Eur J Plant Pathol (2009) 125:603–616
DOI 10.1007/s10658-009-9509-0

Identification of bacterial protein markers and enolase as a plant response protein in the infection of *Olea europaea* subsp. *europaea* by *Pseudomonas savastanoi* pv. *savastanoi*

Alexandre Campos · Gonçalo da Costa · Ana Varela Coelho · Pedro Fevereiro

Received: 30 December 2008 / Accepted: 29 June 2009 / Published online: 17 July 2009

Abstract Olive knot disease is characterised by the development of galls on *Olea europaea* stems as a result of infection by *Pseudomonas savastanoi* pv. *savastanoi*. Protein differential accumulation during the first week of infection was studied using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry to investigate the biochemical changes occurring in infected tissues and to understand the factors involved in bacteria pathogenesis and plant response to infection. Common infection symptoms were obtained in 1 year-old plants of two Portuguese cultivars, 'Galega' and 'Cordovil de Serpa' using the strain NCPPB 2327. The comparison of protein patterns of non-inoculated stem tissues, stem tissues inoculated with water or with the strain NCPPB 2327 led to the detection of differentially expressed infection-related proteins. Moreover a distinct protein pattern was obtained between cultivars in response to infection. The differential protein expression was characterised by qualitative and quantitative variation. Among the differentially expressed proteins were the bacterial *P. savastanoi* orthologues of outer membrane porin F, the tellurium resistance protein, aconitate hydratase 2 and a hypothetical protein with unknown function. From *O. europaea*, protein orthologues of enolase and calcium-dependent protein kinase were found to be differentially expressed. Results are discussed in the context of the molecular basis of plant–pathogen interactions in the search for markers for the presence of the bacterium in plant tissues.

Keywords Olive knot disease · *Pseudomonas savastanoi* · Proteomics · Enolase · OprF

Abbreviations

DPE	Differential protein expression
MALDI-TOF	Matrix assisted laser-desorption ionisation—time of flight
IS	Inoculated stems
NIS	Non-inoculated stems
WIS	Water-inoculated stems
2-DE	Two-dimensional gel electrophoresis

A. Campos · G. da Costa · A. V. Coelho · P. Fevereiro
Instituto de Tecnologia Química e Biológica,
Universidade Nova de Lisboa,
Apart. 127,
2781-901 Oeiras, Portugal

P. Fevereiro (✉)
Departamento de Biologia Vegetal,
Faculdade de Ciências da Universidade de Lisboa,
Campo Grande,
1700 Lisboa, Portugal
e-mail: psalema@itqb.unl.pt

A. V. Coelho
Departamento de Química, Universidade de Évora,
7000-671 Évora, Portugal

Introduction

Olive knot disease is caused by the bacterium *Pseudomonas savastanoi*. Typical symptoms are the development of knots or galls in wounded sites of young twigs, branches and stems (Iacobellis et al. 1994). *Olea europaea* is a species with high resistance to bacterial attack, and *P. savastanoi* is one of the few pathogens able to cause serious injury to these trees. The natural resistance of *O. europaea* is attributed, in part, to the existence of a physical barrier of oleanolic acid crystals at the leaf surface and the production of secoiridoid glucosides, oleuropein and ligustroside antimicrobial derivatives (Cayuela et al. 2006). Verbascoside is a phenolic compound abundant in olive knots in contrast to other plant tissues. This compound may also play a role in the defence response of olive tree to *P. savastanoi* infection (Cayuela et al. 2006). Phenolic and/or polysaccharidic compounds have been shown to accumulate in outgrowths and xylem vessels of infected olive explants possibly as a reaction to hinder infections (Marchi et al. 2009).

Olive knot disease was reported to lower the yield and fruit quality in orchards (Schroth et al. 1968, 1973), and is present in many Portuguese olive orchards. The epiphytic bacterial population, as well as knots, function as bacterial reservoirs. Infection occurs in the rainy seasons (around April and November) when water transports the inoculum to wounded tissues. Infection can occur with temperatures ranging from 4–38°C but optimum temperatures are between 23–24°C (Cayuela et al. 2006). Bacterial cells in knots were shown to be able to migrate, infecting new wounds in plants (secondary infections) (Penyalver et al. 2006). A recent histological investigation on the systemic spread of *P. savastanoi* in olive explants (Marchi et al. 2009) described an interaction between *O. europaea* and *P. savastanoi* that closely resembles the formation of knots *in vivo*. The bacterium was also located in xylem vessels of the explants, suggesting a capacity for systemic invasion and induction of secondary infections in different parts of the plants.

Several factors such as the plant genotype, plant age, concentration of *P. savastanoi* at the infection sites and the interaction of this bacterium with other bacterial species have been shown to influence the development of the disease (Marchi et al. 2006; Penyalver et al. 2006). Knot formation is believed to be the consequence of local accumulation of indole-3-acetic acid (IAA) and cytokinin, both synthesised by the bacterium (Iacobellis et al. 1994). It was verified that hypersensitive response and pathogenicity genes (hrp) and hypersensitive response and conserved genes (hrc), responsible for encoding components of the type III secretion system are active in *P. savastanoi* and play a key role in pathogenicity and symptom development (Sisto et al. 2004). After a global genetic analysis of native plasmids from several *P. savastanoi* strains, Pérez-Martínez et al. (2008) reported the existence of putative new genetic determinants of *P. savastanoi* pathogenicity, namely a variety of insertion sequences and other gene sequences that hybridised with known *P. syringae* effector genes.

The biochemical changes that take place in plant tissues in response to *P. savastanoi* infection have not been studied and are essential to understand this disease and the genetic factors involved in the response of olive tree against this infection. Proteomics has been a suitable strategy to study overall biochemical changes in biological systems in response to stimuli. Proteomics has been applied to the study of plant–pathogen interactions with important achievements in the discrimination of plant and pathogen differentially expressed proteins (Marra et al. 2006; Natera et al. 2000), identification of pathogenesis-related (PR) proteins (Campo et al. 2004; Castillejo et al. 2004; Marra et al. 2006; Rep et al. 2002) and proteins involved in signal transduction and cellular stress (Campo et al. 2004; Pérez-Bueno et al. 2004; Subramanian et al. 2005).

The present work aims to characterise the response of ‘Galega’ and ‘Cordovil de Serpa’ plants to infection with *P. savastanoi* and to analyse protein variations in the infected tissues by two-dimensional gel electrophoresis (2-DE) and MALDI-TOF mass spectrometry to investigate the biochemical events occurring during infection. ‘Galega’ is a worldwide-known Portuguese cultivar that represents >50% of the total olive trees grown in Portugal, and is less susceptible to this phytopathogenic bacterium when compared with ‘Cordovil de Serpa’, a characteristic cultivar from the south of Portugal (Oliveira and Luz 2003). Within this study we were able to verify that protein variations in infected tissues are significantly distinct for both cultivars. Several proteins

from host and pathogen were identified by MALDI-TOF analysis that represent (1) early markers of the presence and growth of *P. savastanoi* in infected plant stems and (2) the differential molecular response of 'Galega' and 'Cordovil de Serpa'. This work describes an investigation into the molecular basis of the *O. europaea* and *P. savastanoi* interaction, involving a search for protein markers of the bacterial presence in infected plant tissues, therefore contributing to the control of the disease and the economic losses associated with it.

