Priming of protein expression in the defence response of *Zantedeschia aethiopica* to *Pectobacterium carotovorum*

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SUMMARY

The defence response of *Zantedeschia aethiopica*, a natural rhizomatous host of the soft rot bacterium *Pectobacterium carotovorum*, was studied following the activation of common induced resistance pathways—systemic acquired resistance and induced systemic resistance. Proteomic tools were used, together with *in vitro* quantification and *in situ* localization of selected oxidizing enzymes. In total, 527 proteins were analysed by label-free mass spectrometry (MS) and annotated against the National Center for Biotechnology Information (NCBI) nonredundant (nr) protein database of rice (*Oryza sativa*). Of these, the fore most differentially expressed group comprised 215 proteins that were primed following application of methyl jasmonate (MJ) and subsequent infection with the pathogen. Sixty-five proteins were down-regulated following MJ treatments. The application of benzothiadiazole (BTH) increased the expression of 23 proteins; however, subsequent infection with the pathogen repressed their expression and did not induce priming. The sorting of primed proteins by Gene Ontology protein function category revealed that the primed proteins included nucleic acid-binding proteins, cofactor-binding proteins, ion-binding proteins, transferases, hydrolases and oxidoreductases. In line with the highlighted involvement of oxidoreductases in the defence response, we determined their activities, priming pattern and localization *in planta*. Increased activities were confined to the area surrounding the pathogen penetration site, associating these enzymes with the induced systemic resistance afforded by the jasmonic acid signalling pathway. The results presented here demonstrate the concerted priming of protein expression following MJ treatment, making it a prominent part of the defence response of *Z. aethiopica* to *P. carotovorum*.

INTRODUCTION

Zantedeschia sp. (calla lilies) are herbaceous monocotyledonous ornamental geophytes that are highly sensitive to *Pectobacterium*

carotovorum, the causal agent of soft rot disease. Currently, there is no effective way to control *P. carotovorum* in ornamental geophytes. The use of common bactericidal formulations to decrease *P. carotovorum* proliferation is relatively ineffective and these compounds are often phytotoxic (Gracia-Garza *et al.*, 2002; Snijder and van Tuyl, 2002). Currently, the only way to control *P. carotovorum* in *Zantedeschia* is through the use of strict sanitation measures and selection for more resistant cultivars (Luzzatto-Knaan and Yedidia, 2009; Snijder *et al.*, 2004a, b; Snijder and van Tuyl, 2002).

Evidence suggests that *Zantedeschia aethiopica* is capable of activating induced defence mechanisms to fight bacterial soft rot (Luzzatto *et al.*, 2007a, b). The defence pathways and mechanisms involved have been found to depend on the plant's ability to recognize the pathogen and initiate defence signalling pathways, including a number of defence-related genes. Similar to other plant systems, once recognition occurs, a large number of genes are activated and a systemic response is triggered (Rushton and Somssich, 1998; Walters and Heil, 2007). A similar systemic response has been shown recently for *Z. aethiopica* (Luzzatto-Knaan *et al.*, 2013). However, a defence response may be referred to as priming only if the plant displays a rapid and/or stronger activation of defence-related genes when subsequently challenged by a pathogen (Conrath, 2011; Heil and Bostock, 2002; van Hulten *et al.*, 2006; Luzzatto *et al.*, 2007a; Sticher *et al.*, 1997).

Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are the two most recognized defence pathways in plants. These immune responses can be triggered by localized infection or by treatment with particular microbes or any of a diverse group of structurally unrelated organic and inorganic compounds (Heil and Bostock, 2002; Kúc, 2001; Pieterse *et al.*, 1998, 2009, 2012; Proietti *et al.*, 2013; Vallad and Goodman, 2004). SAR involves the salicylic acid (SA) signalling molecule and is characterized by elevated levels of pathogenesis-related proteins and the hypersensitive response, whereas ISR is characterized by the jasmonic acid (JA)/ethylene signalling pathway and by increased levels of secondary metabolites and oxidizing enzymes (Constabel and Ryan, 1998; Glas *et al.*, 2012; Kim *et al.*, 2006; Li and Steffens, 2002; Luzzatto *et al.*, 2007a; Pourcel *et al.*, 2007).

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Previously, we have shown that methyl jasmonate (MJ), acting via the JA signalling pathway, provides effective and long-lasting resistance to *P. carotovorum* infection of *Z. aethiopica*, when compared with a control or the SA analogue benzothiadiazole (BTH). The MJ-induced resistance was accompanied by the priming of polyphenolic compounds and increased antimicrobial activity, triggered by challenge inoculation with *P. carotovorum* (Luzzatto *et al.*, 2007a, b). However, unlike in model plant systems, the activated expression of a large number of defence-related genes and the roles of these genes in pathogen restriction have not been studied in *Zantedeschia*.

Microarray technology has become a powerful tool for the analysis of induced resistance and genome-scale analyses of gene expression in model plants (Proietti *et al.*, 2013; von Rad *et al.*, 2005). However, the study of evolutionarily distant plants whose genomes have not been sequenced and for which few genomic data are available requires a different methodology. Proteomics is such an approach (Carpentier *et al.*, 2008; Liska and Shevchenko, 2003). Proteomics allows the identification of the functional components of the gene (i.e. the protein), unravelling post-translational modifications, even when a complete set of genomic data is not available (Carpentier *et al.*, 2008; Deepak *et al.*, 2008).

