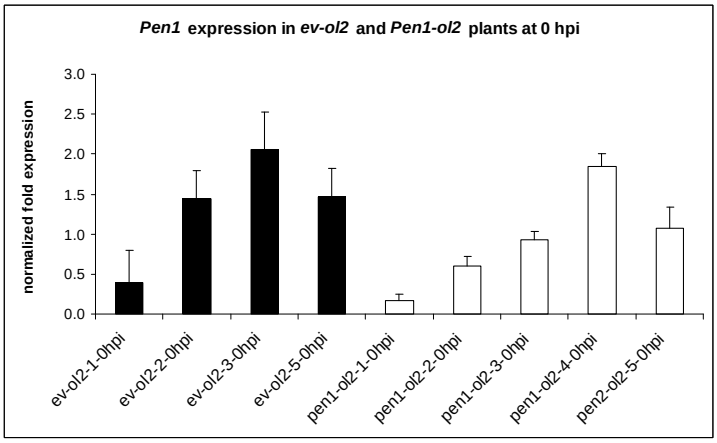
The presence of a first haustorium is higher in *M12E62-620-ol2* than in ev-*ol2* 24 hpi. No slides were made available 48 hpi. Nevertheless, similarly as shown in Figure 3, *M12E62-620-ol2* susceptibility is included between ev-*ol2* and *Pen1-ol2*. (Figure 7).

Aniline blue staining shows the presence of papillae from four hpi in *Pen1-ol2*, as well as in ev-*ol2*.

RT-PCR results:

Expression of *Pen1* gene was tested by RT-PCR in silenced plants, in control S-MM, in inoculated S-MM, and in *ev-ol2* plants. Elongation factor (EF) gene was used as the reference gene (Figure 8).

Figure 8: Normalized fold expression graphs of *Pen1* in ev-ol2 and *Pen1-ol-2* 0hpi and 4hpi.



Pen1 expression displays some variations between replicates. Replicate 4 shows a high expression of *Pen1*. This is explained by the method of silencing. VIGS does not lead to a complete silencing of the gene. Some leaves are fully silenced, for others the silencing is patched. For replicate 4 of *Pen1-ol2*, we may have taken a not completely silenced leaf. This can be illustrated by the pattern obtained with PDS silencing (Figure 9).

Figure 9: S-MM plant silenced for PDS gene. Some leaves are completely white, others are green and white, some are even completely green.

Conclusions and Discussion

In this study *Pen1*, *Pen3* and *M12E62-620* have been proven to be involved in *ol-2* mediated resistance. Plants lacking expression of *Pen1* and *M12E62-620* genes show a decrease in prepenetration resistance as well as colonization of the leaves upon infection with the tomato powdery mildew. Plants silenced for *Pen3* gene show less prepenetration resistance, and no symptoms are visible on the leaves.
Those three genes, *Pen1*, *Pen3* and *M12E62-620* play a role in the resistance of *ol-2* plants.

***Pen1* involvement in prepenetration resistance.**
Pen1 genes are syntaxins, members of the SNARE family (soluble N-ethylmaleimide sensitive factor attachment protein receptor) involved in membrane fusion events (25).

Pen1 and its homologues have been proven to play a role in prepenetration resistance: timing and composition of papillae formation, accumulation at attempted entry sites and secretion of antimicrobial compounds and cell wall components, low basal resistance in susceptible background:
- Non host resistance studies have demonstrated that PEN 1 is recruited to the papillae formation upon *Arabidopsis* infection with *Bgh*. Loss of PEN 1 results in a 2-hour delay in papillae (28). This late formation could even be responsible for the non detection of the spore by the plant, the cell wall being sealed by this late papillae (16). *Pen 1-Arabidopsis* mutant plants allowed a seven fold higher incidence of *Bgh* penetration compared with wild type plants, as well as an increase in the incidence of HR like cell death (29, 33).
In our study papillae formations do not seem affected by the silencing of *Pen1*. Papillae formed at the fungus entry sites are as big and similarly shaped as control *ol-2* plants, but *Pen1*-silenced plants showing susceptibility, the papilla, despite being formed, should display a key difference with an effective papilla, either in timing, or in composition. In our study, first papillae were observed at four hpi in *Pen1-ol2* as well as in ev-*ol2* plants, so no delay was observed, differently from *Arabidopsis* data. We argued that papillae formed in *Pen1-ol2* plants lack an essential component for resistance therefore allowing more penetration. In a research aiming at looking for *ror1* and *ror2* function in *mlo*-mediated resistance in barley, association of papillae formation with H_2O_2 accumulation at the attempted entry site has been demonstrated to be determinant of the level of resistance (35; 36). In future experiments, H_2O_2 should be monitored in *Pen1-ol2* plants
- Syntaxin PEN1 accumulates at the adapted fungus (*G. cichoracearum*) entry site in *Arabidopsis* at the same frequency than for non-adapted fungi (38, 39), contributing to the papillae formation in host and non host resistance. It is believed nowadays that PEN1 forms a SNARE complex together with SNAP 33 and VAMP721/722, this complex secreting antimicrobial compounds and cell wall components at sites of attempted fungal infection (33, 37, 31).
- It has been demonstrated that in barley and *Arabidopsis*, *Ror2* and *Pen1* respectively contribute to low-level basal penetration rate in a MLO genetic background (29). In the first experiment of this study, controlled S-MM silenced for PEN1 displayed an over susceptible phenotype. Symptoms on the leaves appeared after seven days

of inoculation compared with eleven days for the controlled S-MM. Nevertheless, no significant differences have been observed at the microscopic level, and this phenomenon has not been observed in the second experiment. That can be the fact of the higher amount of inoculum sprayed for the second experiment, masking the difference between *Pen1*-MM and EV-MM.

***Pen1* and post penetration resistance.**

Pen1 also seems to be involved in post penetration resistance: negative regulation of SA, JA, and ET, negative regulation of necrotic cell death, and upregulation in expression after 48 hours of inoculation.