Materials and methods

Bacterial strains and culture

Pseudomonas savastanoi type strains NCPPB 1506 and NCPPB 2327 from the National Collection of Plant Pathogenic Bacteria (York, UK) were grown in nutrient agar (NA), Luria Bertani (LB) (Bertani 1951) or *Pseudomonas* agar F media (King et al. 1954), in the dark at 28°C. Liquid cultures were grown at 180–200 rpm in nutrient broth (NB) or LB. Suspensions in exponential growth (O.D. $_{600\ nm}$=0.8) were used for plant inoculation.

Plant material and plant infection

One year-old plants of *O. europaea* subsp. *europaea* cvs Galega and Cordovil de Serpa were maintained in the greenhouse, under natural light conditions, at 24± 4°C, in 500 ml buckets containing the substrate 'Terra de Montemor' (A Estufa, Portugal) under adequate phytosanitary conditions.

Plants were infected with *P. savastanoi* following a procedure adapted from Surico et al. (1984). Small wounds were made in stems with a needle and 20 µl of a bacterial suspension in distilled water (10^9 cfu ml^{-1}) were injected. The excess of inoculum outside the wounds was removed. Ten wounds were made in each plant, starting from the fifth internode below the apex and leaving one internode between infections. Analysis of disease development was evaluated by wound visual inspection after three and six weeks of infection. *Pseudomonas savastanoi* pathogenicity was verified by the ability to induce knots or necrotic swellings in infected stem sites. Disease development was monitored by determining the percentage of wounds that produced knots or necrotic swellings in each plant, and registering the wound diameter.

For the analysis of protein expression the plants were: (1) wounded in stems and inoculated with *P. savastanoi* (2) wounded and inoculated with sterile distilled water or (3) not wounded or inoculated. Four plants were used as replicates, for each treatment and time of sampling. Stem samples (approximately 0.5 cm×0.5 cm×0.2 cm) from inoculated sites were collected at one, four and seven days after inoculation, frozen in liquid nitrogen and stored at −80°C.

Protein extraction

Plant material was ground in liquid nitrogen and proteins extracted as described by Costa et al. (1998) with the following adaptations: 2 ml of protein precipitation solution was used per 0.1 g of plant material and UKS buffer was replaced by dithiothreitol (10 mM), thiourea (2 M), triton X-100 (0.4%, *v*/*v*), urea (7 M), 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS) (4%, *w*/*v*), IPG buffer pH 3–10 (1%, *v*/*v*) as described by Gion et al. (2005) plus polyvinylpolypyrrolidone (PVPP) (0.025 mg ml^{-1}) in a ratio of 1 ml of solubilisation solution per 0.05 g pellet. Protein samples were clarified at 10,000 *g* for 30 min (4°C) and stored at −80°C. Protein concentration was determined by the Bradford method, using the Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as standard.

Two-dimensional gel electrophoresis of proteins

Samples with 80 µg of protein (analytical gels) or 600 µg of protein (preparative gels) were diluted to 470 µl in CHAPS (2%, *w*/*v*), urea (8 M), IPG buffer pH 3–10 (0.5%, *v*/*v*) dithiothreitol (0.02 mM) and used in Isoelectric Focusing (IEF) as the first-dimension protein separation technique. Electrophoresis was performed according to Görg et al. (2000) in the IPGphor system (Amersham Pharmacia Biotech, Uppsala, Sweden), using immobiline drystrips of 24 cm with a linear pH gradient from 3 to 10 (Amersham Pharmacia Biotech, Uppsala, Sweden) and started with 30 V for 12 h followed by 1 h at 200 V, 1 h at 500 V, 1 h at 1,000 V, a voltage gradient

of 30 min up until 8,000 V and finally 8,000 V h^{-1} to achieve 50,000 V h^{-1} (adapted from Gion et al. 2005). After IEF the strips were stored at −80°C. For the second-dimension protein separation IEF strips were equilibrated according to Görg et al. (2000) and using 10 mg ml^{-1} dithiothreitol and 25 mg ml^{-1} iodoacetamide. IEF strips were placed on top of 12% (*w*/*v*) acrylamide SDS-PAGE slab gels (20 cm×25 cm× 1 cm) and the second-dimension protein separation performed in a Hoeffer DALT electrophoresis system (Amersham Pharmacia Biotech, Uppsala, Sweden) at 125 V. Analytical gels were silver-stained according to Blum et al. (1987) and preparative gels stained by Coomassie Blue Colloidal as described by Consoli and Damerval (2001).

Gel analysis

Images of analytical gels were acquired in the Computing Densitometer, model 325E (Molecular Dynamics, Sunnyvale, CA, USA) and protein spots detected with the 2D Image Master, version 4.1 (Amersham Biosciences, Uppsala, Sweden) automatically reproducing the sensitivity parameters for every gel image. Initial spot volumes determined by the programme were normalised automatically by multiplying the total spot area in the gel by the ratio between the volume of each particular spot and total spot volume of the gel. A linear scaling method (Burstin et al. 1993) was also used to obtain protein spot volume normalisation. The method consisted in applying Eqs. 1 and 2 below:

$$K_j = Y_{..}/Y_{.j} \quad (1)$$

$$Y_{ij}corr = Y_{ij} \times K_j \quad (2)$$

where Y_{ij} *corr* is the normalised volume of spot *i* in gel *j*; Y_{ij}, the volume of spot *i* in gel *j*, K_j the linear scaling factor to reduce the global experimental variation between gels, $Y_{..}$ the mean volume of the spots from all gels in study and $Y_{.j}$ the mean volume of the spots from gel *j*. Normalisation methods were reproduced for every gel image.

Protein expression analysis

Protein samples from plant replicates were subjected to 2-DE. A set of four 2-DE gels, relating to four biological replicates (four plants), was obtained for each tissue condition, cultivar and sampling time-point. The first analysis consisted of the detection of a reproducible protein pattern (qualitative information) regarding each tissue condition, cultivar and sampling time, after 4 days (t1) and 7 days (t2) of infection, and named as 2-DE maps. A mean normalised volume was calculated for every reproducible protein spot (quantitative information). Data from 2-DE gels regarding the first day of infection were not subjected to subsequent analysis and used only to assist the analysis of the expression of some proteins displaying variations at t1 and t2. The second analysis consisted of the comparison of non-inoculated stems (NIS), water-inoculated stems (WIS) and inoculated stems (IS), 2-DE maps regarding one cultivar and one sampling time (t1 or t2). From this analysis we detected protein qualitative (presence/absence of proteins) and quantitative variations (changes in the volumes of protein spots). Mean volume comparison was performed with the Kruskal-Wallis test and *t*-test for unequal variances ($P<0.05$). Significant differences were taken into account when, at least, accordance was verified for the *t*-test regarding the two normalisation methods.