Plant proteomics lags behind other kingdoms, such as fungi (yeast), bacteria and animals (mammals), in terms of research and publications, mostly because of the relatively small amount of plant genome data available (Agrawal and Rakwal, 2006). The dicot *Arabidopsis thaliana* and the monocot *Oryza sativa* (rice) serve as model plants with sequenced genomes, as well as starting points for proteomic studies in other plant systems of interest (Lin *et al.*, 2009). In some cases, the proteomic data analysis is performed against the National Center for Biotechnology Information (NCBI) nonredundant (nr) green plant database (Macarasin *et al.*, 2009). With the benefits and pitfalls of this background, we have used proteomic tools to study the defence response of the ornamental monocot *Z. aethiopica* to the necrotrophic pathogen *P. carotovorum*. Based on previous studies, a label-free quantification method for differential protein expression based on liquid chromatography-mass spectrometry (LC-MS) was utilized, and the MS data were analysed using the MaxQuant software and searching the *O. sativa* part of the NCBI nr database. Although this method has not been used very often in nonmodel plants, it has been employed to determine the relative abundance of proteins in different samples, such as poplar and wheat (Chen *et al.*, 2012; Mak *et al.*, 2006).

The plant responses were analysed following elicitation with two plant activators: BTH, which is associated with the SA-dependent signalling pathway leading to SAR, and MJ, which is associated with the JA-dependent signalling pathway leading to ISR (Pieterse *et al.*, 2009, 2012; Vallad and Goodman, 2004). The overall objective of this study was to investigate protein expres-

sion following the elicitation of the two signalling pathways and following infection with the necrotrophic pathogen *P. carotovorum* in a nonmodel host *Z. aethiopica*. It is expected that the identification of expressed proteins according to their class and function might help us to better understand the mechanisms underlying the defence response of *Z. aethiopica* to *P. carotovorum*.

RESULTS

Comparative quantitative proteomics

Extracts of three independent biological repeats were pooled in each treatment, and compared with a similar pool of another treatment. The proteins from each sample were trypsinized, resolved by reversed-phase chromatography and analysed by tandem MS (MS/MS) analysis. The identification of proteins was performed by analysing the MS and MS/MS data against the closest related model organism for which a complete database was available, as the genome of *Z. aethiopica* was not available. Quantification was performed by label-free analysis, calculating the peak volume of each identified peptide.

Six leaf treatments were compared: a distilled deionized water (ddw) control treatment; ddw followed by *P. carotovorum* infection; BTH treatment (5 µg/mL) with or without subsequent *P. carotovorum* infection; MJ treatment (10 mg/mL) with or without subsequent *P. carotovorum* infection. Analysis of all treatments was performed using MaxQuant software (Cox and Mann, 2008), resulting in 1371 peptide sequences that were identified and assigned to 527 proteins annotated against the *Oryza* part of the NCBI nr database. The threshold for differential expression was set at a two-fold change (increase or decrease) in the relative intensities.

The expression of 134 of the 527 proteins was up-regulated (two-fold or greater) following *P. carotovorum* infection relative to each of the induction treatments (control, BTH or MJ), whereas 147 proteins were down-regulated (two-fold or greater) relative to the same induction treatments (Fig. 1). When the induction treatments with BTH or MJ were analysed relative to the control treatment, 54 and 93 up-regulated (two-fold or greater) proteins were observed, whereas 37 and 56 proteins were down-regulated (two-fold or greater), respectively. The same analysis (relative to control) following infection with *P. carotovorum* after either BTH or MJ treatment revealed similar numbers (58) of up-regulated proteins (two-fold or greater) for BTH + pathogen, whereas MJ + pathogen resulted in the up-regulation of 120 proteins (two-fold or greater) relative to the control. The largest group of up-regulated proteins following infection with *P. carotovorum* was found among MJ-treated plants (70 proteins). This group represented more than one-half of all up-regulated proteins following infection (Fig. 1). The smallest number of up-regulated

Fig. 1 Venn diagrams showing the number of proteins whose expression was induced by the plant activators methyl jasmonate (MJ) and benzothiadiazole (BTH) and by *Pectobacterium carotovorum* infection. *Zantedeschia aethiopica* plants received the following treatments: C, distilled deionized water (ddw), BTH (5 $\mu\text{g/mL}$), MJ (10 mg/mL), followed by inoculation with *P. carotovorum* (Pc). Differences in expression between the inoculated and uninoculated treatments were calculated (threshold value, ≥ 2). Data are based on pooled protein samples from three independent experiments.

proteins following infection with *P. carotovorum* was observed for BTH-induced plants. In plants treated only with the pathogen, 48 proteins were up-regulated relative to the control. With the threshold used (two-fold or greater), only a few proteins were common among the different treatments. The largest number of down-regulated proteins was found in leaves that were only inoculated with the pathogen (83 proteins). The MJ + pathogen treatment repressed the expression of 51 proteins, relative to the treatment in which leaves were only induced with MJ, similar to the BTH + pathogen treatment with 52 repressed proteins relative to BTH alone (Fig. 1).