- In the study of Zhang et al, 2007, it has been demonstrated that mutation in SYP121 together with mutation in SYP 122 (both close homologues to PEN1) lead to negative regulation of defence signaling pathways such as SA, JA, ET, and an increase in pathogenic induced cell death (30). In our study, the formation of necrotic cells at 15% of the attempted entry site of the powdery mildew in *Pen1*-silenced plants 15 dpi seems to confirm that PEN1 is a negative regulator of programmed cell death (few necrotic cells were found for unsilenced *ol2* plants). At 24 hpi, *Pen1-ol2* plants show 5% of necrotic cells, and at 48 hpi, 10% of HR are observable. Despite VIGS technique being a transient method of transformation, the increasing ratio of HR shows that the silencing is still active in the plants.
- In a study of Zheng et al (2008, unpublished), it was demonstrated that *mlo*-mediated resistance in *Arabidopsis* was jeopardized by the silencing of key genes in the SA pathway, using in their study the triple mutant of *Arabidopsis*, *Atmlo2/Atmlo6/Atmlo12*. Interestingly, in the study of Consonni et al (2006) the simple mutant used (*Atmlo2*) did not show any enhanced symptoms when SA genes were silenced (14), maybe because of the simple mutant genetic background used.

Non-expression of *Pen1* in our study should have increased SA production, and therefore triggered another layer of resistance. Level of SA production should be monitored to measure the impact of PEN1 in its regulation in tomato plants. Pathogenesis related proteins (PR1) expression should also be monitored, to have an idea on the role of *Pen1* in post penetration resistance in *ol-2*-mediated resistance in tomato.

- In *Arabidopsis*, *Pen1* expression show a 5-fold increase upon infection with host and non host powdery mildew 48hpi (16). It will be interesting to know the expression of *Pen1* in ev-*ol2* and *Pen1-ol2* 48 hpi. Expression of *Pen1* in infected tomato *ol-2* plants will be monitored in next experiment.

***Pen3* involvement in resistance.**

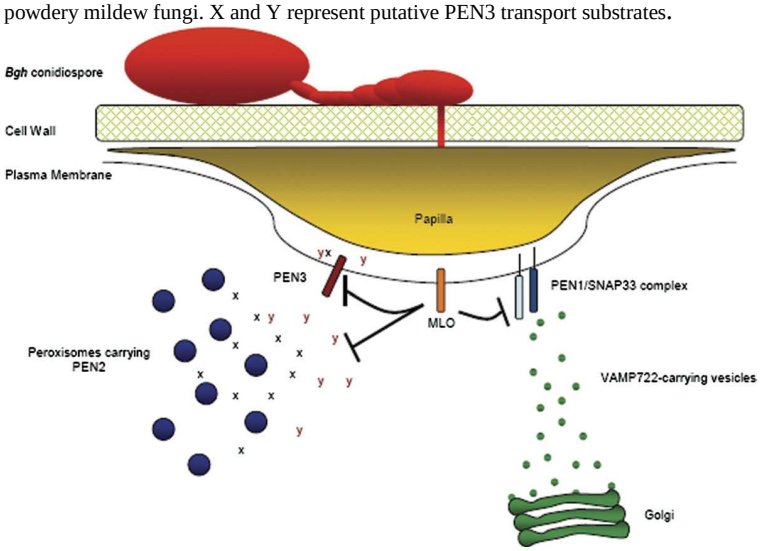
Pen3 gene in *Arabidopsis* encodes an ATP-binding-cassette (ABC transporter), which transport substrates across cellular membranes. It exported toxic compounds at attempted fungal infection sites, while accumulation of those toxins activates the SA pathway. PEN3 and PEN2 (a β-glycosyl hydrolase) proteins work in concert, but in a distinct secretory pathway than the one in which PEN1 is involved (34).

In our study, we also observed that silencing of *Pen3* increases the penetration ratio. Despite the low increase in first haustorium formation incidence, the observation of secondary haustoria is twice as much as in non silenced plants fifteen dpi. Similar observations have been made on *Pen3* mutant *Arabidopsis* challenged with *Bgh*. A striking feature is the lack of HR. Indeed, if the role of the homologue of PEN3 in tomato is similar to the *Arabidopsis* one, the cells would not be able to export toxic compound to the site of fungal penetration. They would retain the toxins, it would kill the cells. Further experiments have to be carried out on the role of PEN3 in tomato.

***Pen1/Pen2/Pen3* in host and non-host *Arabidopsis* resistance**

In *Arabidopsis*, Underwood et al (2008) summarized what is known about *mlo*-mediated resistance (Figure 10). PEN3 transporter exports PEN2 enzymatic products at the attempted fungus entry site, while PEN1/SNAP33, in a distinct pathway, mediates the fusion of Vamp722/721 golgi vesicles at the plasma membrane. Those vesicles transports components important for the papillae formation (Figure 10). MLO inhibits both defense pathways (31).

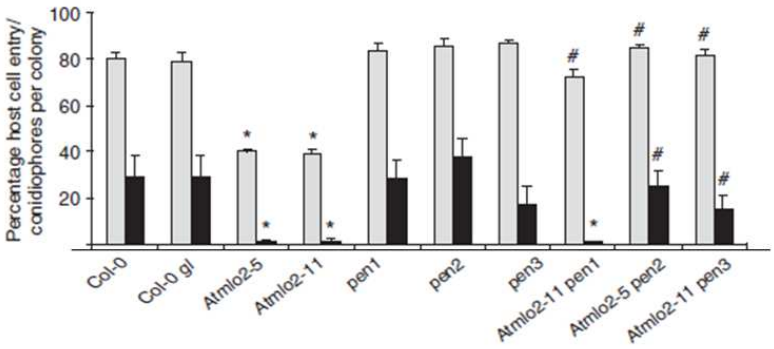
Figure 10: From Underwood and Somerville (2008). Model depicting the roles of the PEN proteins and MLO in resistance to penetration by powdery mildew fungi. X and Y represent putative PEN3 transport substrates**.**



Comparison of *Pen1 and Pen2/Pen3* resistance pathways involvement in resistance in *Arabidopsis* and tomato.

In the study of Consonni et al (2006), *Pen1*, *Pen2* and *Pen3* genes were silenced in *Arabidopsis Atmlo2* resistant mutant. Plants were tested against *Erysiphe cichoracearum*. Figure 11 shows the results they found (14).

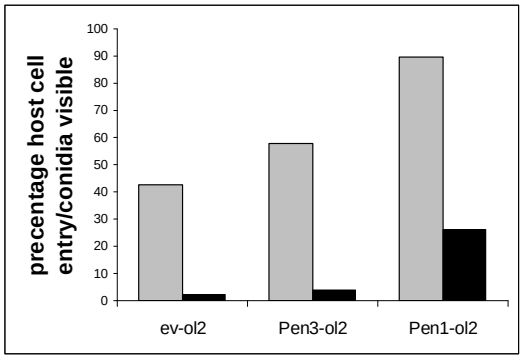
Figure 11: From Consonni et al (2006). Quantitative analysis of host cell entry (determined at 48 h (light gray bars)) and conidiophore formation (6 d post inoculation; black bars) on wild-type Col-0 and Atmlo mutant plants.



