

Full Length Research Paper

## Involvement of a hypersensitive-like reaction in tolerance to fire blight in pear (*Pyrus communis* L.)

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**Infection by *Erwinia amylovora*, the causal agent of fire blight (FB) disease, induces in apple and pear host plants, the generation of reactive oxygen species (ROS). We analyzed at molecular level, the link between ROS production and susceptibility to bacterial infection. Gene expression time course showed that expression of chloroplast, mitochondrial and nuclear genes, whose transcription is redox-dependent, was down regulated or suppressed in tolerant cultivar, Harrow Sweet, in comparison with susceptible cultivar, Williams. Monitoring of oxidative burst by localization of hydrogen peroxide showed that oxidative burst was triggered faster in tolerant cultivar in response to infection. These results suggest that transcription of some redox-dependent genes of cytoplasmic organelles and nucleus, in the two cultivars, is regulated faster in the tolerant cultivar than in the susceptible one.**

**Key words:** Chloroplast, electron transport Chain, *Erwinia amylovora*, mitochondria, *Pyrus communis*, redox.

### INTRODUCTION

*Erwinia amylovora* in both compatible and incompatible interactions secretes three types of effector proteins: HrpN, HrpN/W and DspA/E (Bogdanove et al., 1998; Kim and Beer, 1998; Wei et al., 1992). HrpN inhibits electron transport chain (ETC) of mitochondria and triggers an

oxidative stress in its incompatible interaction with tobacco cells (Xie and Chen, 2000). Venisse et al. (2001, 2002) also demonstrated the role of oxidative stress in compatible interactions of *E. amylovora* with susceptible hosts. Moreover, plant cells have a second ETC in their

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**Abbreviations:** DAB, 3,3'-Diaminobenzidine; DEPC, diethyl pyrocarbonate; ETC, electron transport chain; FB, fire blight; Fd, ferredoxin; DspA/E, disease specific type III effector protein; GSSG, glutathione disulfide; Hrp, harpin protein N and W; PCD, programmed cell death; PEP, plastid encoded RNA polymerase-PEP enzyme; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; PTK, plastid transcription kinase; ROS, reactive oxygen species; SLFs, sigma-like transcription factors.

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chloroplasts that in many aspects are similar to the ETC of mitochondria. There is biochemical evidence indicating the role of chloroplast ETC in oxidative burst of plant-pathogen interactions (Abdollahi and Ghahremani, 2011), and likewise Samuilov et al. (2002) proved that mitochondria and chloroplast are required in programmed cell death (PCD) of guard cells in pea leaves. They showed that illumination stimulated the CN-induced destruction of guard cells that contain both mitochondria and chloroplasts, but not of epidermal cells that contain mitochondria only.

Transcription in of cytoplasmic organelle genomes is dominantly under control of oxidative-reductive (redox) state. The role of redox state of chloroplasts on transcription of chloroplast and nuclear photosynthetic genes are well documented (Danon, 2002; Oswald et al., 2001; Pfannschmidt et al., 2001; Surpin et al., 2002). Trebitsh et al. (2000) showed a role for chloroplast thioredoxin in redox signaling and light-regulated translation of the *psbA* gene through disulfide bridge of RNA-binding complex and binding to 5' region of *psbA* mRNA. Recent studies have identified two distinct RNA polymerases in chloroplasts; one out of two is an eubacteria-like multi-subunit type, with core components encoded by chloroplast genes (plastid encoded RNA polymerase-PEP enzyme) (Maliga, 1998). The major PEP core is surrounded by sigma-like transcription factors (SLFs). SLFs properties can be reversibly altered by phospho/dephosphorylation of a kinase (Baginsky et al., 1999), named plastid transcription kinase (PTK) (Baginsky et al., 1997). PTK phosphorylation of SLFs that is under control of redox state of chloroplasts leads to decrease transcription of chloroplast genes (Baginsky et al., 1999). In addition, there is evidence indicating that redox controls the transcription rate in the genome of mitochondria through activity of DNA-topoisomerase I (Konstantinov et al., 2001). Konstantinov and Tarasenko (1999) showed that redox poise of mitochondria controls the activity of DNA-topoisomerase I via the reduction/oxidation of a critical disulfide bridge. Venisse et al. (2001, 2002) demonstrated that *E. amylovora* invades host tissues by production of reactive oxygen species (ROS). Oxidative stress leads to a more oxidizing cell redox potential and consequently switches on or turns off some pathways, or transcription and translation of genetic information. Plant cells can distinguish different degrees of oxidant exposure (Cooper et al., 2002), and intensity of oxidative stress can control molecular events in the host cells.

To date, however, the biochemical evidences are not supported by molecular evidences on expression of mitochondrial and chloroplast genes whose regulation is redox-dependent, and nuclear genes encoding for chlorophyll synthesis, therefore still remain unclear whether the biochemical differences observed between tolerant and susceptible pear cultivars are regulated at gene expression level.

The aim of this study was to compare specific molecu-

lar events in a susceptible and a tolerant pear cultivar in responses to infection by *E. amylovora*. Our results provide useful hints to analyze the mechanisms leading to susceptibility and resistance in pears to fire blight, and develop more appropriate strategies to reduce damage caused by activation of host defense mechanisms.

