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Short communication

Involvement of iron and ferritin in the potato–*Phytophthora infestans* interaction

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Abstract

This work shows that the infection of potato (*Solanum tuberosum*) detached leaves by the late blight pathogen *Phytophthora infestans*, was drastically reduced by adding deferoxamine, an exogenous iron chelator. Reactive oxygen species in leaves inoculated with *P. infestans* were also reduced after adding deferoxamine. A leaf ferritin cDNA fragment was obtained by PCR and used as probe for screening a tuber cDNA library. A cDNA (named *StF1*) encoding the iron-storing potato ferritin was cloned. *StF1* is 915 bp in length and has an open reading frame of 230 amino acids that contains the information for the mature 28 kDa subunit of potato ferritin. *StF1* was used as probe in northern blot hybridizations to analyze expression of the ferritin gene. In leaves, ferritin mRNA accumulated in response to pathogen attack. In tubers, ferritin mRNA increased upon treatment with the elicitor eicosapentaenoic acid. These results suggest that iron plays a role in the potato-*P. infestans* interaction.

Abbreviations: DFO – deferoxamine; EPA – eicosapentaenoic acid; ROS – reactive oxygen species; DAB – diaminobenzidine.

Iron is one of the most abundant metals in the earth crust. It plays a key role in living organisms due to its chemical versatility. Iron can also react with reactive oxygen species (ROS). This reaction produces hydroxyl radicals ($OH^{\bullet}$), which induce lipid peroxidation, protein denaturation and degradation, and DNA mutation (Halliwell and Gutteridge, 1984; Harrison and Arosio, 1996). There are indications that iron may act as a virulence factor in mammalian infections (Guerinot, 1994; Long, 1996; Payne, 1994). For example, low iron concentrations are an obstacle for pathogen growth in mammalian infectious diseases (Long, 1996). Thus, pathogen growth, as well as the deleterious effects on host cells derived from ROS production, could be limited through low-iron availability.

Deferoxamine (DFO), an iron-specific chelator, inhibits the iron-catalyzed generation of $OH^{\bullet}$ (Gutteridge et al., 1979) and protects pea leaves from oxidative stress generated by paraquat (Zer et al., 1994). Likewise, some intracellular molecules are also able to bind iron. Among them, ferritin is considered as an intracellular iron-buffer. It is a 500 kDa protein composed of 24 identical subunits. It can store up to 4500 Fe(III) ions in an inorganic complex (Harrison and Arosio, 1996). Plant ferritin is located in plastids and ferritin gene expression is influenced by factors such as iron concentration and oxidative stress (Lobreaux and Briat, 1997; Lobreaux et al., 1992; 1995). Moreover, Deak et al. (1999) have demonstrated a role for ferritin in the tolerance to oxidative damage and pathogen attack in transgenic tobacco plants.

Although iron is known to be necessary for growth of *Phytophthora infestans* (Cuppet and Lilly, 1973), no data are available concerning the role of iron in the interaction of *P. infestans* with potato, its natural host. It

is also known that ROS production occurs when potato is infected by *P. infestans* (for a review see Doke et al., 1996). So far, however, knowledge on potato ferritin is lacking. There are no reports about cloning potato ferritin genes nor about the regulation of potato ferritin gene expression. In this work, we describe the impact of DFO-mediated iron depletion on the disease severity caused by *P. infestans* on potato leaves, as well as cloning of a potato ferritin cDNA and the potato ferritin gene expression during the disease development.