Protein classification

Gene Ontology (GO) categories were used to evaluate the potential functions of the differentially expressed proteins. The identified proteins were classified by molecular function, biological processes and cellular component, according to annotations in the Gene Ontology Annotation (UniProt-GOA) Database (<http://www.ebi.ac.uk/GOA/>). The identified proteins corresponded to 13 functional groups and 18 biological processes associated with seven cellular components, covering a wide range of pathways and functions (Fig. 2). The cellular component analysis revealed a high percentage of genes corresponding to the cell, membrane, organelle, macromolecular complexes and extracellular region. For categories based on molecular function, the five most represented GO terms were binding, catalytic activity, structural molecule activity, antioxidant activity and electron carrier activity. For biological processes, the five most represented GO terms were metabolic progress, cellular progress, response to stimulus, biological regulation and cellular component organization or biogenesis (Fig. 2).

Clustering of protein expression patterns based on expression intensities

The expression levels of proteins may vary between treatments, when the cell undergoes specific processes. The analysis of protein expression data requires the clustering of proteins into groups with similar expression patterns. We used the clustering algorithm called CLICK (CLuster Identification via Connectivity Kernels), which is applicable to gene and protein expression analyses (Sharan and Shamir, 2000). Our data yielded nine distinct expression patterns across all six treatments, four of which were selected according to their association with one of the defence pathways, and are presented here (Fig. 3).

Cluster 1 includes 215 proteins that were observed at elevated levels in the MJ treatment and whose expression was further increased following *P. carotovorum* infection, presenting a primed mode of action. These primed proteins were associated with 101 GO functions in 24 categories; 31 displayed ion binding activity, 21 nucleic acid binding activity, 20 oxidoreductase activity, 17 transferase activity, 17 were identified as structural constituents of the ribosome and 16 were associated with hydrolase activity.

Cluster 2 includes 65 proteins whose expression was repressed in the MJ treatments. These were associated with 20 GO functions in 14 categories; five were associated with ion binding activity, five with oxidoreductase activity, four with transferase activity, three with hydrolase activity and three with structural constituents of the ribosome.

Cluster 3 includes 47 proteins whose expression was repressed following *P. carotovorum* infection or MJ treatment. These were associated with 22 GO functions in 13 categories: six with oxidoreductase activity, four with transferase activity, four with cofactor binding, three with hydrolase activity and three with structural constituents of the ribosome.

Cluster 4 includes 23 proteins that were up-regulated following BTH treatment and repressed following inoculation with *P. carotovorum*. These were associated with 11 GO functions in 12 categories: four with nucleic acid binding activity, three with structural constituents of the ribosome, two with oxidoreductase activity, two with tetrapyrrole activity and two with hydrolase activity.

Identification of differentially expressed proteins

Based on the LC-MS data, of the 215 cluster 1 proteins whose expression levels increased following MJ treatment and increased further after pathogen infection, 88 differentially expressed proteins revealed a two-fold or greater increase in expression (relative to the control). The protein data presented in Table 1 represent the first report of *Z. aethiopica*-induced proteins. Table 2 shows the 30 proteins of cluster 2 whose expression was down-regulated in response to MJ + *P. carotovorum* treatment. The expression levels of these 30 proteins were repressed by over 40%; the

Fig. 2 Level 2 Gene Ontology (GO) classification of all proteins. The identified proteins were classified on the basis of the molecular function term, biological processes term and cellular component term for each protein, from the annotations in the Gene Ontology Annotation (UniProt-GOA) Database. The GO term distribution was extracted using BLAST2GO.

expression of nine was repressed by 40%–50%, the expression of nine others was repressed by over 50% and 12 were fully suppressed.

Table 3 lists the 20 of the 47 annotated proteins whose expression levels decreased by $\geq 40\%$ relative to the control following *P. carotovorum* infection (cluster 3). Table 4 lists the five of the 23 annotated proteins whose expression levels increased by two-fold or greater relative to the control after BTH treatment and decreased following infection with the pathogen (cluster 4).

***In vitro* analysis of peroxidase (POD) and polyphenol oxidase (PPO) activity**

Cluster 1 represents the priming of *Z. aethiopica*-induced proteins with intense levels of expression following induction with MJ, and even higher expression levels following inoculation with the pathogen. Of the 215 primed proteins, 101 were assigned to GO functional groups; 20 of these primed proteins were associated with oxidoreductase activity (Fig. 3a,b). Oxidoreductases include

oxidases and dehydrogenases, namely PODs, phenoloxidases, hydroxylases, oxygenases and reductases (Baker *et al.*, 1997). The high proportion of oxidoreductases among the cluster 1 proteins led to the investigation of these activities *in vitro* and *in situ* by examining POD (EC 1.11.1.7) and PPO (EC 1.14.18.1) activity using colorimetric assays and semi-denaturing polyacrylamide gels.