## MATERIALS AND METHODS

### Bacterial strain, plant materials and infection

*E. amylovora* Ea273 was obtained from American Type Culture Collection (ATCC No. 49946). Pathogenicity of this bacterium was tested by inoculating *in vitro* shoots of Williams cultivar, through surgical removal of apices by a sterile scalpel, previously soaked in the bacterial suspension. Optimum concentration of inoculum and *in vitro* propagation of plant materials have been previously described (Abdollahi et al., 2004).

Five to six cm long *in vitro* grown shoots of two pear cultivars, Williams (susceptible) and Harrow Sweet (tolerant), were inoculated by adding 100  $\mu$ l of a bacterial suspension (OD = 2 at  $A_{600}$ , in phosphate buffer pH = 7) to the surface of growth medium in 10 replications. All shoots were transferred to 16 h light photoperiod at  $23 \pm 1^\circ\text{C}$ . Evolution of necrosis was compared in dark and constant light conditions (using cool white fluorescent lamps at  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux) after basal inoculation of the shoots.

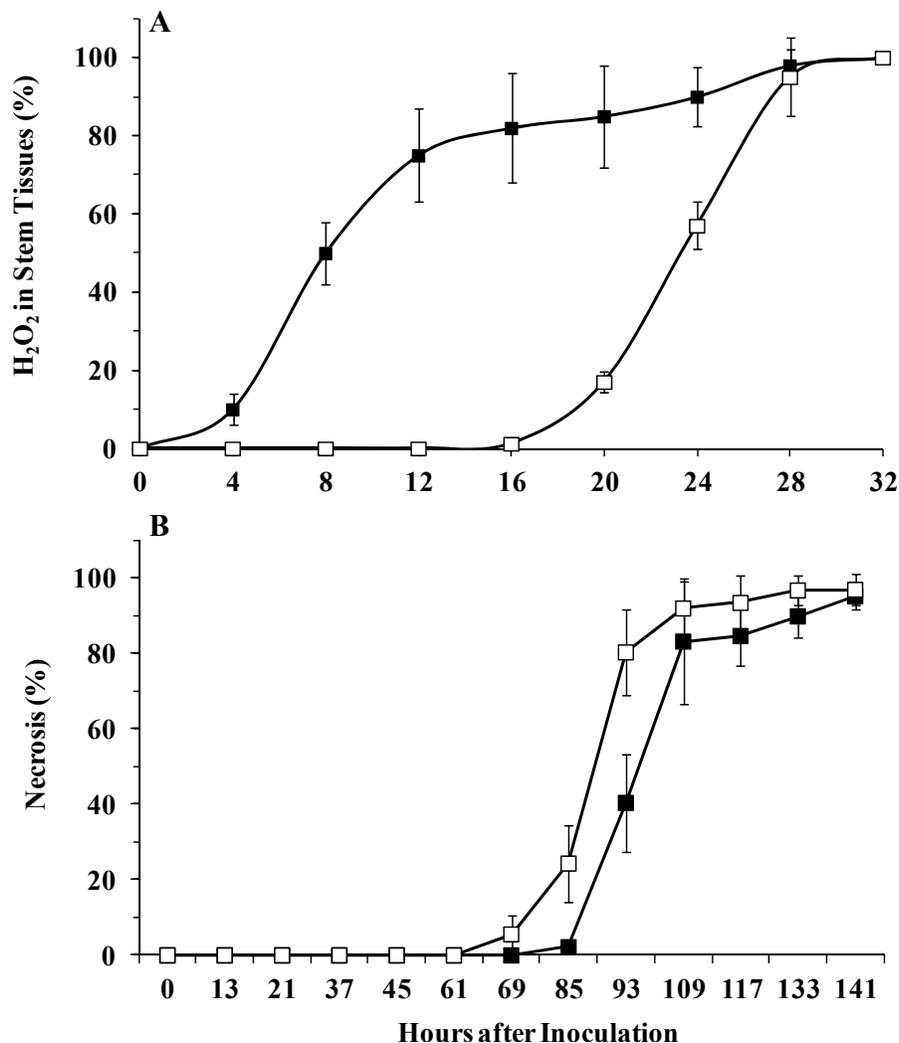
### Detection of $\text{H}_2\text{O}_2$ by DAB staining method

$\text{H}_2\text{O}_2$  generation in the inoculated and control shoots of each cultivar was localized, using 3,3'-diaminobenzidine (DAB)-HCl (Sigma-Aldrich, Italy) as described by Thordal-Christensen et al. (1997). The shoot axes was catted in 5-mm-long pieces, in three replications, which were soaked in an acidic water solution (pH = 3.8) containing 1 mg/ml DAB and 0.1% (v/v) Triton and incubated at  $30^\circ\text{C}$  (120 rpm) for 6 h. Samples were catted lengthwise and  $\text{H}_2\text{O}_2$  traces were detected under stereomicroscope by red, purple and brown colors of the stem tissues. Data were expressed in percentage of produced  $\text{H}_2\text{O}_2$ .