Potato plants (*Solanum tuberosum* L cv. Pampeana) were grown for 30 days (Laxalt et al., 1996). After that, detached potato leaves were treated with 10 μM DFO (an iron chelator) for 5 days at 18 °C in the dark, in order to diminish the iron availability. Control detached leaves were maintained in water. Then, all the leaves were sprayed with a suspension of 10^4 sporangia · ml^{-1} of *P. infestans* (race 1, 4, 7, 8, 10, 11; mating type A2). Leaflets were maintained at 18 °C in a moist chamber, with a 14 h light photoperiod. Two days after inoculation, the water treated leaves contained severe lesions (Figure 1A). In contrast, the DFO treated leaves showed a reduction of lesion development (Figure 1B). Since non-infected potato leaves treated for 10 days with DFO showed symptoms of chlorosis (not shown), we had clear evidence of effective iron sequestration. The protection obtained by using DFO could be derived from the inhibition of hydroxyl radical generation, as is the case for paraquat-treated pea leaves (Zer et al., 1994). To test this hypothesis, we stained infected leaves with diaminobenzidine (DAB), a histochemical reagent for ROS (Beligni and Lamattina, 2000). Little DAB staining was evident in the infected DFO treated leaves (Figure 1D), whereas infected water treated leaves induced strong DAB staining (Figure 1C). These results suggest that the regulation of iron availability in potato might counteract the deleterious consequences of *P. infestans* infection.

Expert (1999) has reviewed the influence of iron uptake mechanisms in phytopathogenicity of *Erwinia* spp. Production of pectinases, which are critical for infection development, is tightly associated with the ability of the bacterium to acquire iron. However, this does not seem to be the case for other bacterial and fungal phytopathogens (Riquelme, 1996; Mei et al., 1993). In our case, results are consistent with the idea that iron may be acting as a virulence factor during the development of late blight in potato.

To be able to analyze ferritin gene expression we cloned a potato ferritin cDNA. PCR was performed using potato leaf cDNA as template. The degenerated

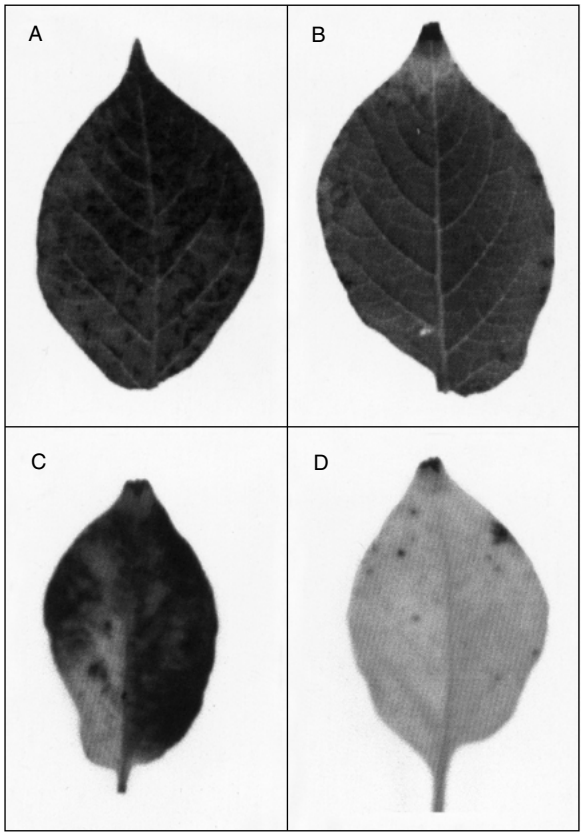

Figure 1. Effect of deferoxamine on the severity of lesions produced by *P. infestans* in potato leaflets and on the production of ROS. Detached potato leaves were maintained in water (A,C) or 10 μM deferoxamine (B,D) for 5 days. Then, the leaflets were sprayed with a suspension of 10^4 sporangia · ml^{-1} of *P. infestans*. Pictures shown in A and B were taken 2 days after infection. Two days after infection, leaves were stained overnight with DAB and destained in boiled ethanol in order to detect ROS. Pictures shown in C and D illustrate an example of one of the three separate experiments performed in duplicate.