Atmlo2-11 pen1 displays an enhanced host cell entry percentage, but no increase in conidiophore per colony, whereas *Atmlo2-5 pen2* and *Atmlo2-11 pen3* show a big increase in conidiophore formation. Zheng et al (2008, unpublished) have done the same study, but they used a non host pathogen: the tomato powdery mildew *O.neolycopersici*, the results they found were identical.

In our case, the contrary was observed. *Pen3-ol2* showed no increase in conidiophores formation, whereas *Pen1-*ol2 displayed a big increase in conidiophores (Figure 12).

Figure 12: Quantitative analysis of host cell entry (determined at 16 h (light gray bars)) and conidiophore formation (16 d post inoculation; black bars) on *ol-2* plants.



Although both in tomato and *Arabidopsis*, PEN1 and PEN3 are involved in prepenetration resistance, the two pathways do not have the same impact on resistance for each plant species. In *Arabidopsis*, PEN2 and PEN3 work in concert in the same secretory pathway. To verify this statement in *ol2*-mediated resistance in tomato, *Pen2* gene has to be silenced as well.

Mlo gene is an universal gene found in several species. *Pen1* and *Pen3* are conserved requirements for *mlo*-mediated resistance in artificial or natural *Mlo*-mutants.

Chloroplast light harvesting complex II protein in *ol-2* genotype resistance.

The chloroplast light harvesting complex II gene found upregulated when tomato *ol-2* plants are challenged with powdery mildew has been described as a senescence associated/down regulated gene. Upon infection on tomato with *Xanthomonas campestris*, mRNA level of this gene is upregulated (26, 27). Role of this protein in infection process has not been demonstrated yet.

Over this study, a 1.8-fold increase in first haustorium formation in *ol2*-tomato plants silenced for this gene has been found as well as a 3.8-fold augmentation of secondary haustoria and colonies, and the presence of HR 15 dpi. Some colonies were visible on the leaves from 18 dpi. Those findings are similar to *Pen1* silencing to a great extend. *M12E62-620* therefore seems to play a role in penetration, at the first layer of defense.

The involvement of this gene in *Xanthomonas campestris*-infected tomato leaves as well as in powdery mildew-infected tomato leaves suggests a broad role for this gene.

The full length of *M12E62-620* TDF has to be cloned, in order to know with exactitude the role of this protein, as well as its belonging to any family of genes. Is this *M12E62-620* part of a large family therefore leading to a nonspecific silencing? Or silencing of *M12E62-620* specific gene only lead to this dramatic decrease in resistance?

Acknowledgments

I thank a lot Yuling Bai for giving me the opportunity to work in her lab. I thank her for her help in writing this report, and for all the discussions we had. I also thank Zheng Zheng for his help in the lab, his suggestions and discussions. I thank Nini for sharing the work with me from April and for being a very nice workmate.

References

(1) Jones H, Whipps J.M, Gurr S.J (2001) The tomato powdery mildew fungus *Oidium neolycioperisici* . *Molecular Plant Pathology* 2(6), 303-309.

(2) Whipps J.M, Budge S.P Fenlon J.S (1998) Characteristics and host range of tomato powdery mildew. *Plant Pathology* 47, 36-48.

(3) Huang C.C, Hoefs-Van de Putte P.M, Haanstra-Van Der Meer J.G, Meijer-Dekens F, Lindhout P. (2000) Characterization and mapping of resistance to *Oidium lycopersicum* in two *Lycopersicon hirsitum* accessions: evidence for close linkage of two Ol-genes on chromosome 6 of tomato. *Heredity* 85, 511-520

(4) Bai Y, van der Hulst B, Huang C.C, Wei L, Stam P, Lindhout P. (2004) Mapping Ol-4, a gene conferring resistance to *Oidium neolycopersici* and originating from *Lycopersicon peruvianum* LA2172, requires multi-allelic, single-locus markers *Theoritical and Applied Genetics* 109, 1215–1223.

(5) Bai Y, Van der Hulst R, Bonnema G, Marcel T.C, Meijer-Dekens F, Niks R.E, Lindhout P. (2005) Tomato Defense to *Oidium neolycopersici*: Dominant *Ol* Genes Confer Isolate-Dependent Resistance Via a Different Mechanism Than Recessive ol-2. *MolecularPlant Microbe Interaction* 18(4), 354–362.

(6) Ciccarese F, Amenduni M, Schiavone D, Cirulli M. (1998) Occurance and inheritance of resistance to powdery mildew (*Oidium lycopersici*) in *Lycopersicon* species. *Plant Pathology* 47, 417-419.

(7) De Giovanni C, Dell'Orco P, Bruno A, Ciccarese F, Lotti C, Ricciardi L. (2004) Identification of PCR-based markers (RAPD, AFLP) linked to a novel powdery mildew resistance gene (ol-2) in tomato. *Plant Science* 166 41–48

(8) Bai Y, Huang C.C, van der Hulst R, Meijer-Dekens F, Bonnema G, Lindhout P (2003) QTLs for Tomato Powdery Mildew Resistance (*Oidium lycopersici*) in *Lycopersicon parviflorum* G1.1601 Co-localize with Two Qualitative Powdery Mildew Resistance Genes. *Molecular Plant Microbe Interaction* 16 (2), 169–176.

(9) Li C, Bonnema G, Che D, Dong L, Lindhout P, Visser R,1 Bai Y. (2007) Biochemical and Molecular Mechanisms Involved in Monogenic Resistance Responses to Tomato Powdery Mildew. *Molecular Plant Microbe Interaction* 20(9), 1161–1172.

(10) Lu R., Montserrat Martin-hernandez A., Peart J.R, Malcuit I. Baulcombe D.C. (2003) Virus induced gene silencing in plants. *Methods* 30, 296-303.