### Plant DNA and mRNA extraction

One-week-etiolated shoots of plant materials were used for DNA extraction in three replications by modified Sul and Korban (1996) method and quantified at  $A_{260}$ . Plant mRNAs were extracted from 0.1 g of plant materials in three replications by QuickPrep™ Micro mRNA purification kit (Amersham Pharmacia Biotech, Italy). This was done after the acidification of the medium indicated adequate activity of bacteria but before the appearance of necrotic symptoms of disease (Figure 1A). mRNAs were extracted from plant materials at least 3 h after beginning the light photoperiod, and dissolved in 10  $\mu$ l diethyl pyrocarbonate (DEPC) treated water and quantified at  $A_{260}$ .

cDNA were synthesized, using 115 M-MuLV Reverse Transcriptase (Amersham Pharmacia Biotech, Italy). Each cDNA synthesis reaction was performed by using 100 ng mRNA, 0.5  $\mu$ g of pd(T)12-18 as primer for the first strand cDNA synthesis in a total volume of 50  $\mu$ l. cDNA synthesis was carried out at  $42^\circ\text{C}$  for 30 min, followed by deactivation of M-MuLV Reverse Transcriptase at  $95^\circ\text{C}$  for 5 min. Absence of DNA in the mRNA solutions was verified by following the expression of *eIF1- $\alpha$*  housekeeping gene (elongation factor) that on its genomic tested fragment has a 100 bp intron. All PCR reactions were conducted in the presence of 10 ng cDNA or 100 ng DNA, 1  $\mu$ M of each forward and reverse primers (Table 1),



**Figure 1.** Progress of necrosis in the basal inoculated shoots to determine appropriate time for mRNA extraction (A), and localization of H<sub>2</sub>O<sub>2</sub> generation in the stem tissues of the basal inoculated shoots of pear cvs. Williams (susceptible) and Harrow Sweet (tolerant) triggered by attack of *E. amylovora* (B).

0.75 mM MgCl<sub>2</sub>, 2.5 μM dNTP, 1X PCR buffer and 1 unit Taq-DNA polymerase (Amersham Pharmacia Biotech, Italy). The PCR products were separated and visualized on 1% (w/v) agarose-ethidium bromide gel.

#### Designing of primers and DNA sequencing

DNA sequences of candidate genes were aligned by Multalin software (Corpet, 1988) and the primers were designed on the most conserved regions of the sequences. Partial sequences of chloroplast genes *psbA*, *psbB*, *psbC* and *psbD* (on DNA) and mitochondrial gene *nad4* (on cDNA) were sequenced to verify their identity and deposited in Gene Bank (Table 1).

#### Expression of candidate nuclear, chloroplast and mitochondrial genes

The gene expression studies were done in two gene groups, the

housekeeping and redox-dependent-transcription genes (Table 1). Expressions of genes were studied at least in two replications, through PCR-amplification, by using cDNA as templates. PCR profiles were adjusted by using genomic DNA to obtain a single amplicon with expected length, except the *actin* gene.

#### Topology prediction of effector proteins of *E. amylovora*

The amino acid sequences of the effector proteins of *E. amylovora* (Accession of *hrpN*, *hrpW* and *dspA/E*, Q01099, AAF63402 and AAC62315, respectively) were obtained from NCBI database. Putative secondary structures of HrpN, HrpW and DspA/E proteins were predicted by GOR3 (Gibrat et al., 1987), GOR4 (Garnier et al., 1996), HNN (Guermeur, 1997), SOPM (Geourjon and Deléage, 1994) and SOPMA (Geourjon and Deléage, 1995) Internet software. Putative transmembrane α-helices in these proteins were predicted, using TMHMM (Moller et al., 2001), DAS (Cserzo et al., 1997), HMMTOP (Tusnady and Simon, 1998) and TMpred (Hofmann and Stoffel, 1993) programs.

**Table 1.** Sequences of primer used for amplification of genomic DNA and study of expression of different chloroplastic, mitochondrial and nuclear genes in the interaction between pear genotypes and *E. amylovora*.