Protein identification

Proteins from preparative gels were isolated and subjected to trypsin digestion as described by Pandey et al. (2000). Sample peptides were co-crystallised in MALDI-TOF sample plates according to Da Costa et al. (2008) using the matrix α-cyano-4-hydroxycinnamic acid (CHCA) or, as an alternative, 2,5- dihydroxybenzoic acid (DHB). Peptide mass spectra acquisition, in the Voyager-DESTR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA), and protein identification were performed essentially as described by Da Costa et al. (2008) using the Mass Spectrometry protein sequence DataBase (MSDB; 20060529x) (http://csc-fserve.hh.med.ic.ac.uk/msdb.html). In the case of unsuccessful identifications the homology search was repeated restricted to databases of the taxonomy groups eubacteria and viridiplantae or the species *Arabidopsis thaliana*, performing internal calibration and elimination of keratin and trypsin contaminant masses, or submitting the sum of peptide mass lists from PMF obtained with matrix CHCA or DHB for the same protein.

Results

Pseudomonas savastanoi pathogenicity

The pathogenicity of two *P. savastanoi* strains (NCPPB 1506 and NCPPB 2327) were first assessed to select a strain able to induce the disease symptoms in the cultivars under study. Only strain NCPPB 2327 was able to induce symptoms on 1 year-old 'Galega' and 'Cordovil de Serpa' plants, promoting the development of knots and necrotic swellings at the inoculation sites (Fig. 1). The pathogenicity of this strain was confirmed by comparing the symptoms registered in the controls (inoculated with water). In this case only a slight swelling at the inoculation point followed by lignification and no tissue proliferation was observed, as shown in Fig. 1. Strain NCPPB 2327 was therefore used in subsequent experiments.

Fig. 1 Symptoms produced in olive plants (Galega cultivar) after a 6 week infection interval with *P. savastanoi* strain NCPPB 2327. Control wound (**a**); knot (**b**); necrotic swelling (**c**). The *arrows* indicate inoculation sites

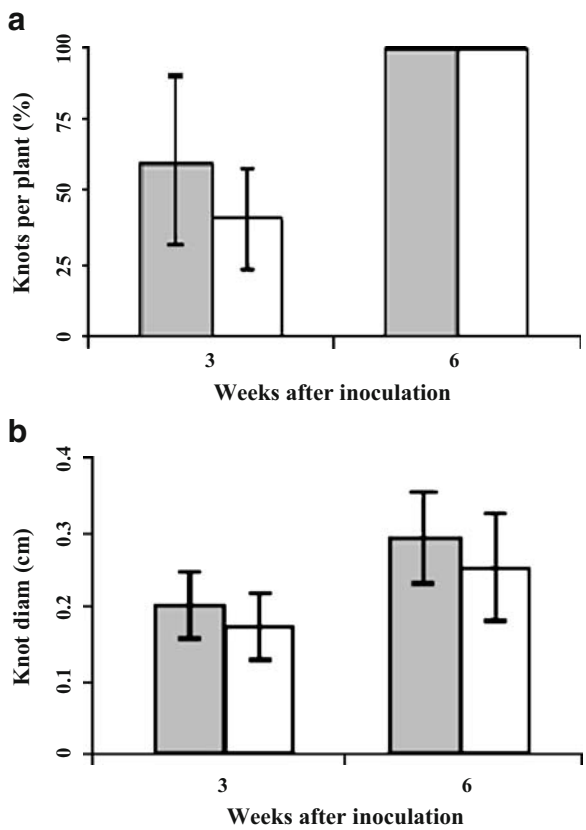

Fig. 2 Disease development in 'Cordovil de Serpa' (*grey bars*) and 'Galega' (*white bars*) plants upon infection with *P. savastanoi* strain NCPPB 2327 after 3 and 6 weeks of infection. (**a**) Percentage of inoculation sites developing knots per plant; (**b**) knot mean diameter. In (**b**) bar values are means of three repetitions (n=3) for 'Cordovil de Serpa' and four repetitions (n=4) for 'Galega'. *Bars* represent standard deviations

The response of 'Galega' and 'Cordovil de Serpa' plants to infection with the strain NCPPB 2327 is shown in Fig. 2. After 3 weeks of infection all plants developed knots or necrotic swellings. However the percentage of inoculation sites that developed these symptoms in each plant was highly variable (Fig. 2a). After 6 weeks all infected plants developed knots (Fig. 2a). Significant increases in knot diameter were verified in infected plants of both cultivars, after 6 weeks of infection (Fig. 2b).

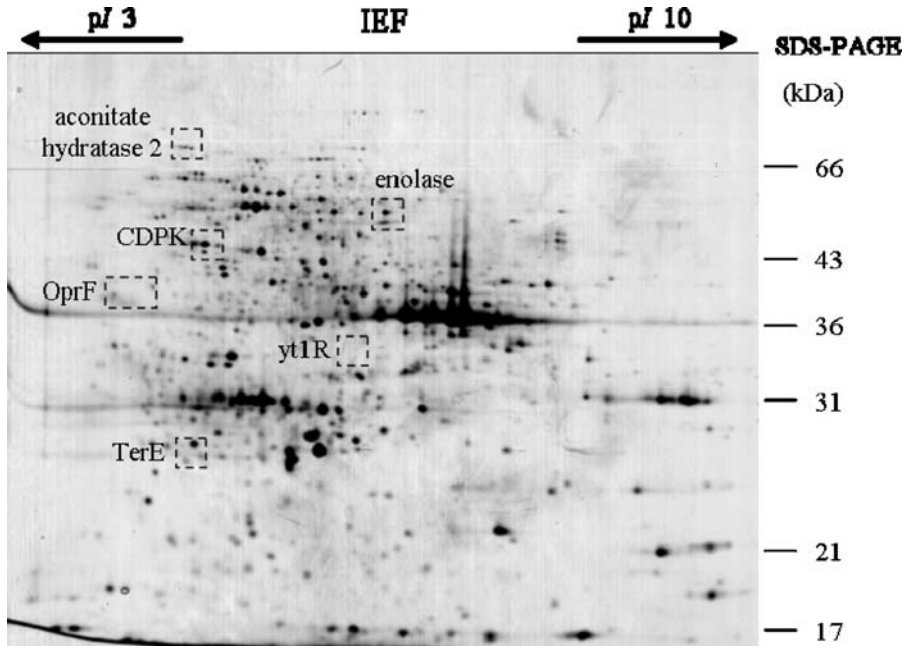

Fig. 3 2-DE gel from 'Codovil de Serpa' non-inoculated stems (NIS). The gel was loaded with 80 μg protein and silver-stained. Proteins were first separated by IEF in a pH range of 3 to 10 and further by SDS-PAGE in 12% (*w*/*v*) acrylamide gels. Protein variation displayed by identified proteins is documented in subsequent figures of this work from magnified images of gel areas in squares

Protein differential expression in infected 'Galega' and 'Cordovil de Serpa' and protein identification

For each cultivar, a comparative analysis of 2-DE maps was performed considering the fourth (t1) and seventh (t2) day after infection of the NIS, WIS and IS. A characteristic 2-DE gel from stems of olive plants is presented in Fig. 3.

A total of 35 and 42 proteins showing quantitative and qualitative variation associated with infection and/or wounding, were detected in 'Galega' and 'Cordovil de Serpa' plants, respectively (results not shown). Qualitative variations represented 69% and 31% of overall DPE detected in 'Galega' and 'Cordovil de Serpa' stem tissues, respectively. Qualitative DPE was cultivar-specific except for a group of six proteins that were detected in IS in both cultivars (this protein group is discussed further in this work). On the other hand all quantitative protein variation obtained was cultivar-specific (results not shown). In 'Galega' IS the majority of qualitative DPE was detected after 4 days of infection and reproduced after 7 days of infection. Quantitative DPE was mainly detected after 7 days of infection. In 'Cordovil de Serpa' the majority of proteins displaying qualitative variation was detected after 7 days of infection. As opposed to 'Galega', 14 proteins displaying quantitative variation were detected in 'Cordovil de Serpa' IS after 4 days of infection.