(11) Li C, Bai Y, Jacobsen E, Visser R, Lindhout P, Bonnema G. (2006) Tomato defense to the powdery mildew fungus: differences in expression of genes in susceptible, monogenic- and polygenic resistance responses are mainly in timing. *Plant Molecular Biology* 62, 127–140.
(12) Achuo E.A, Audenaert K, Meziane H, Höfte M. (2004) The salicylic acid-dependent defense pathway is effective against different pathogens in tomato and tobacco. *Plant Pathology* 53, 65–72.
(13) Bai Y, Pavan S, Zheng Z, Zappel N.F, Reinstädler A, Lotti C, De Giovanni C, Ricciardi L, Lindhout P, Visser R, Theres K, Panstruga R (2007) Naturally Occurring Broad-Spectrum Powdery Mildew Resistance in a Central American Tomato Accession Is Caused by Loss of Mlo Function. *Molecular Plant Microbe Interactions* 21, 30-39.
(14) Consonni C, Humphry M. E, Hartmann A, , Livaja M, , Durner J, Westphal L, John Vogel J, Lipka V, Kemmerling B, Schulze-Lefert P, Somerville S.C, Panstruga R. (2006) Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. *Nature Genetics* 38, 716-720.
(15) Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant Journal* 30(4), 415-429.
(16) Assaad F.F, Qiu J.L, Youngs H, Ehrhardt D, Zimmerli L, Kalde M, Wanner G, Peck S.C, Edwards H, Ramonell K, Somerville CR, Thordal-Christensen H (2004) The *PEN1* syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. *Molecular biology of the cell* 15, 5118-5129.
(17) Pavan S, Zheng Z, Borisova M, van den Berg P, Lotti C, De Giovanni C, Lindhout P, de Jong H, Ricciardi L, Visser R.G.F, Bai Y. (2008) Map- vs. homology-based cloning for the recessive gene *ol-2* conferring resistance to tomato powdery mildew. *Euphytica.* 162, 91-98.
(18) Bai Y, Lindhout P. Review (2007). New challenges for durable resistance breeding in tomato. International Society for Horticultural Sciences.
(19) Martin G.B, Bogdanove A.J, Sessa G. (2003) Understanding the functions of plant disease resistance proteins. Annual review *Plant Biology*. 54, 23-51.
(20) Xiao S, Brown S, Patrick E, Brearley C, Turner J.G. (2003). Enhanced transcription of *Arabidopsis* disease resistance genes *RPW8.1* and *RPW8.2* via a salicylic acid-dependent amplification circuit is required for hypersensitivity cell death. *The Plant Cell*. 15, 33-45.
(21) Hein I, Campbell E.I, Woodhead M, Hedley P.E, Young V, Morris W.L, Ramsay L, Stockhaus J, Lyon G.D, Newton A.C, Birch P.R.J. (2003). Characterization of early transcriptional changes involving multiple signaling pathways in the Mla13 barley interaction with powdery mildew (*Blumera graminis f.sp.hordei*). *Planta*. 218, 803-813.
(22) Freialdenhoven A, Peterhänsel C, Kurth J, Kreuzaler F, Schulze-Lefert P. (1996) Identification of genes required for the function of non-race-specific *mlo* resistance to powdery mildew in barley. *Plant Cell.* 8(1) 5-14.
(23) Hückelhoven R, Fodor J, Trujillo M, Kogel K.H. (2000) Barley *Mla* and *Rar* mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f.sp. *hordei* are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion. *Planta*. 212 (1) 16-24.
(24) Jones J.D.G, Dangl J.L. (2006) The plant immune system. Review. *Nature* 444
(25) Gjetting T, Hagedorn P.H, Schweizer P, Thordal-Christensen H, Carver T.L.W, Lyngkjær M.F. (2006) Single-Cell Transcript Profiling of Barley Attacked by the Powdery Mildew Fungus. *MolecularPlant Microbe Interaction* 20 (3), 235-246.
(26) John I, Hackett R, Cooper W, Drake R, Farrell , Grierson D (1997) Cloning and characterization of tomato leaf senescence-related cDNAs. *Plant Molecular Biology* 33, 641–651.
(27) Jung-Gun Kim,a Kyle W. Taylor,a Andrew Hotson,b Mark Keegan,a Eric A. Schmelz,c and Mary Beth Mudgetta1 XopD SUMO (2008) Protease Affects Host Transcription, Promotes Pathogen Growth, and Delays Symptom Development in Xanthomonas-Infected Tomato Leaves. *Plant Cell* 20(7) 1915–1929.
(28) Maor R, Shirasu K (2005) The arms race continues: battle strategies between plants and fungal pathogens *Current Opinion in Microbiology* 8, 399-404.
(29) Collins N.C, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu J.L, Hu¨ ckelhoven R, Stein M, Freialdenhoven A, Somerville S.C, Schulze-Lefert P (2003) *Nature* 425, 973-976.
(30) Zhang Z, Feechan A, Pedersen C, Newman M.A, Qiu J.L, Olesen K.L, Thordal-Christensen H (2007) SNARE-protein has opposing functions in penetration resistance and defence signaling pathways. *The Plant Journal* 49, 302-312.
(31) Underwood W, Somerville S.C (2008) Focal accumulation of defenses at sites of fungal pathogen attack. *Journal of Experimental Botany* 59, 3501-3508.
(32) Huang C. C, Groot T, Meijer-Dekens F, Niks R. E, Lindhout, P (1998) The resistance to powdery mildew (*Oidium lycopersicum*) in Lycopersicon species is mainly associated with hypersensitive response *European Journal of Plant Pathology* 104, 399-407.