|              | Gene            | Gene product                    | Bases from start codon <sup>a</sup> | Primer Forward               | Fragment length (mRNA) | Primer reverse               | Ta (°C) |
|--------------|-----------------|---------------------------------|-------------------------------------|------------------------------|------------------------|------------------------------|---------|
| Chloroplast  | <i>psbA</i>     | D1 in PSII                      | 243                                 | 5'-GAAAACCGTCTTTACATTGG-3'   | 942                    | 5'-GTTGTGAGGATTACGTTTCAT-3'  | 48      |
|              | <i>psbB</i>     | PS II 47 kDa protein            | 9                                   | 5'-CCTTGGTATCGTGTTTCATAC-3'  | 601                    | 5'-CAATATGATGAGARGCTGTTC-3'  | 48      |
|              | <i>psbC</i>     | PS II 44 kDa protein            | 3                                   | 5'-GAAACGCTCTTTAATGGAAC-3'   | 641                    | 5'-ACACTAACAATCCAHCCTTC-3'   | 48      |
|              | <i>psbD</i>     | D2 in PSII                      | 300                                 | 5'-GATTTTACTCGTTGGTGTCA-3'   | 752                    | 5'-GTTTCCACGKGGTARAACCTC-3'  | 48      |
|              | <i>23S rRNA</i> | 23S ribosomal RNA               | 410                                 | 5'-TGTGAATCAGCAAGGACCAC-3'   | 386                    | 5'-TAACCACAACCTCATCCGCTG-3'  | 55      |
| Mitochondria | <i>atp1</i>     | ATPase subunit 1                | 728                                 | 5'-TCCGCGATAATGGAATGCAC-3'   | 384                    | 5'-AAGCCGACGTTAATAGCAGG-3'   | 55      |
|              | <i>nad1</i>     | NADH dehydrogenase subunit 1    | 37                                  | 5'-CACTTCTACTAGGAGTAGCC-3'   | 266                    | 5'-CGGATCTGACAATACCATAACC-3' | 55      |
|              | <i>nad3</i>     | NADH dehydrogenase subunit 2    | 88                                  | 5'-CCAATAGTTCGACCTATCCAG-3'  | 207                    | 5'-CATAGAGAGATCCAATCGTC-3'   | 55      |
|              | <i>nad4</i>     | NADH dehydrogenase subunit 4    | 212                                 | 5'-CCTTCGATGGCTTCCTTATG-3'   | 324                    | 5'-CTGATATGCTGCCTTGATCT-3'   | 55      |
|              | <i>26S rRNA</i> | 26S ribosomal RNA               | 1305-2291                           | 5'-AACCATGTCTGAAGGAAGCTCG-3' | 301                    | 5'-TTACACCATTTCGTGCAGGTC-3'  | 55      |
| Nucleus      | <i>eIF1-α</i>   | Elongation factor 1             | 608                                 | 5'-ATTGTGGTCATTGGYCAAGT-3'   | 707                    | 5'-CCAATCTTGATAYACATCCTG-3'  | 55      |
|              | <i>act</i>      | actin                           | 163                                 | 5'-ACNGGNATGGTNAAGGCTGG-3'   | 594                    | 5'-GTCNCKNACAATTTCCCGCTC-3'  | 55      |
|              | <i>cab</i>      | Chlorophyll a/b binding protein | 0                                   | 5'-ATGGCTDCYKCHACWATGGC-3'   | 659                    | 5'-CCATTCTTRAKCTCCTTYACC-3'  | 48      |
|              | <i>18S rRNA</i> | 18S ribosomal RNA               | 2641-2654                           | 5'-GTGCTCAAAGCAAGCCTACG-3'   | 760                    | 5'-CGATCAGATACCGTCCTAGT-3'   | 55      |

<sup>a</sup> mRNA shows the housekeeping genes in nucleus, mitochondria and chloroplasts.

### Cytoplasmic organelles targeting analysis of *E. amylovora* effector proteins

Two programs, Predotar (Somanchi and Mayfield, 1999) and TargetP (Emanuelsson et al., 2000) were used to predict the existence of cytoplasmic and mitochondrial presequences in effector proteins of *E. amylovora*. In addition, all known presequences of mitochondrial protein import presequences, listed by Whelan and Glaser (1997), were aligned manually and their homologies were compared with effector proteins of *E. amylovora* by Multalin Internet software (Corpet, 1988). The amphipathicity of putative  $\alpha$ -helices were predicted by PepTool software.

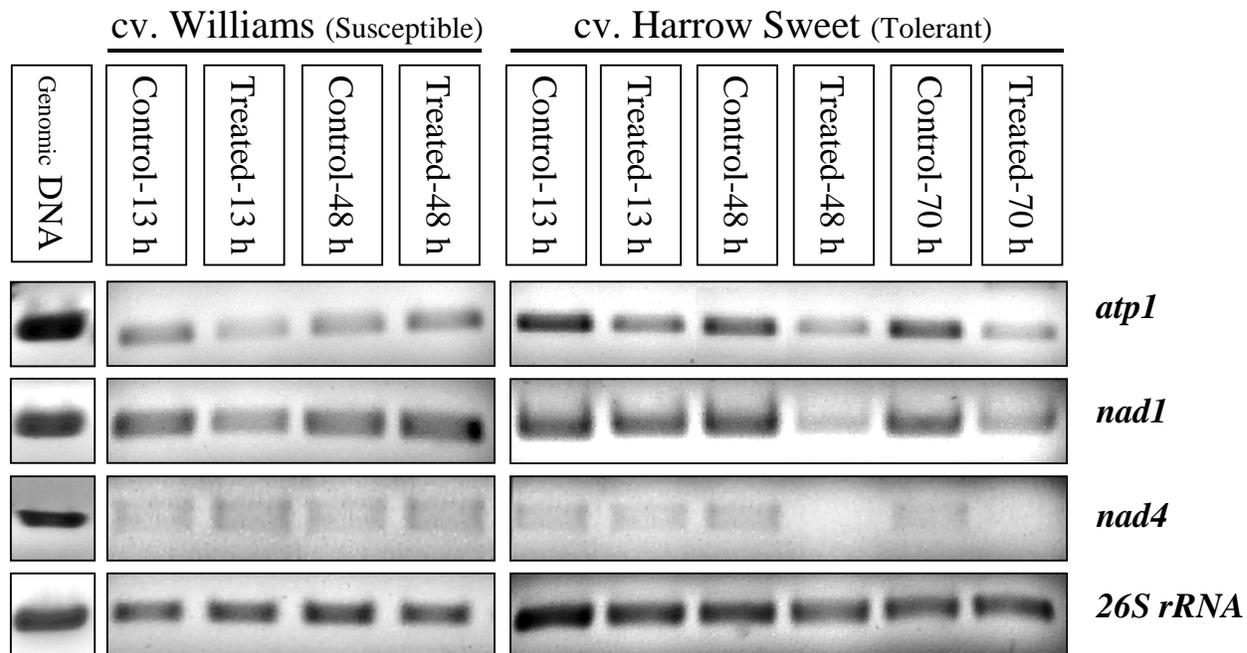
## RESULTS and DISCUSSION

### Hydrogen peroxide accumulation in infected shoots

Monitoring of oxidative burst, by histochemical detection of hydrogen peroxide using DAB-staining, indicated that infection induced an increase in H<sub>2</sub>O<sub>2</sub> in the shoots of both cultivars, although time-course profiles were quite different (Figure 1A). In Harrow Sweet cultivar, cells responded with a rapid generation of H<sub>2</sub>O<sub>2</sub> 4 h after