Nevertheless such DPE was not reproduced after 7 days of infection, instead 15 different proteins, according to their position in the gels, displayed quantitative variation in 'Cordovil de Serpa' IS (results not shown). To assess the function of differentially expressed proteins and relate its expression with the biological system under study, we conducted protein identification by MALDI-TOF peptide mass fingerprint (PMF). A putative identification of seven proteins displaying variation upon infection, using MALDI-TOF peptide mass fingerprint (PMF), was possible out of fifteen analysed (Table 1). Four proteins, outer membrane porin F (OprF), aconitate hydratase 2, unknown protein yt1R and tellurium resistance (TerE) protein were putatively identified as bacterial proteins. Three plant proteins, two enolase isoforms and a calcium-dependent protein kinase (CDPK), were also identified (Table 1).

Bacterial proteins in infected stem tissues

All *P. savastanoi* protein orthologues putatively identified, with the exception of TerE orthologue,

Table 1 Differentially expressed proteins identified by peptide mass fingerprint in both Galega and Cordovil de Serpa cultivars

Spot # [a]	Peptides: total submitted/matched[b]	Estimated MW[c]	Theor.MW (kDa)/pI[d]	Coverage (%)[e]	Homology score/ minimum score[f]	Protein name (AC number)[g]	Species
CC1	21/9	42	36.8/4.77	25	107/77	OprF (Q48JY8_PSE14)	*Pseudomonas syringae*
CC3	33/17	Nd[h]	94.9/5.2	14	74/74	Aconitate hydratase 2 (Q4ZVP8_PSEU2)	*Pseudomonas syringae*
CC5	19/7	35	35.7/9.7	31	74/74	hypothetical protein ytlR (H69995)	*Bacillus subtilis*
G7	43/8	26	20.2/4.8	48	90/77	Tellurium resistance protein TerE (Q48NA2_PSE14)	*Pseudomonas syringae*
G27	23/10	52.7	48/5.5	20	109/77	Enolase (JQ1187)	*Arabidopsis thaliana*
G28	39/12	51.6	47.8/6.1	27	80/77	Enolase (Q6WB92_GOSBA)	*Gossypium barbadense*
CS36	46/10	47	59.1/6.1	24	60/60	probable calcium dependent protein kinase (F85059)	*Arabidopsis thaliana*

[a] *CS* cv. Cordovil de Serpa; *G* cv. Galega; *CC* present in both cultivars

[b] Number of peptides masses: submitted to PMF search/match with those from the theoretical PMF

[c] Estimated molecular weight from protein standards in 2DE gels and image analysis tools

[d] Determined molecular weight and isoelectric point for orthologous protein

[e] Percentage of protein sequence covered by matched peptides

[f] Top score for the homology considered/minimum score for significant identification ($P<0.05$)

[g] Accession number from mass spectrometry protein sequence database (MSDB)

[h] Not determined due to the absence of high MW protein standards that allow reliable estimation of protein MW

Accession numbers are from MSDB database

were detected in 'Galega' and 'Cordovil de Serpa' IS (Fig. 4). The TerE orthologue was only detected in 'Galega' IS. Nevertheless all proteins were absent in NIS and WIS of both cultivars as shown in Fig. 4.

The presence of these proteins was investigated by 2-DE in stem tissues after 1 day of infection. OprF orthologue was the only protein detected in 'Galega' and 'Cordovil de Serpa' IS 1 day after infection. This protein increased significantly in abundance (evaluated as normalised volume) in the seventh day after infection (200 times the normalised volume in 'Galega') but in the IS no significant differences were found in the levels of the protein between both cultivars.

Plant proteins

Enolase orthologues in 'Galega' stem tissues increased in abundance (evaluated as normalised volume) during infection with *P. savastanoi* (Fig. 5). Moreover we verified that the two isoforms present a distinct expression pattern, G28 being the predominant form in stem tissues but G27 isoform displaying the highest increase in abundance upon infection (4.8-fold increase of the normalised volume determined for NIS, in comparison to the 1.5-fold increase in G28 isoform). This increase in abundance of G27 isoform was significant in respect to NIS and WIS (Fig. 5).

The calcium-dependent protein kinase in 'Cordovil de Serpa' stem tissues displayed a significant decrease in abundance upon infection (2.7-fold decrease of the normalised volume determined in NIS). This decrease did not occur in WIS tissues indicating that the quantitative variation is specific for the infection condition.

Discussion

Pseudomonas savastanoi attack is easily detected in the field by the development of knots in stems of olive trees. In this work we were able to reproduce the typical disease symptoms by infecting 1 year-old *O. europaea* subsp. *europaea* plants from 'Galega' and