The results revealed priming of PPO activity following MJ treatment. *Pectobacterium carotovorum* infection of these plants was associated with a more than two-fold increase in PPO activity relative to the uninfected MJ-treated plants and a 12-fold increase relative to the noninduced, infected plants. PPO activity was detected only in the MJ treatments (with or without the pathogen), with an evident priming response following challenge with the pathogen (Fig. 4a). The activity gel revealed different isoenzymes in the MJ treatments with two common bands (~75 and 45 kDa) and an additional band (~20 kDa) observed solely in the priming treatment (MJ + pathogen treatment) (Fig. 4c).

Fig. 3 Clusters of leaf proteins expressed in the different treatments: C, distilled deionized water (ddw); B, benzothiadiazole (BTH; 5 µg/mL); M, methyl jasmonate (MJ; 10 mg/mL); Pc, *Pectobacterium carotovorum*; BPc, BTH + *P. carotovorum*; MPc, MJ + *P. carotovorum*. The pie charts on the right show the number of proteins in different functional classes [Gene Ontology (GO) level 3]. The CLICK (CLuster Identification via Connectivity Kernels) algorithm was used to cluster the annotated proteins on the basis of their expression levels. (a, b) Cluster 1. (c, d) Cluster 2. (e, f) Cluster 3. (g, h) Cluster 4.

Table 1 Proteins with increased differential expression (cluster 1) identified in *Zantedeschia aethiopica* leaves following treatment with methyl jasmonate and subsequent infection with *Pectobacterium carotovorum* (two-fold or greater increase in expression relative to the control).

Protein ID	Matched protein*	Access ID†	Fold change‡	Protein function§	Process/cellular component§
Two- to five-fold change					
14	ATP-dependent Clp protease proteolytic subunit	gjl108708097	2.81	No assigned function	
20	Phosphoglycerate kinase	gjl114386666	2.02	Transferase activity	
38	Putative 40S ribosomal protein	gjl115436514	2.19	RNA binding	
48	Proteasome subunit α type	gjl12229919	2.28	Hydrolase activity	
56	Ubiquitin-fold modifier 1	gjl20140685	2.96	No assigned function	
58	Putative dnaK-type molecular chaperone BiP	gjl41052596	2.82	ATP binding	
65	Tubulin β -5 chain	gjl1174600	2.05	GTPase activity	
67	Putative elongation factor 1- γ	gjl46806490	3.75	Copper ion binding	
83	Putative aspartate transaminase	gjl47497041	2.06	Transaminase activity	
84	Vacuolar H ⁺ -pyrophosphatase	gjl1747294	2.49	Hydrolase activity	
99	40S ribosomal protein S3a	gjl1350986	2.00	Structural constituent of ribosome	
100	Tubulin α -2 chain, putative	gjl108706880	3.23	Nucleotide binding	
103	Rhamnose biosynthetic enzyme 1, putative	gjl108707482	2.36	UDP-L-rhamnose synthase activity	
105	Os03g0319300	gjl115452695	4.99	Calcium ion binding	
123	Hypothetical protein Osl_13874	gjl125546022	2.10	Oxidoreductase activity	Flavonol biosynthetic process
133	26S proteasome non-ATPase regulatory subunit 6	gjl20978545	2.70	No assigned function	Response to mechanical stimulus
150	Putative TGF- β receptor interacting protein	gjl54287644	3.23	Receptor activity	Oxidation-reduction process
159	Vacuolar ATPase B subunit	gjl14150751	2.98	ATP binding	Proteasome complex
161	Putative vacuolar protein-ATPase	gjl52075907	2.02	ATP binding	Response to salt stress
168	Probable histone H2A.2	gjl75294328	2.12	No assigned function	ATP metabolic process
173	Elongation factor 1- β	gjl90110019	2.32	Translation elongation factor activity	Response to wounding
190	Nuclear transport factor 2	gjl15214179	3.50	Ran GTPase binding	Defence response to bacterium
196	Senescence-associated protein-like protein	gjl29367553	2.58	No assigned function	Membrane
198	60S ribosomal protein	gjl109940148	3.52	rRNA binding	Translation
208	Mitochondrial chaperonin-60	gjl22758324	2.11	Copper ion binding	Response to heat
212	Pyruvate kinase family protein	gjl77548686	2.26	Transferase activity	Response to cadmium ion
225	5-Methyltetrahydropteroyl/triglutamate-homocysteine methyltransferase	gjl77556632	4.99	Methyltransferase activity	Methylation
243	Hypothetical protein Osl_02864	gjl125526853	2.49	No assigned function	
249	Hypothetical protein Osl_04319	gjl125528274	2.87	No assigned function	
250	Triose phosphate isomerase	gjl149390895	2.14	No assigned function	
253	Hypothetical protein Osl_35570	gjl125533843	3.15	Structural constituent of ribosome	Wound healing
256	Hypothetical protein Osl_35999	gjl125534271	2.18	No assigned function	
258	Hypothetical protein Osl_37065	gjl125535351	2.64	No assigned function	
261	Hypothetical protein Osl_37384	gjl125535694	2.37	Copper ion binding	
281	Hypothetical protein Osl_08139	gjl125540364	2.36	No assigned function	
312	Hypothetical protein Osl_19723	gjl125552095	3.05	No assigned function	
323	Hypothetical protein Osl_24355	gjl125556650	2.78	No assigned function	
328	Hypothetical protein Osl_25867	gjl125558201	2.75	No assigned function	
332	Isopentenyl pyrophosphate	gjl6856560	2.08	No assigned function	
338	Hypothetical protein Osl_29415	gjl125561721	4.70	Oxidoreductase activity	Metabolic process
360	Oxygen-evolving enhancer protein 1	gjl149392519	3.70	No assigned function	
361	Hypothetical protein Osl_17694	gjl218195711	2.49	No assigned function	
374	Hypothetical protein Osl_33537	gjl218184502	2.17	No assigned function	
396	Hypothetical protein Osl_01750	gjl218188143	2.74	No assigned function	
402	Hypothetical protein Osl_03305	gjl218188864	2.26	No assigned function	
405	Hypothetical protein Osl_04213	gjl218189276	2.01	No assigned function	