inoculation and H<sub>2</sub>O<sub>2</sub> level reached the highest value after 28 h. In contrast, in Williams shoots the production of H<sub>2</sub>O<sub>2</sub> was delayed up to 15 h and extended over the entire length of the shoots 28 h after inoculation.

It is worth noting that in both cultivars, evident symptoms of necrosis appear several hours after the H<sub>2</sub>O<sub>2</sub> level reached the highest value, but the time of onset of these symptoms was cultivar dependent and was delayed in Harrow Sweet cultivar (Figure 1B).



**Figure 2.** Expression of different mitochondrial genes, in control and inoculated shoots, of pear cvs. Harrow Sweet and Williams.

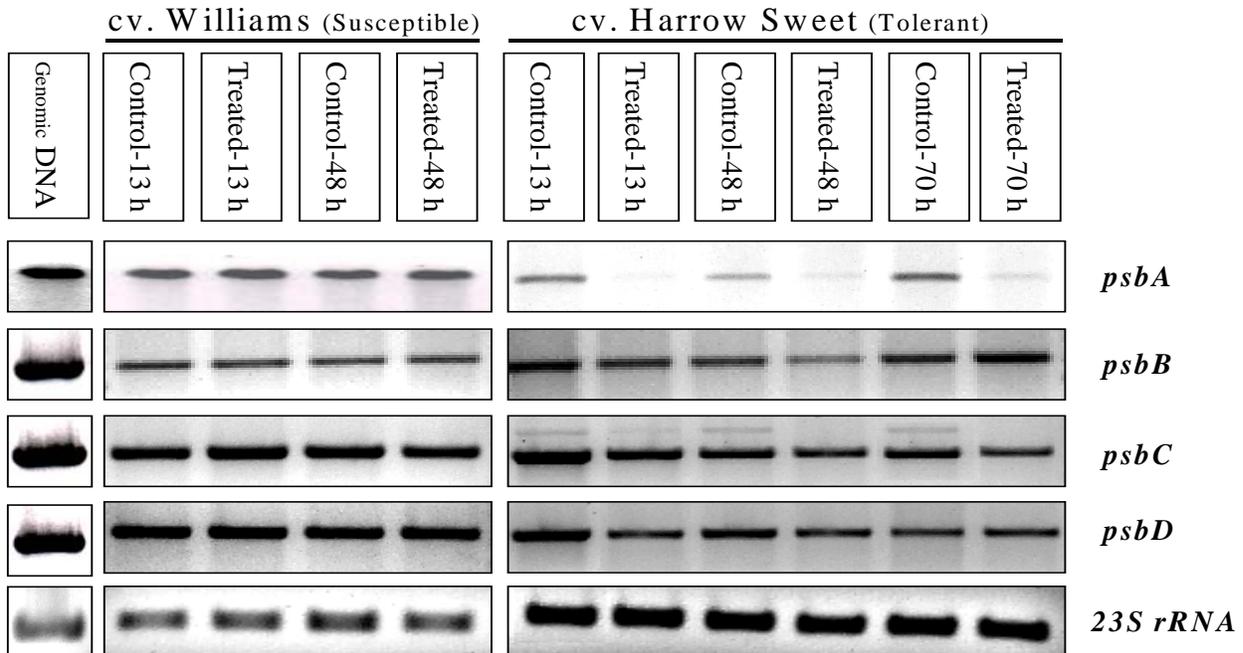
As reported in the literature,  $H_2O_2$  can generate a redox poise in the organelles and affects the expression of redox-depending genes in chloroplast and mitochondria (Konstantinov and Tarasenko, 1999). We intended to test whether ROS production stimulated by *E. amylovora* infection affects the gene regulation of some organelles and nuclear genes.

#### Expression of mitochondrial and chloroplast genes in pear cultivars

In Harrow Sweet inoculated shoots, expression of mitochondrial *atp1* and *nad1* genes was gradually reduced while expression of *nad4* gene was completely suppressed after 48 h (Figure 2). In contrast, in Williams shoots no difference was observed in the expression of tested genes in both inoculated and control shoots (Figure 2). Transcription analysis of chloroplast genes (Figure 3) indicated that expression of *psbA* gene was strictly linked to the capacity of cultivars to tolerate the pathogen and early production of  $H_2O_2$  (Figure 1); it resulted to dramatic decrease, 13 h after inoculation, in Harrow Sweet shoots, while it was slightly affected, 48 h after inoculation, in Williams shoots. No difference in the transcript level of *psbB*, *psbC* and *psbD* genes was detected in inoculated and control shoots of both cultivars (Figure 3).