primers Fer5 (5′-GAG CAR ATI AAT GTG GAA TAC AA-3′) and Fer3 (5′-CCI CAA ACC GTG AAA CTT GA-3′), in which R represents a purine and I is an inosine, were deduced from consensus sequences of the ferritin cDNAs of several plants (Gaymard et al., 1996). By PCR a 455-bp fragment was obtained and cloned in a pGem-T-easy (Promega, USA) cloning vector. The insert was amplified by PCR, isolated from agarose gels, and radioactively labeled by random priming using ^{32}P-dCTP as radiolabeled nucleotide. With the aim to obtain a full-length ferritin cDNA this probe was used to screen a tuber cDNA library from potato cultivar Spunta (cloned in Lambda-Zap II and

kindly provided by V. Godoy, Mar del Plata University, Argentina). Screening was performed according to the instructions of the manufacturer (Stratagene). The sequence of the clone, named *StF1*, is shown in Figure 2. The insert is 915 bp in length, there is a polyadenylation signal at positions 869–874, and a polyA+ tail starting at position 893. The sequence of the potato ferritin leaf cDNA obtained by PCR amplification (not shown) is exactly identical to the sequence present in *StF1* between nucleotides 223–677 of *StF1*. *StF1* has an open reading frame of 230 amino acids with no 5′ UTR. A 3′ UTR of 202 nucleotides is found downstream of a TAG stop codon at position 688. *StF1* is shorter than other known ferritin sequences. Based on sequence alignments with other plant ferritin cDNA sequences, it is estimated that 25 amino acids of the plastid transit peptide are missing. The 28 kDa subunit of the mature potato ferritin begins at position 31. The first 28 amino acids of the mature ferritin constitute an extension peptide, typical of plant ferritins (Harrison and Arosio, 1996; Lobreaux and Briat, 1997). The deduced amino acid sequence of *StF1* is high homologous to other plant ferritins (not shown). Potato ferritin contains the amino acid residues E80, Y87, E110, E113, E114, E115, H118, E164, E197 (Figure 2) that were identified as metal ligands at the ferroxidase centres in *A. thaliana* ferritin (Gaymard et al., 1996).

StF1 expression was analyzed in tubers and leaves. Tuber discs (20 mm diameter and 5 mm thickness) were inoculated with a suspension of 10^4 sporangia $\cdot$ ml^{-1} of *P. infestans.* In other trial, tuber discs were treated with 50 µl of a solution containing 1 mg $\cdot$ ml^{-1} of eicosapentaenoic acid (EPA), a commercially available elicitor from *P. infestans*. Water-treated tuber discs were used as control. All the tuber discs were incubated at 18 °C in the dark, at 100% of relative humidity, for 48 h. The upper 1–2 mm treated surfaces were cut for RNA extraction. Potato leaves were treated as described above. RNA was isolated as described previously by Laxalt et al. (1996). When northern blots containing the RNA were hybridized with the 455 bp PCR fragment, a predominant transcript of 1.3 kb was detected (Figure 3). This is the length of other reported plant ferritin mRNAs (Lobreaux et al., 1992). *StF1* mRNA did not accumulate in unwounded tubers but in water-treated tubers a slight accumulation was observed. Treatment with EPA resulted in increased *StF1* mRNA levels. In contrast, *StF1* mRNA did not increase after *P. infestans* inoculation. In water treated potato leaves and DFO-treated infected leaves the *StF1* expression was similar to that in unwounded tubers. However, infection with *P. infestans* caused accumulation of *StF1* mRNA in potato leaves. Treatment of leaves with 300 µM $FeSO_4$ was used as a control. For iron overloading, detached leaflets were first iron-starved in tubes with water for 14 days. Subsequently, the leaflets were transferred to a tube containing a solution of 300 µM $FeSO_4$ and incubated for another 48 h. Under these conditions, *StF1* expression is strongly induced.