Table 1 Continued.

Protein ID	Matched protein*	Access ID†	Fold change‡	Protein function§	Process/cellular component§
428	Hypothetical protein Osl_09120	gij 218191664	4.21	No assigned function	
437	Hypothetical protein Osl_10600	gij 218192372	2.64	No assigned function	
446	Hypothetical protein Osl_22416	gij 218197916	2.98	No assigned function	
454	Hypothetical protein Osl_17386	gij 218195569	3.26	No assigned function	
484	Hypothetical protein Osl_24541	gij 218198925	2.99	No assigned function	
489	Hypothetical protein Osl_26084	gij 218199659	2.02	No assigned function	
494	Hypothetical protein Osl_28098	gij 218200594	2.38	No assigned function	
509	Hypothetical protein Osl_31865	gij 218202373	2.89	No assigned function	
516	S-Adenosylmethionine synthase 2	gij 3024122	2.29	Transferase activity	Lignin biosynthetic process
523	Putative superoxide dismutase [Cu-Zn]	gij 42408425	2.61	No assigned function	
≥Five-fold change					
12	30S ribosomal protein S1, chloroplast precursor	gij 108707824	5.09	No assigned function	
134	Hypothetical protein Osl_07937	gij 218191112	5.20	Oxidoreductase activity	Oxidation–reduction process
259	Hypothetical protein Osl_37152	gij 125535486	25.54	No assigned function	
439	Hypothetical protein Osl_10996	gij 218192543	12.38	No assigned function	
458	Hypothetical protein Osl_18129	gij 218195939	6.09	No assigned function	
Detected following treatment					
27	Ribosomal protein L16	gij 114812068	¶	Structural constituent of ribosome	Translation
36	ATP-citrate synthase β chain protein	gij 75249275	¶	Transferase activity	Metabolic process
116	α-Tubulin	gij 10441016	¶	Transferase activity	Purine-nucleoside phosphorylase
126	Probable methylenetetrahydrofolate reductase	gij 75294984	¶	Oxidoreductase activity	Oxidation–reduction process
192	Putative poly(A)-binding protein	gij 46389987	¶	Nucleotide binding	
204	Putative myo-inositol-1-phosphate synthase	gij 20043019	¶	Inositol-3-phosphate synthase activity	Defence response to bacterium
205	Hypothetical protein Osl_29960	gij 218201483	¶	Kinase binding	Protein transport
219	ATP-AMP transphosphorylase A	gij 585337	¶	Transferase activity	Response to cadmium ion
224	5-Methyltetrahydropteroyltryglutamate-homocysteine methyltransferase	gij 77556631	¶	Methyltransferase activity	Methylation
265	Hypothetical protein Osl_38175	gij 125536468	¶	No assigned function	
274	Hypothetical protein Osl_06046	gij 125538279	¶	No assigned function	
285	Hypothetical protein Osl_09265	gij 125541449	¶	No assigned function	
289	Hypothetical protein Osl_10220	gij 125542606	¶	No assigned function	
292	Hypothetical protein Osl_10803	gij 125543162	¶	No assigned function	
322	Hypothetical protein Osl_23958	gij 125556326	¶	No assigned function	
336	Hypothetical protein Osl_29091	gij 125561408	¶	No assigned function	
388	Hypothetical protein Osl_38794	gij 218187100	¶	No assigned function	
398	Hypothetical protein Osl_02106	gij 218188296	¶	No assigned function	
399	Hypothetical protein Osl_02961	gij 218188712	¶	No assigned function	
403	Hypothetical protein Osl_04088	gij 218189221	¶	No assigned function	
442	Hypothetical protein Osl_12134	gij 218193097	¶	No assigned function	
452	Hypothetical protein Osl_16908	gij 218195338	¶	No assigned function	
467	Hypothetical protein Osl_20905	gij 218197235	¶	No assigned function	
468	Hypothetical protein Osl_21033	gij 218197290	¶	No assigned function	
481	Hypothetical protein Osl_23984	gij 218198689	¶	No assigned function	
505	Hypothetical protein Osl_30663	gij 218201808	¶	No assigned function	
515	Thioredoxin M5	gij 11135471	¶	No assigned function	

*Matched proteins were identified using ion-trap liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Orbitrap, Thermo Fisher Scientific).