It has been shown that a discrete number of ESTs involved in redox system and photosynthesis were modulated in responses to *E. amylovora* invasion (Sarowar et al., 2011; Baldo et al., 2010). Vrancken et al. (2013) suggested that rapid increase of ROS is one of the earliest

plant responses to *E. amylovora* invasion. Observations made in inoculated shoots of Harrow Sweet cultivar where an early increasing of  $H_2O_2$  production occurred few hours after inoculation, and down-regulation of *psbA* expression was detected 13 h after inoculation, indicates that a photo-production of  $H_2O_2$  occur in the infected tissues. *psbA* gene encodes the protein D1 that plays a pivotal role in photosystem II (PSII). Light-sensing network and oxygen-evolving complex in PSII have been shown to be linked to plant defense against pathogen infection (Genoud et al., 2002; Abbink et al., 2002). The PSII plays an important role in preventing the accumulation of ROS (Krieger-Liszkay, 2005) therefore to activate the protective responses and to induce systemic acquired resistance by ROS are needed to down-regulate PSII activity (Fryer et al., 2003; Kulheim et al., 2002). Although in apple young leaves, photosynthetic activity was inhibited and chlorophyll fluorescence was changed by ferredoxin (Fd) dependent way, prior to the development of disease symptoms (Bonasera et al., 2006), however an up-regulation of some photosynthetic genes have been observed by Heyens and Valcke (2006) and Baldo et al. (2010), in apple invaded tissues. Bonasera et al. (2006) showed that pathogen effector DspE/A interacts with precursor-ferredoxin in the cytoplasm, thereby preventing its transfer into chloroplast, where it is usually converted to ferredoxin (Fd) and it serves as an electron carrier in photosystem I (PSI). Moreover, Singh et al. (2010) suggested that FIBRILLIN4, which is associated with photosystem II, could also play a part in fire blight infections, as the disease is more expressed in the knock-



**Figure 3.** Expression of different chloroplast genes, in control and inoculated shoots, of pear cvs. Harrow Sweet and Williams.

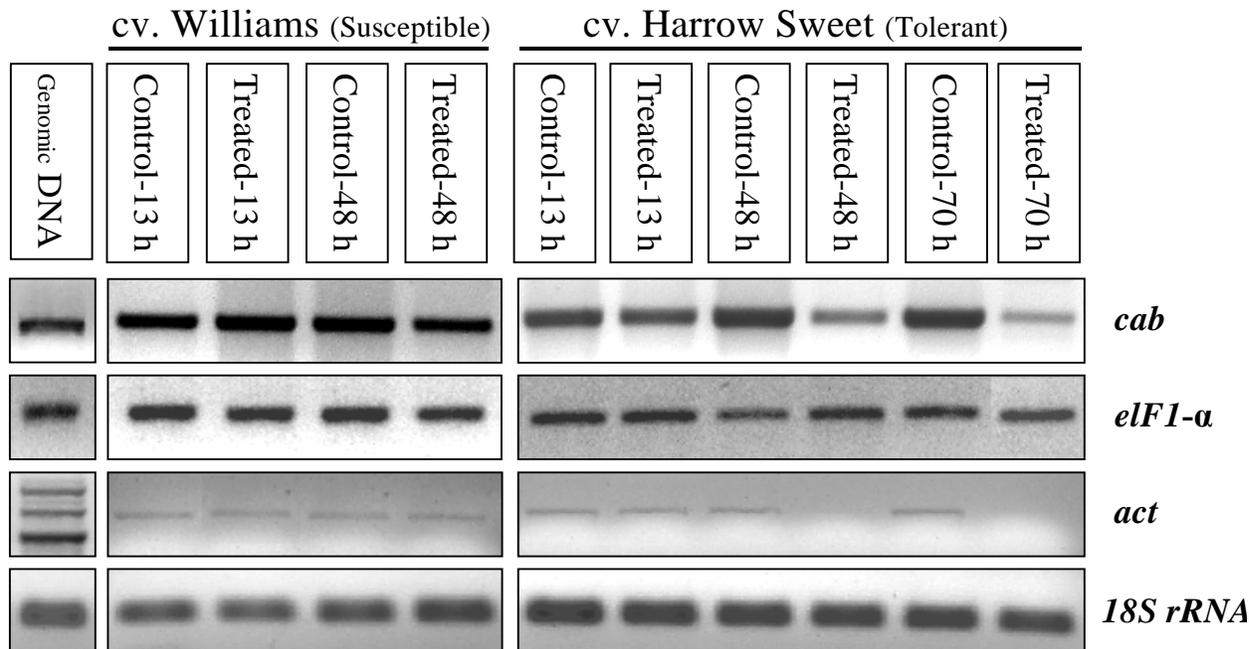
down mutant. Oxidation and reduction of glutathione (GSH), described by Baginsky et al. (1999) can explain changes in *psbA* transcription by the effects of oxygen radicals, generated during infection by the pathogen. *In vivo*, most of the chloroplast glutathione is in the reduced GSH form that serves as a redox buffer and ROS scavenger. During oxidative stress of pathogen, the GSH oxidizes and forms glutathione disulfide (GSSG) and the chloroplast GSH:GSSG ratio decreases significantly, with a transient increase of GSSG and concomitant decrease in GSH redox state, resulting in decrease activity of PTK. Lower PTK activity leads to lower phosphorylation of SLFs in RNA-polymerase complex of chloroplasts and decreases its activity for transcription of genes such as *psbA*. In addition, expression of other photosynthetic genes, *psbC*, *psbB* and *psbD*, were not suppressed like the *psbA* gene, indicating different mechanism, which control the transcription and reduce the redox sensibility of these genes. These results are also in accordance with Pfannschmidt et al. (1999), which showed that the redox state of plastoquinone (PQ) effectively controls the redox poise of chloroplasts and transcription of the *psbA* gene, but transcription of other chloroplast genes were not affected by the PQ redox state.