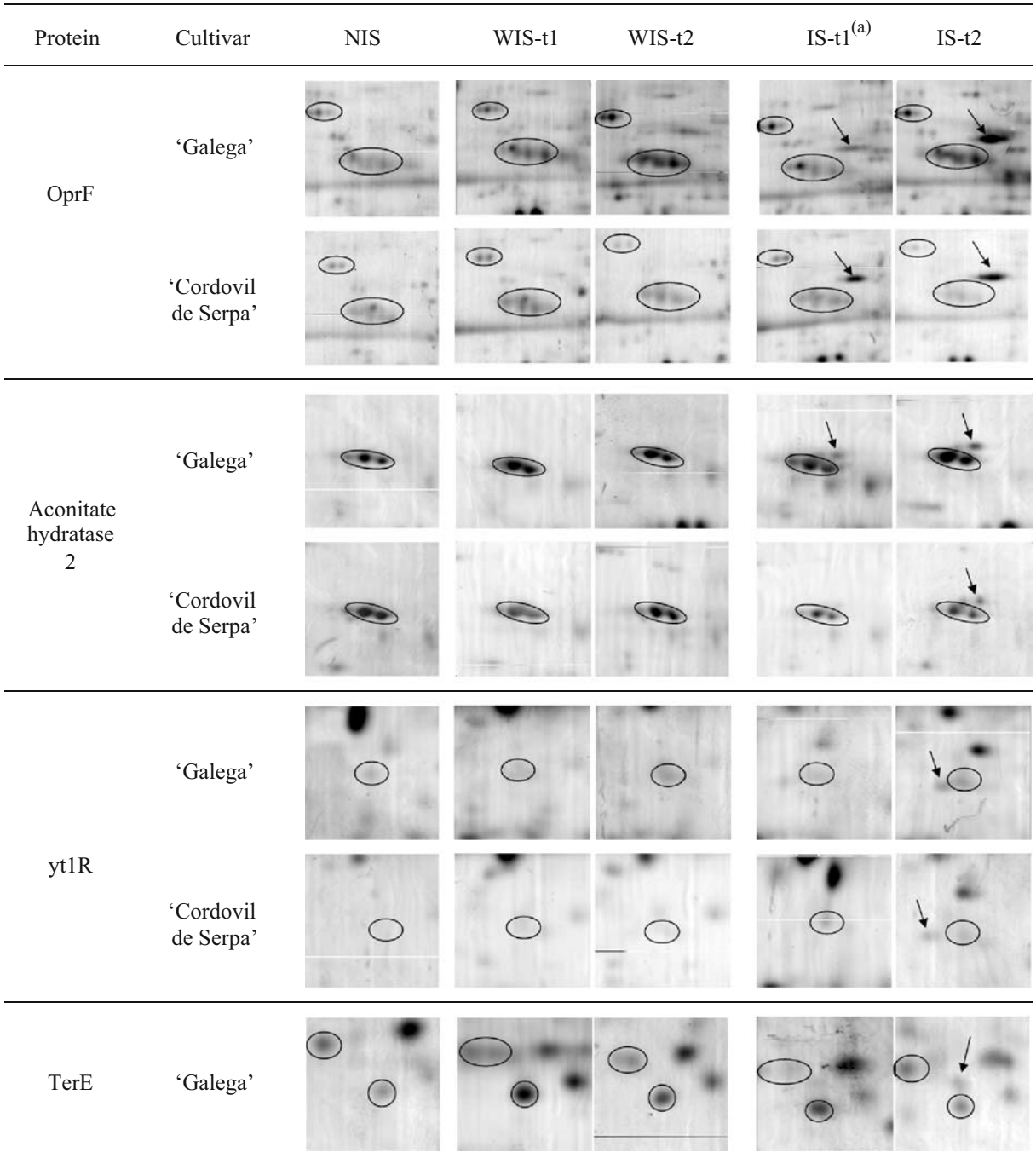

Fig. 4 Differential expression of OprF, aconitate hydratase 2, yt1R and TerE orthologues in 'Galega' and 'Cordovil de Serpa' stem tissues during infection by *P. savastanoi*. Non-inoculated stems (NIS), water-inoculated stems after 4 (WIS-t1) and 7 days of inoculation (WIS-t2) and inoculated stems (IS) after 4 days (IS-t1) and 7 days of infection (IS-t2). (a) The orthologous protein was considered absent in IS-t1 when its detection was not confirmed in the four biological replicates. Reference protein spots are marked in a circle and orthologous proteins with an arrow

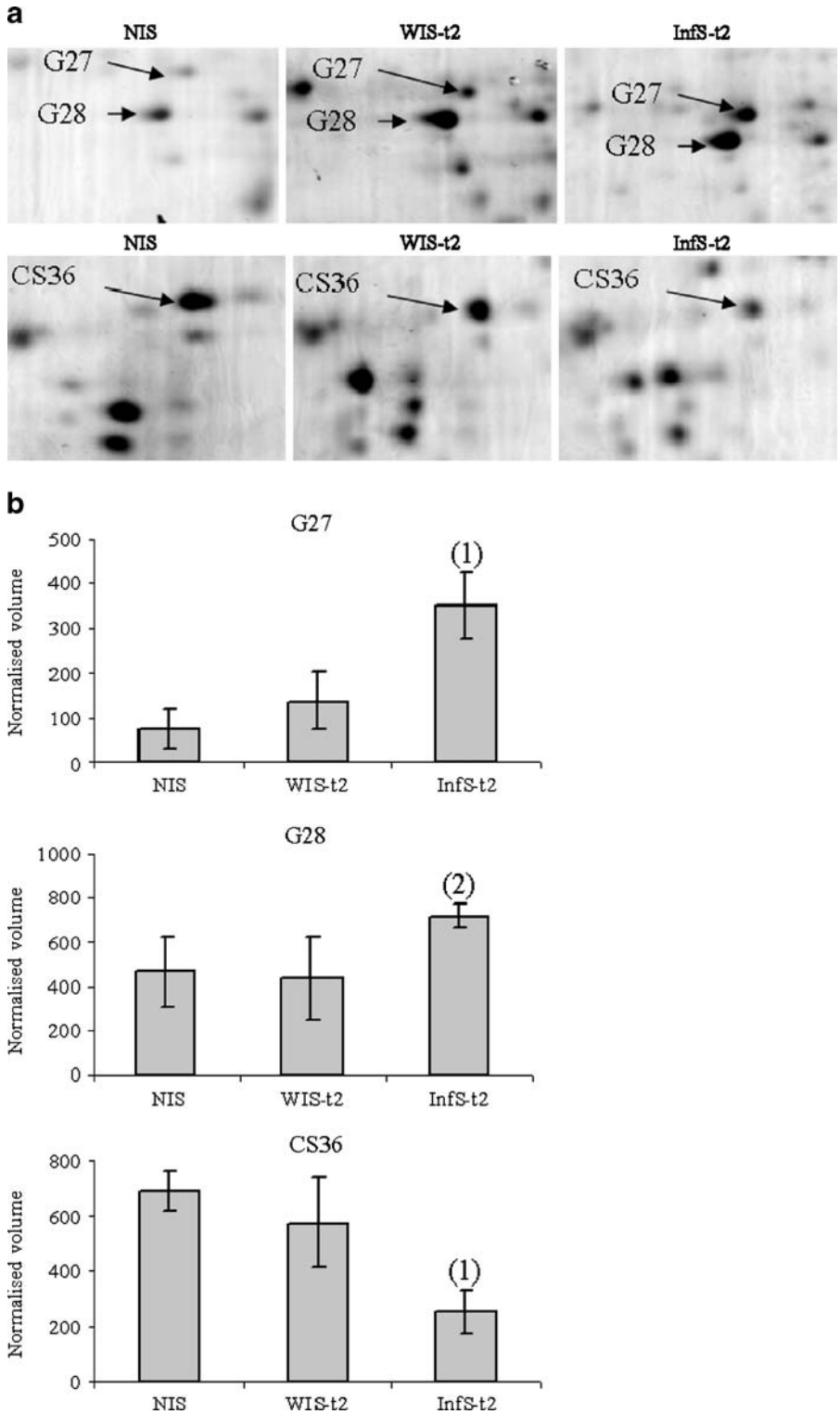

Fig. 5 Expression of enolase (G27, G28) and CDPK (CS36) orthologues in 'Galega' and 'Cordovil de Serpa' stem tissues respectively. Gel images reporting protein expression (**a**) and representation of normalised volumes (**b**). Non-inoculated stem tissues (NIS), water-inoculated stem tissues collected after 7 days of inoculation (WIS-t2) and inoculated stem tissues after 7 days of infection (IS-t2). Differences regarding NIS and WIS-t2 (1), and differences regarding NIS (2) were considered significant at $P<0.05$