(33) Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and Post-invasion Defenses Both Contribute to Nonhost Resistance in *Arabidopsis Science* 310 (5751) 1180 -1183.
(34) Stein M, Dittgen J, Sánchez-Rodríguez C, Houa B.H, Molina A, Schulze-Lefert P, Lipka V,Somerville S (2006) *Arabidopsis PEN3*/PDR8, an ATP binding cassette transporter, contributes to non host resistance to inappropriate pathogens that enter by direct penetration. *Plant Cell* 18, 731–746.
(35) Li A.L, Wang M.L, Zhou R.H, Kong X.Y, Huo N.X, Wang W.S, Jia J.Z. (2005)
Comparative analysis of early H2O2 accumulation in compatible and incompatible wheat-powdery mildew interactions. *Plant Physiology* 54 (3) 308-316.
(36) Hückelhoven R, Trujillo M, Kogel K.H (2001) Mutations in Ror1 and Ror2 genes cause modification of hydrogen peroxide accumulation in mlo-barley under attack from the powdery mildew fungus *Molecular Plant Pathology* 1, 287-292.
(37) Kwon C, Neu C, Pajonk S, Yun H.S, Lipka U, Humphry M, Bau S, Straus M, Kwaaitaal M, Rampelt H, El Kasmi F, Jurgens G, Parker J, Panstruga R, Lipka V, Schulze-Lefert P (2008) Co-option of a default secretory pathway for plant immune responses *Nature* 451, 835-840.
(38) Bhat R.A, Miklis M, Schmelzer E, Schulze-Lefert P, Panstruga R (2005) Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. *Proceeding of the National Academy of Sciences*, USA, 102, 3135–3140.
(39) Meyer D, Pajonk S, Micali C, O'Connell R, Schulze-Lefert P (2009) Extracellular transport and integration of plant secretory proteins into pathogen-induced cell wall compartments. *The Plant Journal* 57, 986–999.
(40) Buschges R, Hollricher K, Panstruga R, Simon, G, Wolter M, Frijters A, van Daelen R, van der Lee T, Diergaarde P, Groenendijk J, Topsch S, Vos P, Salamini F, Schulze-Lefert P (1997) The barley *Mlo*
gene: a novel control element of plant pathogen resistance. *Cell* 88, 695–705.
(41) Piffanelli P, Ramsay L, Waugh R, Benabdelmouna A, D'Hont A, Hollricher K, Jørgensen J.H, Schulze-Lefert P, Panstruga R (2004) A barley cultivation-associated polymorphism conveys resistance to powdery mildew *Letter to Nature, Nature* 430, 887-891.
(42) Devoto A, Hartmann H A, Piffanelli P, Elliott C, Simmons C, Taramino G, Goh C. S, Cohen F. E, Emerson B. C, Schulze-Lefert P, Panstruga R (2003) Molecular phylogeny and evolution of the plant-specific seven-transmembrane MLO family. *Journal of Molecular Evolution* 56, 77-88.
(43) Panstruga R. (2005) Discovery of novel conserved peptide domains by ortholog comparison within plant multi-protein families. *Plant Molecular Biology* 59, 485-500.
(44) Briggs G. C, Osmont K. S, Shindo C, Sibout R, Hardtke C. S. (2006) Unequal genetic redundancies in *Arabidopsis*—A neglected phenomenon? *Trends in Plant Sciences* 11,492-498.
(45) Bai Y, Pavan S, Zheng Z, Zappel N.F, Reinstädler A, Lotti C, De Giovanni C, Ricciardi L, Lindhout P, Visser R, Theres K, Panstruga R (2008) Naturally Occurring Broad-Spectrum Powdery Mildew Resistance in a Central American Tomato Accession Is Caused by Loss of Mlo Function. *Molecular Plant Microbe Interactions* 21(1), 30-39.

Appendix

Complement on constructs protocol:

- Fragments of the chosen TDFs (table 5) were amplified by PCR on cDNA of each genotypes which will be used for the silencing, to avoid non silencing because of small sequence differences between two genotypes.
- 5 µL of the amplified cDNA were ligated into pGem plasmid with 1 µL of T4 ligase and 2 µL of 5 x enzyme buffer. The reaction was incubated overnight at 4 ºC.
- The next day, 50 µL of competent E.coli cells DHα5 were added to 5 µL of the ligation solution, for the transformation process to take place by mean of a heat shock. This above solution was put on ice for 30 min, then for 30 sec at 42ºC, and 2 min on ice. 950 µL of modified LB medium was added to the reaction and the bacteria were incubated for one hour at 37ºC, 225 rpm. 50 µL and 100 µL of solution were plated on 100 mg/µL Ampicilin and 100 mg/µL X-gal agarose plates and incubated overnight at 37ºC.
- White transformants were selected against the blue ones, taken up with a toothpick and checked by PCR. (94ºC 5 min, 94ºC 30 sec, 56ºC 30 sec, 72ºC 1 min 34 times 72ºC 7 min) Primers were designed on the plasmid to avoid picking up colonies having taken up linear constructs. Positive bacterial clones were grown on liquid LB medium with 100 mg/µL ampicilin at 37ºC overnight.
- Pgem plasmid containing the insert were extracted with a Qiagen kit, following the manufacturer instruction with 1.5 mL of E.coli culture. Concentration of plamids was measured wih nanodrop.
- 1 µg/ µL of the above recombinant plasmid was used for the digestion with EcoRI. The reaction was done in a total volume of 50 µL, and let digesting 3 hours at 37ºC.
- An electophoresis was run with the 50 µL of the above solution in a 0.8% agarose TBE gel.
- The band containing the insert was isolated from the gel with a Zymoclean gel DNA recovery kit, following the manufacturer protocol.
- Then the purified DNA containing the insert and EcoRI ends was ligated into TRV2 plasmid following the rapid ligation kit protocol.
- Competent E.coli cells were transformed as described in step 3, plated on 100 mg/µL kanamycin Petri dishes, and incubated overnight at 37ºC.
- Visible individual colonies were selected and checked by PCR (primers designed on the multiple cloning site of TRV2).
- TRV2 plasmid containing the insert were purified from the bacteria, and transformed into Agrobacterium culture by electroporation.

Microscopic slide data experiment1 :

slide no	No IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	Sporulation	HR
TDF ol2-6-4th 1	9	5	2	2	2	0	0
TDF ol2-6-4th 2	11	8	4	3	4	2	0
TDF ol2-5-4th 13	11	11	5	5	6	0	0
TDF ol2-5-4th 14	11	8	5	5	5	0	0
TDF ol2-5-4th 15	11	9	8	8	8	0	0
TDF ol2-6-3rd 17	12	8	8	8	8	1	0
TDF ol2-6-3rd 18	14	11	7	7	4	3	0
TDF ol2-6-3rd 19	7	6	5	5	5	2	0
TDF ol-2-6-4th 2	8	3	1	1	1	0	0
TDF ol2-5-3rd 8	17	15	12	12	12	7	4
TDF results	**111 IU**						
%		**76**	**50**	**50**	**50**	**14**	**4**
Pen3-ol2- 3rd 4	4	1	1	1	1	0	0
Pen3-ol2- 3rd 5	13	6	5	4	6	1	0
Pen3-ol2- 3rd 6	9	7	4	4	5	1	0
Pen3 ol-2-1-4th 7	19	10	6	5	6	0	0
Pen3 ol-2-1-4th 8	10	5	2	2	2	0	0
Pen3-ol2-4th 10	10	8	5	5	6	1	0
Pen3 ol2-7-4th 11	10	7	3	3	3	0	0
Pen3 ol2-7-3rd 17	10	6	3	3	3	1	0
Pen3 ol2-7-3rd 16	6	3	2	2	2	0	0
Pen3 ol2-7-3rd 15	4	2	1	1	1	0	0
Pen3 results	**95 IU**						
%		**58**	**34**	**32**	**36**	**4**	
ev -ol2-3rd 11	18	9	4	4	5	1	0
ev-ol2-4th 13	23	8	1	1	1	0	0
ev -ol2-4th 14	11	2	1	0	1	0	0
ev ol2-2-4th 1	19	10	2	2	2	0	0
ev-ol2- 3rd 1	13	5	2	2	2	0	0
ev-ol2-2-3rd 2	16	8	4	3	3	0	0