It is known that an increase in photosynthetic activity usually induces the production of sugar and ATP that might prevent in *Malus* the colonization by *E. amylovora* by increasing host plant defense through the light sensing signaling pathway and by activation of additional defense related genes (Baldo et al., 2010). In our experiments, pear plantlets of both cultivars are grown in medium with

a large amount of assimilate carbohydrate. This condition makes easier to highlight the differences existing between cultivars regarding fire blight susceptibility, because a suitable amount of energy source is available for both susceptible and tolerant cultivars shoots. In shoots of susceptible Williams cultivar exposed to light, necrotic symptoms of fire blight are delayed with respect to those exposed to darkness, thus supporting a role in the mechanisms of plant defense against pathogen infection of the light signaling pathways.

It has been reported that redox state of mitochondria controls transcription of mitochondrial genome by affecting the activity of DNA-topoisomerase I (Konstantinov et al., 2001). Therefore, it is possible that ROS generation, due to infection by the pathogen, alters the redox poise of mitochondria and changes the transcription rate of this organelle in the pear cells. Activity of DNA-topoisomerase I opens supercoiled mitochondrial DNA by introducing a transient single-strand break in the duplex and acts in a number of different DNA metabolisms, such as DNA replication, transcription and repair (Champoux, 2001). Therefore, it will be expected that any variation in redox poise of mitochondria and activity of DNA topoisomerase I, could alter the transcription of all mitochondrial genes similarly.

Our results showed that infection by the pathogen suppressed expression of some mitochondrial genes and concomitantly did not affect expression of 26S ribosomal-RNA gene in the inoculated shoots of either cultivar. This indicates a role for at least a second mechanism controlling the transcription rate of mitochondrial genes. Oxygen



**Figure 4.** Expression of different nuclear genes in control and inoculated shoots of pear cvs. Harrow Sweet and Williams.

radicals generated in the mitochondria are very mobile and diffuse rapidly elsewhere in the cytoplasm and organelles (Moller, 2001). Subsequently, production of ROS by pathogen infection and penetration in the chloroplasts alters redox state and expression of some redox-dependent-transcription genes such as *psbA* in this organelle.

#### Expression of nuclear genes

Similar to the chloroplast genes, expression of nuclear genes varied with the genotypes and gene kind (Figure 4). Results showed that expression of nuclear genes of Williams cultivar were not affected by the infection, only the expression of the *cab* gene was slightly reduced at 48 h from inoculation. In Harrow Sweet cultivar, two house-keeping genes, *eIF1-α* and *18S ribosomal-RNA*, expressed constantly up to 70 h in both control and inoculated shoots, while expression of *cab* and *act* genes showed a gradual reduction in the inoculated shoots (Figure 4). The constant expression of *eIF1-α* and *18S ribosomal-RNA* showed that even after 70 h after inoculation, RNA-polymerase of the nucleus had not been deactivated by pathogen infection.

Recent findings have confirmed the role of chloroplast signaling on transcription of photosynthetic nuclear genes (Somanchi and Mayfield, 1999). Several factors have been proposed as signaling intermediates, like tetrapyrrole, sugars and redox state of chloroplasts (Surpin et al., 2002). Oswald et al. (2001) demonstrated that transcription of nuclear photosynthetic *rbcs* and *cab* genes are under control of redox state of thioredoxin or glutathione system in plant chloroplasts, although the nature of this

signaling is still unknown. In our work, the reduction of *cab* gene in the inoculated shoots could be a consequence of the chloroplast signaling to the nucleolus through changes in the redox poise of thioredoxin or GSH system of chloroplasts by oxidative stress of pathogen.

#### Topology prediction of effector proteins of *E. amylovora*

Xie and Chen (2000) showed that the effector proteins of *E. amylovora* interact with tobacco cells affecting the complex III and/or IV of mitochondrial ETC. However, the mechanism allowing these proteins to pass through mitochondrial membranes are not known, yet. Most of the proteins located in the chloroplasts and in the mitochondria are encoded by nuclear genes and synthesised in the cytoplasm. They are recognized through the signal sequences present in the N-terminal region of protein and transported through mechanisms of protein trafficking into the appropriate organelles by participation of chaperones (Glaser et al., 1998). In addition, mitochondria and chloroplasts are believed to have evolved from prokaryotic ancestors, and they still exhibit some functional similarities to the bacteria. For instance, at least two out of four protein transport systems in the chloroplasts and one system in the mitochondria are homologous to the translocation system in the inner membrane of bacteria (Moore et al., 1994; Schnell, 1998). It is possible that *E. amylovora* produces effector proteins that target the mitochondria through these protein trafficking systems and affecting ETC of this organelle. Secondary structure analysis of the effector proteins of *E. amylovora* individuate