'Cordovil de Serpa' with *P. savastanoi* type strain NCPPB 2327. Knots or necrotic swellings in infected stem wounds started to appear after 3 weeks of infection and at the sixth week all infected wounds developed knots or necrotic swellings. The disease could be monitored by the percentage of knots per plant or by the increase in knot diameter. The 'Galega' and 'Cordovil de Serpa' plants were both susceptible to the strain NCPPB 2327. 'Galega' is generally accepted to be less susceptible to this disease than 'Cordovil de Serpa'. This statement is supported by observations in the orchards and by a field trial that describes the existence of lower infection levels in 'Galega' by comparison to 'Cordovil de Serpa' trees in the *Beira Interior* region of central Portugal (Oliveira and Luz 2003). The differences between the results achieved and the expected regarding the susceptibility of both cultivars to the bacterium lead us to consider that tolerance to *P. savastanoi* infection in 'Galega' may be related to the plant stage of development. Nevertheless other factors are likely to influence the plant response to infection especially the bacterial strain and inoculum concentration. In a recent study performed by Penyalver et al. (2006) to evaluate the influence of strains, dose of inoculum and plant age in *O. europaea* response to *P. savastanoi*, the authors reported interactions between cultivars and strains and a high dependence of disease severity on pathogen dose applied at the wound sites. The authors were able to discriminate cultivar susceptibility by evaluating the development and weight of primary knots and the presence of secondary knots in 3 year-old plants infected with low inoculum concentrations (10^2 CFU/wound). High inoculum concentrations (>10^4 or 10^5 CFU/wound) can mask differences between cultivars leading to a rate of disease development >75% regardless of the cultivar. They also state that 1 year-old plants were too susceptible to be useful in discriminating susceptibility among cultivars. Taking into consideration Penyalver et al. (2006) observations the infection conditions used in our work are suitable to reproduce the disease symptoms but not to evaluate the differences between 'Galega' and 'Cordovil de Serpa' susceptibility to *P. savastanoi*.

The first week of *O. europaea–P. savastanoi* interaction was further studied by two-dimensional gel electrophoresis of proteins and MALDI-TOF mass spectrometry with the aim to describe protein changes occurring at the infection site and identify proteins that can be associated with bacterial pathogenicity and plant response to infection. Protein variation was detected with this approach that could be related to infection. Although 82.5% and 95% of protein spots were reproducible in NIS 2-DE maps from 'Galega' and 'Cordovil de Serpa' respectively, a considerable percentage of DPE detected was cultivar-specific, indicating that biochemical responses in 'Galega' and 'Cordovil de Serpa' to *P. savastanoi* are distinct and likely to be genotype-dependent. MALDI-TOF mass spectrometry allowed us to putatively identify four *P. savastanoi* and three *O. europaea* protein orthologues from IS. The increase in abundance of the *P. savastanoi* OprF orthologue during the 7 days of infection and the detection of aconitate hydratase 2, yt1R and TerE orthologues after 4 or 7 days of infection, suggests an increase in the bacterial population in IS or an effective up-regulation of the expression of the genes codifying those proteins. These proteins may be regarded as potential markers for the presence of the bacterium in infected plant tissues and to evaluate the progression of infection.

Marchi et al. (2006) reported the growth of *P. savastanoi* population at infected sites in *O. europaea*. Starting from an inoculation with approximately 10^5–10^6 CFU per site, the authors reported an increase of the bacterial population to 1.6×10^7 CFU per site in the first 7 days of infection, increasing steadily to 3.7×10^8 CFU per site after 42 days of infection, further stabilising and achieving 7.2×10^8 CFU g^{-1} after 120 days of infection. Such growth curves can be correlated with the levels of bacterial proteins to assess their potential as markers.

OprF is the major surface protein of the genus *Pseudomonas* (Bodilis et al. 2006). It is also considered a non-specific porin, being involved in other functions such as maintenance of cell shape, growth in low-osmolarity environment and adhesion to supports and molecules (Bodilis and Barray 2006). The OprF gene has been regarded as an important marker for the presence of *Pseudomonas* in plant tissues and to characterise bacterial ecological niches (Bodilis et al. 2006). Our results demonstrate that the OprF gene product can be also detected in infected plant tissues and concomitant with the gene sequence, may function as a molecular marker of bacterial presence in infected plant tissues. Moreover in animal systems, this family of proteins present antigenic properties and is involved in the virulence of pathogenic strains (Malhotra et al.

2000; Price et al. 2001; Williams et al. 2000) raising the question of the potential involvement of the homologous proteins in the virulence of phytopathogenic *Pseudomonas*.

Aconitate hydratase catalyses the isomerisation of citrate to isocitrate via *cis*-aconitate in the citric acid cycle (Artymiuk and Green 2006). In the context of this biological system aconitate hydratase expression may be associated with a change in bacterial metabolism that leads to an up-regulation of the enzymes of the aerobic respiratory pathway and energy production. The *ter* gene family confers resistance to tellurite in several human pathogenic *Escherichia coli* strains allowing their identification through the use of selection media containing potassium tellurite (Orth et al. 2007).

Nevertheless, to the best of our knowledge, the function of the proteins encoded by this gene family in host-pathogen interactions is not known. The *P. savastanoi* terE orthologue was only detected in 'Galega' IS, pointing to the fact that the *P. savastanoi* proteome can change, during infection, as a function of plant genotype. Similarly to the OprF orthologue, these proteins may be early markers of bacterial proliferation in infected olive tissues and of disease progression.

The 'Cordovil de Serpa' CDPK orthologue was repressed to about 1/3 in infected stems when compared with non-infected stems. The CDPKs are calcium-binding serine/threonine protein kinases and are considered to be major primary calcium sensors in plants. The CDPK activation is triggered by biotic and abiotic stresses (Ludwig et al. 2005). In plant–microbe interactions the activities of CDPK have been related to the hypersensitive reaction (HR) and activation of plant resistance genes (Romeis et al. 2001). The decrease in abundance displayed by the CDPK homologue protein in 'Cordovil de Serpa' IS seems to contradict the reported CDPK activation in situations of infection or elicitation. This reduction in expression of a CDPK isoform may have implications in the susceptibility of 'Cordovil de Serpa', nevertheless complementary data is needed to corroborate our results, and to correlate the expression of this isoform with plant physiological response.

Two plant enolase orthologues were identified in stem tissues of *O. europaea* plants. The expression of the two isoforms increased significantly in 'Galega' but at different ratios. Enolase is an enzyme of the glycolytic pathway converting the 2-phosphoglycerate to phosphoenolpyruvate. Glycolysis is a ubiquitous pathway for the production of ATP in anaerobic conditions, and enzymes of this pathway, including enolase, are induced in tissues under these stress conditions (Fukao et al. 2003; Sato et al. 2002). Expression of this protein has been related also with plant response to abiotic stress (Kollipara et al. 2002; Riccardi et al. 1998). Regarding plant–microbe interactions a 2,77-fold increase in enolase activity was found in tumours induced by *Agrobacterium tumefaciens* in *Beta vulgaris* (Scott et al. 1964). Two enolase transcripts were early-induced by the mildew fungus in tomato, one in both a susceptible or resistant genotype and other only in the resistant R-Ol-1 (Li et al. 2006). Two enolase transcripts were also identified at the peribacteroid membrane of *Lotus japonica* root nodules (Wienkoop and Saalbach 2003) and enolase is highly expressed in the pericycle cells of actinorhizal plants (Franche et al. 1998). α-enolase is known to play a role in systemic and invasive autoimmune disorders in vertebrates. Research on this protein suggests multifunctional properties. For example α-enolase is expressed on the surface of a variety of eukaryotic cells as a strong plasminogen-binding receptor and may play an important role in the initiation of disease processes by modulating the pericellular and intravascular firinolytic system (Pancholi 2001). On the other hand α-enolase and its alternative translation initiation product (MBP-1) have been described as potential tumour-suppresser proteins in human cancers controlled by the myelocytomatosis proto-oncogene promoter (c-myc) (Subramanian and Miller 2000). The multifunctional properties of this protein in vertebrates and humans suggest that in plants enolase may have different functions besides its participation in the glycolitic pathway, being probably a relevant component of the plant response to bacterial infections. Since its differential expression could only be detected in 'Galega' and not in the susceptible cv. 'Cordovil de Serpa', it may be an important marker for tolerance to *P. savastanoi* in olive. In this way it will be important to correlate its expression with the tolerant phenotype of 'Galega' to *P. savastanoi* infection.