slide no	No IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	Sporulation	HR
ev results	**99 IU**						
%		**42**	**14**	**12**	**13**	**2**	
Pen1-ol2-2- 4th 3	7	7	6	6	6	1	1
Pen1-ol2-1- 4th 4	11	8	8	8	8	6	1
Pen1-ol2-2-4th 5	12	12	6	6	6	3	0
Pen1-ol2-2-4th 6	1	1	1	1	1	0	0
Pen-1-ol2-4th 20	17	15	14	14	13	3	4
Pen1-ol2-1-3rd 2	10	10	8	8	8	3	0
Pen1-ol2-1-3rd 3	17	16	12	12	12	3	2
Pen1-ol2-2-3rd 18	10	7	7	6	6	4	3
Pen1-ol2-1-4th 12	13	11	10	10	10	4	3
Pen1 ol2-2-3rd 20	6	6	5	5	5	1	2
Pen1 results	**104 IU**						
	%	**89**	**75**	**74**	**76**	**26**	**15**

Layout experiment 2:

PEN1-ol2-1	PEN1-ol2-2	PEN1-ol2-3	PEN1-ol2-4	PEN1-ol2-5
PEN1-ol2-6	PEN1-ol2-7	PEN1-ol2-8	PEN1-ol2-9	PEN1-ol2-10
PEN1-ol2-11	PEN1-ol2-12	PEN1-ol2-13	PEN1-ol2-14	PEN1-ol2-15
PEN1-ol2-16	PEN1-ol2-17	PEN1-ol2-18	PEN1-ol2-19	PEN1-ol2-20
PEN1-ol2-21	PEN1-ol2-22	PEN1-ol2-23	PEN1-ol2-24	PEN1-ol2-25
PEN1-ol2-26	PEN1-ol2-27	PEN1-ol2-28	PEN1-ol2-29	PEN1-ol2-30
PEN1-ol2-31	PEN1-ol2-32	PEN1-ol2-33	PEN1-ol2-34	PEN1-ol2-35
TDF-ol2-1	TDF-ol2-2	TDF-ol2-3	TDF-ol2-4	TDF-ol2-5
TDF-ol2-16	TDF-ol2-17	TDF-ol2-18	TDF-ol2-19	TDF-ol2-20
TDF-ol2-26	TDF-ol2-27	TDF-ol2-28	TDF-ol2-29	TDF-ol2-30
PDS-ol2-1	PDS-ol2-2	PDS-ol2-3	PDS-ol2-4	PDS-ol2-5
EV-MM-1	EV-MM-2	EV-MM-3	EV-MM-4	EV-MM-5
EV-MM-6	EV-MM-7	EV-MM-8	EV-MM-9	EV-MM-10
EV-MM-11	EV-MM-12	EV-MM-13	EV-MM-14	EV-MM-15
EV-MM-16	EV-MM-17	EV-MM-18	EV-MM-19	EV-MM-20
EV-MM-21	EV-MM-22	EV-MM-23	EV-MM-24	EV-MM-25
EV-MM-26	EV-MM-27	EV-MM-28	EV-MM-29	EV-MM-30
EV-MM-31	EV-MM-32	EV-MM-33	EV-MM-34	EV-MM-35
MQ-MM-1	MQ-MM-2	MQ-MM-3	MQ-MM-4	MQ-MM-5
MQ-MM-6	MQ-MM-7	MQ-MM-8	MQ-MM-9	MQ-MM-10

PEN1-MM-1	PEN1-MM-2	PEN1-MM-3	PEN1-MM-4	PEN1-MM-5
PEN1-MM-6	PEN1-MM-7	PEN1-MM-8	PEN1-MM-9	PEN1-MM-10
PEN1-MM-11	PEN1-MM-12	PEN1-MM-13	PEN1-MM-14	PEN1-MM-15
PEN1-MM-16	PEN1-MM-17	PEN1-MM-18	PEN1-MM-19	PEN1-MM-20
PEN1-MM-21	PEN1-MM-22	PEN1-MM-23	PEN1-MM-24	PEN1-MM-25
PEN1-MM-26	PEN1-MM-27	PEN1-MM-28	PEN1-MM-29	PEN1-MM-30
PEN1-MM-31	PEN1-MM-32	PEN1-MM-33	PEN1-MM-34	PEN1-MM-35
TDF-MM-1	TDF-MM-2	TDF-MM-3	TDF-MM-4	TDF-MM-5
TDF-MM-16	TDF-MM-17	TDF-MM-18	TDF-MM-19	TDF-MM-20
TDF-MM-26	TDF-MM-27	TDF-MM-28	TDF-MM-29	TDF-MM-30
PDS-MM-1	PDS-MM-2	PDS-MM-3	PDS-MM-4	PDS-MM-5
EV-ol2-1	EV-ol2-2	EV-ol2-3	EV-ol2-4	EV-ol2-5
EV-ol2-6	EV-ol2-7	EV-ol2-8	EV-ol2-9	EV-ol2-10
EV-ol2-11	EV-ol2-12	EV-ol2-13	EV-ol2-14	EV-ol2-15
EV-ol2-16	EV-ol2-17	EV-ol2-18	EV-ol2-19	EV-ol2-20
EV-ol2-21	EV-ol2-22	EV-ol2-23	EV-ol2-24	EV-ol2-25
EV-ol2-26	EV-ol2-27	EV-ol2-28	EV-ol2-29	EV-ol2-30
EV-ol2-31	EV-ol2-32	EV-ol2-33	EV-ol2-34	EV-ol2-35
MQ-ol2-1	MQ-ol2-2	MQ-ol2-3	MQ-ol2-4	MQ-ol2-5
MQ-ol2-6	MQ-ol2-7	MQ-ol2-8	MQ-ol2-9	MQ-ol2-10