In conclusion, the ferritin gene was expressed differently in distinct potato tissues as a consequence of infection. *P. infestans* inoculation triggered *StF1* expression in leaves but not in tubers. Likewise, in pea, the ferritin gene was expressed differently in several organs during germination (Lobreaux and Briat, 1991). During senescence in *Brassica napus*, ferritin gene expression is probably induced due to release of iron (Buchanan-Wollaston and Aiswort, 1997). It is possible that infection by *P. infestans* causes different levels of iron release in leaves and tuber, and this may explain the differences in ferritin gene expression observed. High concentrations of elicitors like EPA, which is a cell wall component of *P. infestans*, provoke strong ferritin gene expression in potato tubers. It is known that *P. infestans* cell wall components increase H_2O_2 production in potato tubers (Doke et al., 1996; Miura et al., 1995). On the other hand, H_2O_2 induces ferritin gene expression in maize leaf (Lobreaux et al., 1995). Therefore, if EPA increases the H_2O_2 content in potato tuber, ferritin expression will be augmented as a consequence.

Ferritin induction is part of the defence response to pathogen infection in mammals (Beard et al., 1996). Ferritin gene expression increases in plants and animals as a consequence of oxidative stress (Lobreaux et al., 1992; Cairo et al., 1995). However, plant ferritin genes have a different genomic structure and the mechanism of regulation is different from that of its animal counterparts (Lobreaux and Briat, 1997). Little is known about the role of ferritin in response to pathogen attack in plants. Just one report has elegantly demonstrated that transgenic tobacco plants ectopically expressing an alfalfa ferritin gene, exhibited increased tolerance to necrotic toxicity generated by paraquat, iron excess, and viral and fungal infections (Deák et al., 1999). Our report is the first to describe an increase of the endogenous ferritin gene expression as a consequence of pathogen attack in plants.

Although the increased ferritin gene expression observed in potato is not enough to confer resistance to *P. infestans*, our results reinforce the hypothesis that ferritin may be a protective molecule for plant

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1    G  N  K  S  W  S  S  T  A  V  A  A  A  L  E  L  V  D  P  R
1    GGG AAC AAA AGC TGG AGC TCC ACC GCG GTG GCG GCC GCT CTA GAA CTA GTG GAT CCC CGG

21   A  A  G  I  R  H  E  A  T  S  D  V  S  L  T  G  V  V  F  E
61   GCT GCA GGA ATT CGG CAC GAG GCA ACA TCT GAT GTT TCA CTT ACT GGT GTG GTG TTT GAG

41   P  F  D  E  V  N  K  D  E  F  M  V  P  I  T  P  H  T  S  L
121  CCA TTT GAT GAA GTT AAC AAG GAT GAA TTT ATG GTT CCT ATC ACA CCA CAC ACT TCA CTT

61   A  R  Q  R  Y  A  D  E  C  E  G  A  I  N  E  Q  I  N  V  E
181  GCT CGA CAG AGG TAC GCG GAC GAA TGT GAA GGT GCT ATC AAT GAG CAG ATC AAT GTG GAG

81   Y  N  I  S  Y  V  Y  H  A  M  F  A  Y  F  D  R  D  N  V  A
241  TAC AAC ATT TCG TAT GTA TAC CAC GCC ATG TTC GCC TAC TTT GAC AGG GAC AAC GTA GCT

101  L  K  G  L  A  K  F  F  K  E  S  S  E  E  E  K  E  H  A  E
301  CTA AAA GGC CTC GCA AAA TTC TTC AAG GAG TCA AGT GAA GAA GAA AAG GAA CAT GCT GAG

121  K  L  M  H  Y  Q  N  I  R  G  G  R  V  K  L  H  S  I  M  N
361  AAG TTA ATG CAT TAT CAG AAC ATC CGA GGA GGA AGA GTG AAG CTA CAT TCT ATT ATG ATG

141  P  P  S  E  F  D  H  V  D  K  G  D  A  L  Y  A  M  E  L  A
421  CCT CCC TCT GAA TTT GAT CAT GTT GAT AAG GGA GAC GCG TTG TAT GCA ATG GAA CTG GCA