Using proteomics we revealed some of the possible molecular players involved in the interaction between olive and *P. savastanoi*, the pathogen responsible for olive knot disease. The identified proteins are regarded as potential markers of bacterial presence

in plant tissues and of disease progression, as well as in the differentiation of 'Galega' and 'Cordovil de Serpa' responses. Nevertheless their implications in pathogenesis and resistance/tolerance need further validation. This will imply a strong correlation between proteomic analysis and plant resistance/tolerance responses.

This work constitutes, to the best of our knowledge, the first proteomics-driven approach using these two species and is, therefore, a relevant breakthrough in the investigation of *O. europaea–P. savastanoi* interactions. Advances in this research require an increase in the sensitivity of the proteomic approach, including using tandem mass spectrometry and *de novo* protein sequencing for a comprehensive characterisation of protein variation. The differential expression of the identified proteins should also be analysed in incompatible olive-*P. savastanoi* interactions, to confirm specific expressions in the response of a compatible infection-inducting process at the transcriptomic level.

Acknowledgements This work was supported by the project SAPIENS 36239/99, entitled 'Study of the resistance of Portuguese *Olea europaea* subsp. *europaea* to *Pseudomonas savastanoi*'. A. Campos acknowledges a PhD grant from Fundação para a Ciência e Tecnologia (Praxis XXI/BD/19882/1999) of Ministério da Ciência, Tecnologia e Ensino Superior of Portugal. Acknowledgement is due to Dr. Christophe Plomion from INRA-Pierroton, France, for receiving A. Campos and training him in 2-DE and Dr. André Almeida from IICT, Portugal, for the revision of this manuscript and suggestions.

References

Artymiuk, P. J., & Green, J. (2006). The double life of aconitase. *Structure (London, England), 14*, 2–4. doi:10.1016/j.str.2005.12.001.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology, 62*, 293–300.

Blum, H., Beier, H., & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis, 8*, 93–99. doi:10.1002/elps.1150080203.

Bodilis, J., & Barray, S. (2006). Molecular evolution of the major outer-membrane protein gene (oprF) of *Pseudomonas*. *Microbiology, 152*, 1075–1088. doi:10.1099/mic.0.28656-0.

Bodilis, J., Hedde, M., Orange, N., & Barray, S. (2006). OprF polymorphism as a marker of ecological niche in *Pseudomonas*. *Environmental Microbiology, 8*, 1544–1551. doi:10.1111/j.1462-2920.2006.01045.x.

Burstin, J., Zivy, M., De Vienne, D., & Damerval, C. (1993). Analysis of scaling methods to minimize experimental variations in two-dimensional electrophoresis quantitative data: application to the comparison of maize inbred lines. *Electrophoresis, 14*, 1067–1073. doi:10.1002/elps.11501401170.

Campo, S., Carrascal, M., Coca, M., Abián, J., & San Segundo, B. (2004). The defense response of germinating maize embryos against fungal infection: a proteomics approach. *Proteomics, 4*, 383–396. doi:10.1002/pmic.200300657.

Castillejo, M. A., Amiour, N., Dumas-Gaudot, E., Rubiales, D., & Jorrín, J. V. (2004). A proteomic approach to studying plant response to crenate broomrape (*Orobanche crenata*) in pea (*Pisum sativum*). *Phytochemistry, 65*, 1817–1828. doi:10.1016/j.phytochem.2004.03.029.

Cayuela, J. A., Rada, M., Rios, J. J., Albi, T., & Guinda, A. (2006). Changes in phenolic composition induced by *Pseudomonas savastanoi* pv. *savastanoi* infection in olive trees: presence of large amounts of verbascoside in nodules of tuberculosis disease. *Journal of Agricultural and Food Chemistry, 54*, 5363–5368. doi:10.1021/jf060807w.

Consoli, L., & Damerval, C. (2001). Quantification of individual zein isoforms resolved by two-dimensional electrophoresis: genetic variability in 45 maize inbred lines. *Electrophoresis, 22*, 2983–2989. doi:10.1002/1522-2683(200108)22:14<2983::AID-ELPS2983>3.0.CO;2-#.

Costa, P., Bahrman, N., Frigerio, J.-M., Kremer, A., & Plomion, C. (1998). Water deficit responsive proteins in maritime pine. *Plant Molecular Biology, 38*, 587–596. doi:10.1023/A:1006006132120.

Da Costa, G., Lamy, E., Silva, F. C., Andersen, J., Baptista, E. S., & Coelho, A. V. (2008). Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins. *Journal of Chemical Ecology, 34*, 376–387. doi:10.1007/s10886-007-9413-z.

Franche, C., Laplaze, L., Duhoux, E., & Bogusz, D. (1998). Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. *Critical Reviews in Plant Sciences, 17*, 1–28. doi:10.1016/S0735-2689(98)00356-6.

Fukao, T., Kennedy, R. A., Yamasue, Y., & Rumpho, M. E. (2003). Genetic and biochemical analysis of anaerobically-induced enzymes during seed germination of *Echinochloa crus-galli* varieties tolerant and intolerant of anoxia. *Journal of Experimental Botany, 54*, 1421–1429. doi:10.1093/jxb/erg140.

Gion, J.-M., Lalanne, C., Le Provost, G., Ferry-Dumazet, H., Paiva, J., Chaumeil, P., et al. (2005). The proteome of maritime pine wood forming tissue. *Proteomics, 5*, 3731–3751. doi:10.1002/pmic.200401197.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., et al. (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis, 21*, 1037–1053. doi:10.1002/(SICI)1522-2683(20000401)21:6<1037::AID-ELPS1037>3.0.CO;2-V.

Iacobellis, N. S., Sisto, A., Surico, G., Evidente, A., & DiMaio, E. (1994). Pathogenicity of *Pseudomonas syringae* subsp. *savastanoi* mutants defective in P. production. *Journal of Phytopathology, 140*, 238–248. doi:10.1111/j.1439-0434.1994.tb04813.x.

King, O. E., Ward, M. K., & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *The Journal of Laboratory and Clinical Medicine, 44*, 301–307.

Kollipara, K. P., Saab, I. N., Wych, R. D., Lauer, M. J., & Singletary, G. W. (2002). Expression profiling of reciprocal maize hybrids divergent for cold germination and desiccation tolerance. *Plant Physiology, 129*, 974–992. doi:10.1104/pp.000729.

Li, C., Bai, Y., Jacobsen, E., Visser, R., Lindhout, P., & Bonnama, G. (2006). Tomato defence 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. doi:10.1007/s11103-006-9008-z.

Ludwig, A. A., Saitoh, H., Felix, G., Freymark, G., Miersch, O., Wasternack, C., et al. (2005). Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 10736–10741. doi:10.1073/pnas.0502954102.