Number of plants
1-5 tRNA isolation 0 hpi-------check the silencing effect
6-10 tRNA + microscope sample 4hpi---------start germinating
11-15 tRNA+ microscope sample 8hpi papilla formation
16-20 tRNA+ microscope sample 24hpi----haustoria formation
21-25 tRNA+ microscope sample 48 hpi
26-30 tRNA+ microscope sample 21dpi
31-35 phenotyping

third and fourth leaves will be used (depend on the plants 5th could be also consider good)

sowing thursday 12-3-2009
LB 22-3-2009
YEB 23-3-2009
transplanting Monday 23-3-2009
agroinfitration 24-3-2009
inoculation Tuesday 14-4-2009 at 08:00

Macroscopic and microscopic data experiment 2:

	macroscopic data							**microscopic data**					
	6 dpi	**8 dpi**	**10 dpi**	**12 dpi**	**14 dpi**	**16 dpi**	**18 dpi**	**no IU**	**no of 1st haust**	**no of 2nd appre**	**no of 2nd haust**	**no hyphae tips**	**HR**
	DI	**DI**	**DI**	**DI**	**DI**	**DI**	**DI**						
PEN1-ol2-11	0	0	0	0	0	0.5	0.5	-	-	-	-	-	-
PEN1-ol2-12	0	0	0	0	0	0.5	0.5	13	0	-	-	-	-
PEN1-ol2-13	0	0	0	0	0.5	1	1	17	0	-	-	-	-
PEN1-ol2-14	0	0	0	0	1	1	1	6	0	-	-	-	-
PEN1-ol2-15	0	0	0	0	0.5	0.5	0.5	3	0	-	-	-	-
PEN1-ol2-16	0	0	0	0.5	1	1	0.5	21	5	-	-	-	-
PEN1-ol2-17	0	0	0	0	0	0	0	39	13	-	-	-	7
PEN1-ol2-18	0	0	0	0	0.5	1	1	39	15	-	-	-	5
PEN1-ol2-19	0	0	0	0	0.5	0.5	0	15	5	-	-	-	7
PEN1-ol2-20	0	0	0	0	0	0	0	-	-	-	-	-	-
PEN1-ol2-21	0	0	0	0	0	0.5	0.5	25	12	0	0	3	1
PEN1-ol2-22	0	0	0	0	0	0.5	0.5	54	25	6	2	13	5
PEN1-ol2-23	0	0	0	0	0	0.5	0.5	10	3	0	0	0	0
PEN1-ol2-24	0	0	0	0	0.5	0.5	0.5	9	6	1	0	1	1
PEN1-ol2-25	0	0	0	0	0	0	0	21	4	1	0	3	0

	macroscopic data							microscopic data					
	6 dpi	8 dpi	10 dpi	12 dpi	14 dpi	16 dpi	18 dpi	no IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	HR
	DI	DI	DI	DI	DI	DI	DI						
PEN1-ol2-26	0	0	0	0	0.5	0.5	0.5	-	-	-	-	-	-
PEN1-ol2-27	0	0	0	0.5	1	1	1	-	-	-	-	-	-
PEN1-ol2-28	0	0	0	0	0	0	0	-	-	-	-	-	-
PEN1-ol2-29	0	0	0	0	0	0.5	0.5	-	-	-	-	-	-
PEN1-ol2-30	0	0	0	0	0	0.5	0.5	-	-	-	-	-	-
PEN1-ol2-31	0	0	0	0	0	0	0.5	-	-	-	-	-	-
PEN1-ol2-32	0	0	0	0	0.5	0.5	0.5	-	-	-	-	-	-
PEN1-ol2-33	0	0	0	0	0.5	1	0.5	-	-	-	-	-	-
PEN1-ol2-34	0	0	0	0	0	0	0	-	-	-	-	-	-
PEN1-ol2-35	0	0	0	0	1	1	0.5	-	-	-	-	-	-
TDF-ol2-16	0	0	0	0	0	0.5	0.5	27	9	-	-	-	-
TDF-ol2-17	0	0	0	0	0	0	0	28	7	-	-	-	-
TDF-ol2-18	0	0	0	0	0	0.5	0.5	23	6	-	-	-	-
TDF-ol2-19	0	0	0	0	0	0	0	18	5	-	-	-	-
TDF-ol2-20	0	0	0	0	0.5	0.5	0	12	2	-	-	-	-
TDF-ol2-26	0	0	0	0	0	0.5	0.5	-	-	-	-	-	-

	macroscopic data							microscopic data					
	6 dpi	8 dpi	10 dpi	12 dpi	14 dpi	16 dpi	18 dpi	no IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	HR
	DI	DI	DI	DI	DI	DI	DI						
TDF-ol2-27	0	0	0	0	0.5	0	0	-	-	-	-	-	-
TDF-ol2-28	0	0	0	0	0.5	1	1	-	-	-	-	-	-
TDF-ol2-29	0	0	0	0	0.5	1	1	-	-	-	-	-	-
TDF-ol2-30	0	0	0	0	0.5	0.5	0.5	-	-	-	-	-	-
EV-MM-11	-	-	-	-	-	-	-	8	3	-	-	-	-
EV-MM-12	-	-	-	-	-	-	-	-	-	-	-	-	-
EV-MM-13	-	-	-	-	-	-	-	18	2	-	-	-	-
EV-MM-14	-	-	-	-	-	-	-	12	1	-	-	-	-
EV-MM-15	-	-	-	-	-	-	-	10	1	-	-	-	-
EV-MM-16	0	2	2	3	3	3	3	23	17	-	-	15	-
EV-MM-17	1	2	3	3	3	3	3	12	7	-	-	6	-
EV-MM-18	1	2	2	3	3	3	3	30	18	-	-	16	-
EV-MM-19	1	2	3	3	3	3	3	21	15	-	-	14	-
EV-MM-20	1	2	2	3	3	3	3	16	8	-	-	10	-
EV-MM-21	1	2	2	3	3	3	3	12	11	8	3	9	-
EV-MM-22	1	3	3	3	3	3	3	31	25	23	13	7	-