161  L  S  L  E  K  L  T  K  E  K  L  L  T  L  H  S  V  A  D  R
481  TTG TCC TTG GAG AAG TTA ACA AAG GAG AAA CTT TTG ACC CTG CAT AGC GTG GCT GAT CGA

181  N  N  D  S  E  M  Q  D  F  V  E  R  E  F  L  A  E  Q  V  E
541  AAC AAT GAC TCC GAA ATG CAA GAT TTT GTT GAA CGC GAA TTT TTG GCC GAG CAG GTT GAG

201  A  I  K  K  I  A  E  Y  V  S  Q  L  R  R  V  G  K  G  H  G
601  GCT ATT AAG AAA ATT GCA GAA TAT GTA AGC CAG CTA AGG AGG GTT GGA AAA GGA CAT GGA

221  V  W  H  F  D  Q  M  L  L  H
661  GTT TGG CAC TTT GAT CAG ATG CTT TTA CAT tagaattcttaccttaatgcttgaattccttcagtt
728  caggcccctattatctaaagattataagtctctacactatatgttgttgtccaataatgtaatacata
796  tttatgttacttcatattcaagtcatgtataggcatgatcctagaagattaatatggcagcttgcttt
864  caagaaataaaagttgtttgatgaggaattaaaaaaaaaaaaaaaaaaaa
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Figure 2. Nucleotide and deduced amino acid sequence of the *Solanum tuberosum* ferritin cDNA clone *StF1*. The upper lines represent the nucleotide sequences in which capital letters correspond to the coding region and lower case letters to the 3′ UTR. The lower lines represent the deduced amino acid sequence indicated by the one-letter code. Numbers on the left indicate nucleotide or amino acid positions. Oligonucleotide sequences of the primers used for PCR are underlined. Amino acids at the beginning and at the end of the 28 kDa subunit are boxed. Double underlining indicates the putative polyadenylation signal sequence. Residues in bold indicate those amino acids identified as metal ligands in the ferroxidase centres in *A. thaliana* ferritin (see text).

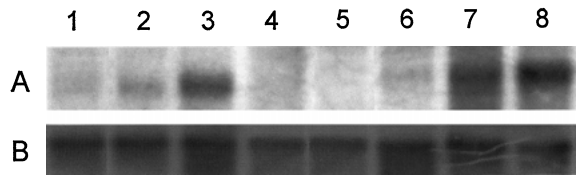

Figure 3. Northern blot analysis of potato ferritin expression. (A) Total RNA (10 μg per lane) was extracted from: lane 1, potato tubers (unwounded control); lane 2, tuber discs treated for 48 h with water (wounded control); lane 3, tuber discs treated for 48 h with eicoisapentaenoic acid (EPA); lane 4, tuber discs 48 h after inoculation with *P. infestans*; lane 5, potato leaves pretreated with DFO and 48 h after infection with *P. infestans*; lane 6, mock inoculated leaves (water treated); lane 7, potato leaves 48 h after infection with *P. infestans*; lane 8, potato leaves treated with 300 μM $FeSO_4$ (iron overloading). After extraction, RNAs were electrophoresed on denaturating agarose gel, transferred and probed with the 455 bp potato ferritin cDNA probe. (B) The filter shown in (A) was stripped and reprobed with sunflower 18S rRNA to check the RNA quantities loaded on gel. The experiment was repeated three times. Representative autoradiographs are shown.

cells. Ferritin may scavenge the intracellular iron and thus limit the generation of the very reactive and toxic hydroxyl radicals (Deák et al., 1999). The results presented here suggest that iron regulating molecules, such as DFO and ferritin, may play a protective role against the deleterious effects of *P. infestans* on potato. In addition, these results support the idea that intensive work on alternative natural molecules, such as DFO and ferritin, may help in designing environmentally friendly methods to fight plant diseases.

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