†Access ID is the access identification of the protein determined via comparison with the *Oryza* part of the National Center for Biotechnology Information (NCBI) nonredundant (nr) database.

‡Fold change according to quantification and analysis carried out using MaxQuant 1.2.2.5 software.

§Protein function/process/cellular component based on Gene Ontology Annotation (UniProt-GOA) Database [Gene Ontology (GO) level 3].

¶Fold could not be calculated; control intensities were below detection level.

Table 2 Proteins with repressed differential expression (cluster 2) identified in *Zantedeschia aethiopica* leaves following treatment with methyl jasmonate and subsequent infection with *Pectobacterium carotovorum* ($\geq 40\%$ reduction relative to the control).

Protein ID	Matched protein*	Access ID†	Fold change‡	Protein function§	Process/cellular component§
40%–50% change					
155	Hypothetical protein Osl_17265	gi 218195501	0.60	No assigned function	Plastid organization
251	Hypothetical protein Osl_05369	gi 125529268	0.56	No assigned function	
214	Fructose-bisphosphate aldolase	gi 78099750	0.56	Lyase activity	Response to abscisic acid stimulus
218	Monothiol glutaredoxin-S12	gi 122063510	0.55	Oxidoreductase activity	Cell redox homeostasis
304	Hypothetical protein Osl_16800	gi 125549171	0.54	No assigned function	
89	Catalase-1	gi 108706015	0.54	Peroxidase activity	Response to oxidative stress
492	Hypothetical protein Osl_27178	gi 218200179	0.54	No assigned function	
163	Putative alanine aminotransferase	gi 50510015	0.52	Transaminase activity	Biosynthetic process
66	26S proteasome ATPase subunit Rpt	gi 11991116	0.51	ATP binding	Protein catabolic process
50%–95% change					
455	Hypothetical protein Osl_17466	gi 218195608	0.47	No assigned function	
391	Hypothetical protein Osl_00149	gi 218187401	0.45	No assigned function	
276	Hypothetical protein Osl_06200	gi 125538439	0.44	No assigned function	
267	Hypothetical protein Osl_38775	gi 125537077	0.43	No assigned function	
233	Hypothetical protein Osl_16423	gi 218195106	0.39	No assigned function	
128	Putative galactose kinase	gi 31249736	0.30	Kinase activity	Metabolic process
95	Pentose-5-phosphate 3-epimerase	gi 109940150	0.29	Isomerase activity	Metabolic process
244	Hypothetical protein Osl_03093	gi 125527088	0.24	No assigned function	
518	Malate dehydrogenase, glyoxysomal	gi 3183079	0.14	Structural constituent of ribosome	
$\geq 95\%$ change					
75	Small GTP-binding protein	gi 642121	0.00	GTP binding	Signal transduction
239	Hypothetical protein Osl_00615	gi 125524634	0.00	No assigned function	
252	Hypothetical protein Osl_34765	gi 125533056	0.00	No assigned function	
309	Hypothetical protein Osl_19495	gi 125551858	0.00	No assigned function	
327	Hypothetical protein Osl_11322	gi 218192705	0.00	No assigned function	
346	Hypothetical protein Osl_32141	gi 125564469	0.00	No assigned function	
414	Hypothetical protein Osl_06478	gi 218190367	0.00	No assigned function	
424	Hypothetical protein Osl_08447	gi 218191345	0.00	No assigned function	
432	Hypothetical protein Osl_09642	gi 218191917	0.00	No assigned function	
469	Hypothetical protein Osl_21076	gi 218197310	0.00	No assigned function	
500	Hypothetical protein Osl_30076	gi 218201535	0.00	No assigned function	
511	Zinc finger protein ZFP252	gi 28849865	0.00	No assigned function	

*Matched proteins were identified using ion-trap liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Orbitrap, Thermo Fisher Scientific).

†Access ID is the access identification of the protein determined via comparison with the *Oryza* part of the National Center for Biotechnology Information (NCBI) nonredundant (nr) database.

‡Fold change according to quantification and analysis carried out using MaxQuant 1.2.2.5 software.

§Protein function/process/cellular component based on Gene Ontology Annotation (UniProt-GOA) Database [Gene Ontology (GO) level 3].

A similar priming trend was observed for POD activity. We observed a 1.5-fold increase in POD activity in plants in the MJ + *P. carotovorum* treatment, relative to the plants that were treated with MJ, but not inoculated. POD activity was also induced following treatment with *P. carotovorum* alone (Fig. 4a). The POD activity gel demonstrates this activation (Fig. 4b). It also shows that MJ activates the same POD as the pathogen, and does so just as strongly. Gel analysis revealed that different isoenzymes were expressed in each treatment, implying the existence of common POD isoenzymes (~35 kDa) and a specific isoenzyme for the MJ treatments (~30 kDa). An additional isoenzyme (~45 kDa) was expressed in the treatments that included both induction and infection (BTH + pathogen and MJ + pathogen; Fig. 4). Notably, the pathogen-free BTH treatment was not associated with any POD activity *in vitro* (Fig. 4b).