Malhotra, S., Silo-Suh, L. A., Mathee, K., & Ohman, D. E. (2000). Proteome analysis of the effect of mucoid conversion on global protein expression in *Pseudomonas aeruginosa* strain PAO1 shows induction of the disulfide bond isomerase, DsbA. *Journal of Bacteriology, 182*, 6999–7006. doi:10.1128/JB.182.24.6999-7006.2000.

Marchi, G., Sisto, A., Cimmino, A., Andolfi, A., Cipriani, M. G., Evidente, A., et al. (2006). Interaction between *Pseudomonas savastanoi* pv. *savastanoi* and *Pantoea agglomerans* in olive knots. *Plant Pathology, 55*, 614–624. doi:10.1111/j.1365-3059.2006.01449.x.

Marchi, G., Mori, B., Pollacci, P., Mencuccini, M., & Surico, S. (2009). Systemic spread of Pseudomonas savastanoi pv. savastanoi in olive explants. *Plant Pathology, 58*, 152–158. doi:10.1111/j.1365-3059.2008.01935.x.

Marra, R., Ambrosino, P., Carbone, V., Vinale, F., Woo, S. L., Ruocco, M., et al. (2006). Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. *Current Genetics, 50*, 307–321. doi:10.1007/s00294-006-0091-0.

Natera, S. H. A., Guerreiro, N., & Djordjevic, M. A. (2000). Proteome analysis of differentially displayed proteins as a tool for the investigation of symbiosis. *Molecular Plant-Microbe Interactions, 13*, 995–1009. doi:10.1094/MPMI.2000.13.9.995.

Oliveira, M. R., & Luz, J. P. (2003). Susceptibilidade varietal de cultivares de oliveira a *Pseudomonas savastanoi* na regiao da Beira Interior. (Poster presented at the III Simpósio Nacional de Olivicultura, Escola Superior Agrária de Castelo Branco, Castelo Branco, Portugal).

Orth, D., Grif, K., Dierich, M. P., & Würzner, R. (2007). Variability in tellurite resistance and the *ter* gene cluster among shiga toxin-producing *Escherichia coli* isolated from humans, animals and food. *Research in Microbiology, 158*, 105–111. doi:10.1016/j.resmic.2006.10.007.

Pancholi, V. (2001). Multifunctional α-enolase: its role in diseases. *Cellular and Molecular Life Sciences, 558*, 902–920. doi:10.1007/PL00000910.

Pandey, A., Andersen, J. S., & Mann, M. (2000). Use of mass spectrometry to study signaling pathways. *Science's STKE*, pl1. Retrieved March 30, 2008, from http://stke.sciencemag.org/cgi/content/abstract/sigtrans;2000/37/pl1.

Penyalver, R., García, A., Ferrer, A., Bertolini, E., Quesada, J. M., Salcedo, C. I., et al. (2006). Factors affecting *Pseudomonas savastanoi* pv. *savastanoi* plant inoculations and their use for evaluation of olive cultivar susceptibility. *Phytopathology, 96*, 313–319. doi:10.1094/PHYTO-96-0313.

Pérez-Bueno, M. L., Rahoutei, J., Sajnani, C., Garcia-Luque, I., & Barón, M. (2004). Proteomic analysis of the oxygen-evolving complex of photosystem II under biotic stress: studies on *Nicotiana benthamiana* infected with tobamoviruses. *Proteomics, 4*, 418–425. doi:10.1002/pmic.200300655.

Pérez-Martínez, I., Zhao, Y., Murillo, J., Sundin, G. W., & Ramos, C. (2008). Global genomic analysis of *Pseudomonas savastanoi* pv. *savastanoi* plasmids. *Journal of Bacteriology, 190*, 625–635. doi:10.1128/JB.01067-07.

Price, B. M., Galloway, D. R., Baker, N. R., Gilleland, L. B., Staczek, J., & Gilleland, H. E. (2001). Protection against *Pseudomonas aeruginosa* chronic lung infection in mice by genetic immunization against outer membrane protein F (OprF) of *P. aeruginosa*. *Infection and Immunity, 69*, 3510–3515. doi:10.1128/IAI.69.5.3510-3515.2001.

Rep, M., Dekker, H. L., Vossen, J. H., De Boer, A. D., Houterman, P. M., Speijer, D., et al. (2002). Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato. *Plant Physiology, 130*, 904–917. doi:10.1104/pp.007427.

Riccardi, F., Gazeau, P., De Vienne, D., & Zivy, M. (1998). Protein changes in response to progressive water deficit in maize. Quantitative variation and polypeptide identification. *Plant Physiology, 117*, 1253–1263. doi:10.1104/pp.117.4.1253.

Romeis, T., Ludwig, A. A., Martin, R., & Jones, J. D. G. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. *The EMBO Journal, 20*, 5556–5567. doi:10.1093/emboj/20.20.5556.

Sato, T., Harada, T., & Ishizawa, K. (2002). Stimulation of glycolysis in anaerobic elongation of pondweed (*Potamogeton distinctus*) turions. *Journal of Experimental Botany, 53*, 1847–1856. doi:10.1093/jxb/erf036.

Schroth, M. N., Hildebrand, D. C., & O'Reilly, H. J. (1968). Off-Flavor of olives from trees with olive knot tumors. *Phytopathology, 58*, 524–525.

Schroth, M. N., Osgood, J. W., & Miller, T. D. (1973). Quantitative assessment of the effect of the olive knot disease on yield and quality. *Phytopathology, 63*, 1064–1065.

Scott, K. J., Craigie, J. S., & Smillie, R. M. (1964). Pathways of respiration in plant tumors. *Plant Physiology, 39*, 323–327. doi:10.1104/pp.39.3.323.

Sisto, A., Cipriani, M. G., & Morea, M. (2004). Knot formation caused by *Pseudomonas syringae* subsp. *savastanoi* on olive plants is hrp-dependent. *Phytopathology, 94*, 484–489. doi:10.1094/PHYTO.2004.94.5.484.

Subramanian, A., & Miller, D. M. (2000). Structural analysis of α-enolase. Mapping the functional domains involved in down-regulation of the c-*myc* protooncogene. *The Journal of Biological Chemistry, 275*, 5958–5965. doi:10.1074/jbc.275.8.5958.

Subramanian, B., Bansal, V. K., & Kav, N. N. V. (2005). Proteome-level investigation of *Brassica carinata*-derived resistance to *Leptosphaeria maculans*. *Journal of Agricultural and Food Chemistry, 53*, 313–324. doi:10.1021/jf048922z.

Surico, G., Comai, L., & Kosuge, T. (1984). Pathogenicity of strains of *Pseudomonas syringae* pv. *savastanoi* and their indoleacetic acid-deficient mutants on olive and oleander. *Phytopathology, 74*, 490–493. doi:10.1094/Phyto-74-490.

Wienkoop, S., & Saalbach, G. (2003). Proteome analysis. Novel proteins identified at the peribacteroid membrane from *Lotus japonicus* root nodules. *Plant Physiology, 131*, 1080–1090. doi:10.1104/pp.102.015362.

Williams, K. M., Bigley, E. C., III, & Raybourne, R. B. (2000). Identification of murine B-cell and T-cell epitopes of *Escherichia coli* outer membrane porin F with synthetic polypeptides. *Infection and Immunity, 68*, 2535–2545. doi:10.1128/IAI.68.5.2535-2545.2000.