	macroscopic data							microscopic data					
	6 dpi	8 dpi	10 dpi	12 dpi	14 dpi	16 dpi	18 dpi	no IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	HR
	DI	DI	DI	DI	DI	DI	DI						
EV-MM-23	1	2	2	3	3	3	3	18	15	13	8	11	1
EV-MM-24	1	2	3	3	3	3	3	32	25	11	5	20	1
EV-MM-25	1	3	2	3	3	3	3	17	11	10	4	10	-
EV-MM-26	1	2	2	3	3	3	3	-	-	-	-	-	-
EV-MM-27	1	2	3	3	3	3	3	-	-	-	-	-	-
EV-MM-28	1	2	3	3	3	3	3	-	-	-	-	-	-
EV-MM-29	1	2	3	3	3	3	3	-	-	-	-	-	-
EV-MM-30	1	3	3	3	3	3	3	-	-	-	-	-	-
EV-MM-31	1	2	2	3	3	3	3	-	-	-	-	-	-
EV-MM-32	1	3	3	3	3	3	3	-	-	-	-	-	-
EV-MM-33	1	2	2	3	3	3	3	-	-	-	-	-	-
EV-MM-34	1	3	3	3	3	3	3	-	-	-	-	-	-
EV-MM-35	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-11	1	2	2	3	3	3	3	-	-	-	-	-	-
PEN1-MM-12	1	2	2	3	3	3	3	-	-	-	-	-	-
PEN1-MM-13	1	2	3	3	3	3	3	-	-	-	-	-	-

	macroscopic data							microscopic data					
	6 dpi	8 dpi	10 dpi	12 dpi	14 dpi	16 dpi	18 dpi	no IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	HR
	DI	DI	DI	DI	DI	DI	DI						
PEN1-MM-14	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-15	1	3	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-16	1	2	3	3	3	3	3	40	30	-	-	28	-
PEN1-MM-17	1	2	3	3	3	3	3	10	8	-	-	9	-
PEN1-MM-18	1	2	3	3	3	3	3	16	9	-	-	5	-
PEN1-MM-19	1	2	3	3	3	3	3	33	22	-	-	19	-
PEN1-MM-20	1	2	3	3	3	3	3	20	15	-	-	11	1
PEN1-MM-21	1	2	3	3	3	3	3	40	25	24	6	30	5
PEN1-MM-22	1	2	3	3	3	3	3	15	14	10	8	13	
PEN1-MM-23	1	3	2	3	3	3	3	50	33	27	17	33	3
PEN1-MM-24	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-25	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-26	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-27	1	2	2	3	3	3	3	-	-	-	-	-	-
PEN1-MM-28	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-29	1	2	3	3	3	3	3	-	-	-	-	-	-

	macroscopic data							microscopic data					
	6 dpi	8 dpi	10 dpi	12 dpi	14 dpi	16 dpi	18 dpi	no IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	HR
	DI	DI	DI	DI	DI	DI	DI						
PEN1-MM-30	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-31	1	1	2	3	3	3	3	-	-	-	-	-	-
PEN1-MM-32	1	1	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-33	1	2	2	3	3	3	3	-	-	-	-	-	-
PEN1-MM-34	1	2	3	3	3	3	3	-	-	-	-	-	-
PEN1-MM-35	1	2	3	3	3	3	3	-	-	-	-	-	-
TDF-MM-16	1	2	3	3	3	3	3	-	-	-	-	-	-
TDF-MM-17	1	2	3	3	3	3	3	14	8	-	-	34	-
TDF-MM-18	1	2	3	3	3	3	3	30	20	-	-	18	-
TDF-MM-19	1	2	3	3	3	3	3	49	36	-	-	34	-
TDF-MM-20	1	2	3	3	3	3	3	-	-	-	-	-	-
TDF-MM-26	1	2	3	3	3	3	3	-	-	-	-	-	-
TDF-MM-27	1	2	3	3	3	3	3	-	-	-	-	-	-
TDF-MM-28	1	2	3	3	3	3	3	-	-	-	-	-	-
TDF-MM-29	1	2	3	3	3	3	3	-	-	-	-	-	-
TDF-MM-30	1	2	3	3	3	3	3	-	-	-	-	-	-

	macroscopic data							microscopic data					
	6 dpi	8 dpi	10 dpi	12 dpi	14 dpi	16 dpi	18 dpi	no IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	HR
	DI	DI	DI	DI	DI	DI	DI						
EV-ol2-11	-	-	-	-	-	-	-	8	0	-	-	-	-
EV-ol2-12	-	-	-	-	-	-	-	-	-	-	-	-	-
EV-ol2-13	-	-	-	-	-	-	-	11	0	-	-	-	-
EV-ol2-14	-	-	-	-	-	-	-	11	0	-	-	-	-
EV-ol2-15	-	-	-	-	-	-	-	20	0	-	-	-	-
EV-ol2-16	0	0	0	0	0	0	0	14	4	-	-	-	-
EV-ol2-17	0	0	0	0	0	0.5	0.5	46	10	-	-	-	-
EV-ol2-18	0	0	0	0	0.5	1	0	20	3	-	-	-	-
EV-ol2-19	0	0	0	0	0.5	0.5	0.5	43	10	-	-	-	1
EV-ol2-20	0	0	0	0	0.5	0.5	0.5	39	4	-	-	-	-
EV-ol2-21	0	0	0	0	0	0	0	12	6	-	-	-	-
EV-ol2-22	0	0	0	0	0	0.5	0	47	12	-	-	6	-
EV-ol2-23	0	0	0	0	0	0	0	34	9	1	-	4	2
EV-ol2-24	0	0	0	0	0.5	0.5	0.5	38	4	1	1	4	-
EV-ol2-25	0	0	0	0	0	0.5	0.5	42	9	1	-	2	-
EV-ol2-26	0	0	0	0	0	0	0	-	-	-	-	-	-

	macroscopic data							microscopic data					
	6 dpi	8 dpi	10 dpi	12 dpi	14 dpi	16 dpi	18 dpi	no IU	no of 1st haust	no of 2nd appre	no of 2nd haust	no hyphae tips	HR
	DI	DI	DI	DI	DI	DI	DI						
EV-ol2-27	0	0	0	0	0	0.5	0	-	-	-	-	-	-
EV-ol2-28	0	0	0	0	0	0	0	-	-	-	-	-	-
EV-ol2-29	0	0	0	0	0	0	0	-	-	-	-	-	-
EV-ol2-30	0	0	0	0	0	1	1	-	-	-	-	-	-
EV-ol2-31	0	0	0	0	0	0	0	-	-	-	-	-	-
EV-ol2-32	0	0	0	0	0	0.5	0.5	-	-	-	-	-	-
EV-ol2-33	0	0	0	0	0	0	0	-	-	-	-	-	-
EV-ol2-34	0	0	0	0	0	0	0	-	-	-	-	-	-
EV-ol2-35	0	0	0	0	0	0	0	-	-	-	-	-	-