In situ localization of POD and PPO activity

Oxidizing enzymes play an active part in defending the plant against pathogens. Diaminobenzidine (DAB) is a common synthetic substrate that polymerizes instantly through the action of PODs and on contact with hydrogen peroxide (H_2O_2) to develop a dark colour (Cohen *et al.*, 2011). In a similar way, following oxidation by PPO, dopa is oxidized to yield dopachrome, which then further polymerizes to form an aggregate of dark melanin (Constabel and Ryan, 1998).

Staining with DAB and dopa was used to localize POD and PPO activity in *Z. aethiopica* leaf tissue. Briefly, following induction and *P. carotovorum* inoculation, leaf discs were incubated in 0.1% DAB or 25 mM dopa. The leaf discs were bleached in boiling ethanol and observed under a light microscope (Fig. 5). The results

Table 3 Proteins with repressed differential expression (cluster 3) identified in *Zantedeschia aethiopica* leaves following *Pectobacterium carotovorum* infection ($\geq 40\%$ reduction relative to the control).

Protein ID	Matched protein*	Access ID†	Fold change‡	Protein function§	Process/cellular component§
40%–50% change					
456	Hypothetical protein Osl_17671	gi 218195699	0.59	No assigned function	Glutamine biosynthetic process
357	Hypothetical protein Osl_09033	gi 218191618	0.55	No assigned function	
145	Glutamine synthetase	gi 121343	0.54	Ligase activity	
379	Hypothetical protein Osl_35121	gi 218185255	0.53	No assigned function	
320	Hypothetical protein Osl_22460	gi 125554832	0.52	No assigned function	
298	Hypothetical protein Osl_13894	gi 125546040	0.51	No assigned function	Interspecies interaction
34	Os01g0253300	gi 115435706	0.50	Binding	
50%–95% change					
122	Glycolate oxidase	gi 122246745	0.48	Oxidoreductase activity	Interspecies interaction
172	Os07g0626400	gi 115473475	0.45	Heat shock protein binding	Protein folding
362	Catalase isozyme	gi 152013381	0.41		
120	Os03g0773800	gi 115455637	0.39	Oxidoreductase activity	Oxidation–reduction process
140	Glycolate oxidase	gi 75326731	0.36	Oxidoreductase activity	Interspecies interaction
169	Putative 40S ribosomal protein	gi 24417185	0.31	Structural constituent of ribosome	Translation
189	Putative aminotransferase	gi 42407771	0.23	Transferase activity	Metabolic process
329	Hypothetical protein Osl_25927	gi 125558262	0.22	No assigned function	
230	Hypothetical protein Osl_16326	gi 125548728	0.21	No assigned function	
≥95% change					
44	Ubiquitin-conjugating enzyme	gi 226492411	0.00	Ligase activity	Protein ubiquitination
275	Hypothetical protein Osl_06150	gi 125538387	0.00	No assigned function	
351	Hypothetical protein Osl_32438	gi 125564750	0.00	No assigned function	
400	Hypothetical protein Osl_03090	gi 218188764	0.00	No assigned function	

*Matched proteins were identified using ion-trap liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Orbitrap, Thermo Fisher Scientific).

†Access ID is the access identification of the protein determined via comparison with the *Oryza* part of the National Center for Biotechnology Information (NCBI) nonredundant (nr) database.

‡Fold change according to quantification and analysis carried out using MaxQuant 1.2.2.5 software.

§Protein function/process/cellular component based on Gene Ontology Annotation (UniProt-GOA) Database [Gene Ontology (GO) level 3].

Table 4 Proteins with increased differential expression (cluster 4) identified in *Zantedeschia aethiopica* leaves following treatment with benzothiadiazole (BTH) and subsequent infection with *Pectobacterium carotovorum* (two-fold or greater change relative to the control).

Protein ID	Matched protein*	Access ID†	Fold change‡	Protein function§	Process/cellular component§
Two- to five-fold change					
15	Photosystem II 47-kDa protein	gi 109156612	2.41	Chlorophyll binding	Electron transport chain
72	40S ribosomal protein	gi 109940154	2.02	No assigned function	Ribosome
504	Hypothetical protein Osl_30475	gi 218201704	2.96	No assigned function	
Detected following treatment					
59	Putative EF-hand Ca ²⁺ binding protein CCD1	gi 41053013	¶	Calcium ion binding	
506	Hypothetical protein Osl_30699	gi 218201824	¶	No assigned function	

*Matched proteins were identified using ion-trap liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Orbitrap, Thermo Fisher Scientific).

†Access ID is the access identification of the protein determined via comparison with the *Oryza* part of the National Center for Biotechnology Information (NCBI) nonredundant (nr) database.

‡Fold change according to quantification and analysis carried out using MaxQuant 1.2.2.5 software.

§Protein function/process/cellular component based on Gene Ontology Annotation (UniProt-GOA) Database [Gene Ontology (GO) level 3].