**Table 2.** Targeting prediction of *E. amylovora* effector proteins to the plant mitochondria and chloroplasts by Predotar (Small 2003) and TargetP (Emanuelsson et al., 2000) software.

| Effector protein | Predotar              |                        |          | TargetP          |                  |                 |                    |          | Predicted location |                 |
|------------------|-----------------------|------------------------|----------|------------------|------------------|-----------------|--------------------|----------|--------------------|-----------------|
|                  | Cp score <sup>a</sup> | Mit score <sup>b</sup> | Location | cTP <sup>c</sup> | mTP <sup>d</sup> | SP <sup>e</sup> | Other <sup>f</sup> | Location |                    | RC <sup>g</sup> |
| HrpN             | 0.012                 | 0.133                  | Neither  | 0.210            | 0.134            | 0.026           | 0.522              | ---      | 4                  | Neither         |
| HrpW             | 0.029                 | 0.062                  | Neither  | 0.469            | 0.094            | 0.040           | 0.465              | C        | 5                  | Chloroplast     |
| DspA/E           | 0.065                 | 0.124                  | Neither  | 0.133            | 0.155            | 0.018           | 0.727              | ---      | 3                  | Neither         |

<sup>a</sup>, <sup>c</sup>Chloroplasts targeting probability; <sup>b</sup>, <sup>d</sup>mitochondria targeting probability; <sup>e</sup>secretory pathway targeting probability; <sup>f</sup>any other location targeting probability; <sup>g</sup>reliability class (RC=1 as the highest reliability).

**Table 3.** Evolution of fire blight necrosis in the basal inoculated shoots of pear cv. Williams under continuous dark and light conditions. Values represent the average of two independent experiments  $\pm$  2SE.

| Hours after inoculation | Light           | Dark            |
|-------------------------|-----------------|-----------------|
| 0                       | 0.0             | 0.0             |
| 12                      | 0.0             | 0.0             |
| 20                      | 0.0             | 0.0             |
| 32                      | 0.0             | 0.0             |
| 40                      | 0.0             | 0.0             |
| 52                      | 0.6 $\pm$ 0.5   | 2.5 $\pm$ 2.1   |
| 60                      | 1.3 $\pm$ 1.0   | 6.3 $\pm$ 5.3   |
| 72                      | 8.1 $\pm$ 5.1   | 15.0 $\pm$ 4.4  |
| 80                      | 25.0 $\pm$ 4.5  | 64.0 $\pm$ 5.4  |
| 92                      | 75.0 $\pm$ 4.4  | 90.0 $\pm$ 6.5  |
| 100                     | 90.0 $\pm$ 4.2  | 100.0 $\pm$ 0.0 |
| 108                     | 100.0 $\pm$ 0.0 | 100.0 $\pm$ 0.0 |

9, 3 and 7 putative  $\alpha$ -helices, in HrpN, HrpW and DspA/E proteins, respectively, as predicted by five different software (see materials and methods). However, only in the HrpN protein a high significant and consistent putative transmembrane helix motive, about 20 amino acids in size, is present. HrpN is a potential effector candidate to elicit either a hypersensitive reaction on non-host plants or a pathogenic reaction on host plants. Therefore, the presence of transmembrane alpha helix motive may explain the role of HrpN in pathogenicity of *E. amylovora*.

Prediction of HrpN, HrpW and DspA/E targeting the chloroplasts and mitochondria by Predotar software did not identify any signal sequences in the N terminal region (Table 2). However, the analyses run using TargetP software predicted a possible HrpW chloroplast-target signal sequence. Since it has been shown that the chloroplasts are involved in PCD of guard cells in pea leaves (Samuilov et al., 2002), it may be postulated that the effector proteins of pathogen interact with photosystem II, probably by down regulation of *psbA* gene, triggering an oxidative stress of cells of infected plants. If this hypothesis is true, we should expect a slower progress of the necrosis in plants exposed to light than those exposed to dark condition.

### Delay of the progress of necrosis under light conditions

Comparison between the evolution of necrosis in the inoculated shoots of Williams cultivar in the dark and in the light showed that necrosis progressed faster in the dark (Table 3). This difference could not be caused by the inhibitory effects of light on pathogen, but in the absence of flux of electrons in the chloroplast ETC can be due to the reduced capacity of plant to contrast the pathogen. This event can be explained by the interaction between HrpW and ETC of chloroplasts, which generates oxidative stress. It means that under light condition, HrpW interacts with chloroplast ETC, supporting mitochondria as a second source of ROS generation and decreasing the invasion of pathogen. Experiments to unravel the role of HrpW and chloroplast on the progress of fire blight are in progress.

### Conclusion

With this study, the relationship between the two events are shown for the first time, but further researches need to be carried out to identify the biological determinants that generate the differences between tolerant Harrow Sweet and susceptible Williams cultivars. In fact, the causal relationship between ROS activity and down-regulation of expression of redox-dependent nuclear, mitochondrial and chloroplast genes is not straightforward, because plant resistance in general, and induced resistance in particular, is an emergent property of a plant that results from the combined action of multiple genetic, biochemical, physiological and morphological traits that interact with one another and that are expressed heterogeneously in space and time.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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