¶Fold could not be calculated; control intensities were below detection level.

show that, in the control and BTH treatments, no polymerized product accumulated in the leaf tissue at the wound or the *P. carotovorum* infection site. Under the same conditions, POD and PPO activities were observed around the wounds in the leaves that had been treated with MJ, with augmented levels observed at and around the penetration area following infection with *P. carotovorum*. This observation implies that POD and PPO activity are involved in the plant's induced defence response to the pathogen (Fig. 5).

DISCUSSION

The use of label-free MS analysis, in combination with bioinformatics tools, facilitates proteomic research in organisms for which the available genomic data are limited at best (Carpentier *et al.*, 2008). We have focused on *Z. aethiopica*, a natural rhizomatous host of the soft rot pathogen *P. carotovorum*, to unravel the proteome underlying the defence response of a less frequently studied monocot to the soft rot bacterium. The

Fig. 4 *In vitro* peroxidase (POD) and polyphenol oxidase (PPO) activity of the total soluble protein extracted from *Zantedeschia aethiopica* leaves. Leaves were treated with distilled deionized water (ddw) (control, C), 5 $\mu g/mL$ benzothiadiazole (B) or 10 mg/mL methyl jasmonate (M) and, 24 h later, inoculated with *Pectobacterium carotovorum* (Pc). Proteins were extracted 24 h after inoculation (10 mM sodium acetate, pH 5.6). (a) *In vitro* enzymatic activity was quantified spectrophotometrically (475 nm) using 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H_2O_2 as the POD substrate and dopa as the substrate for PPO. Bars represent mean POD and PPO activity, based on four biological replicates for each treatment $\pm$ SE. Treatments followed by the same letter are not significantly different according to the Tukey–Kramer multiple range test at $P < 0.01$. (b, c) Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (10%) activity gels for PODs using TMB in the presence of H_2O_2 as the substrate (b) and for PPO using dopa as the substrate (c). Each lane contained 30 μg of protein. In all cases, extracted protein was pooled from the leaves of three plants that received the same treatment. Molecular weight (MW) markers are shown at the left of each gel.

differential expression of proteins following the induction of common plant defence pathways and subsequent inoculation with *P. carotovorum* was analysed using the most similar monocot database available, that of *O. sativa*. The role of the JA/ethylene signalling pathway in the defence response of *Z. aethiopica* to *P. carotovorum* has been documented previously in work based on the use of physiological and biochemical tools (Luzzatto *et al.*, 2007a, b). However, this is the first attempt to investigate the molecular basis of this response using a proteomic approach. A total of 527 proteins were annotated in *Z. aethiopica* leaves in response to the following six treatments: a ddw control, BTH and MJ induction treatments and the same induction treatments followed by challenge inoculation with the pathogen.

The first level of organization of the *Z. aethiopica* proteome was based on Venn diagrams depicting the number of up-regulated and down-regulated proteins in the *P. carotovorum* infected/uninfected state. Our analysis of infected/uninfected treatments revealed 134 up-regulated proteins (with a threshold of two-fold or greater change), the largest number of which were distinct proteins observed in the MJ + pathogen treatment. One hundred and forty-seven proteins were down-regulated and the largest

number of distinct down-regulated proteins was associated with *P. carotovorum* infection. Overlapping proteins were more likely to be among those down-regulated, suggesting a significant role for bacterial suppression of gene expression.

To obtain a better understanding of how proteins are associated with various molecular cell functions, GO terms were used to categorize the proteome. The different categories allowed us to identify proteins associated with several cellular components that are related to the membrane and the extracellular region. These could be related to a defensive state established following the activation of a resistance response. In a similar manner, cellular components, such as signalling, response to stimuli, multi-organism processes and immune system processes, could be congruent with a defensive mode. The next category, molecular function, revealed potential defence responses associated with molecular transducer activity, binding, catalytic activities, transporter activities and antioxidant activity.

Clustering of expression patterns of differential proteins based on the algorithm CLICK, according to the intensity of expression, enabled the visualization of specific proteins with similar behaviours across different manipulations. Each of the four expression patterns presented here demonstrates a defence-associated phe-

Fig. 5 Light microscopy images of peroxidase (POD) and polyphenol oxidase (PPO) activity in leaf discs of *Zantedeschia aethiopica* treated with plant activators: distilled deionized water (ddw) (control), 5 μg/mL benzothiadiazole (BTH) or 10 mg/mL methyl jasmonate (MJ). The discs were inoculated with *Pectobacterium carotovorum* 24 h later. Enzymatic activity was recorded 24 h after inoculation following incubation with diaminobenzidine (DAB) for the localization of POD activity (a) and dopa for the localization of PPO activity (b). POD or PPO activity was visualized via the accumulation of a polymerized product, as shown by the brown/black ring around the wounded or infected site (bar, 500 μm).

nomenon, of which the first and by far the largest cluster may represent the unique physiological state known as priming, with 215 proteins displaying a similar expression trend line (cluster 1). Primed plants show rapid and/or strong activation of defence responses when subsequently challenged by a pathogen (Conrath, 2011; Conrath *et al.*, 2002, 2006; Heil and Bostock, 2002; Sticher *et al.*, 1997).

The results presented here show that induction with MJ, but not BTH, was further augmented following infection with *P. carotovorum*. This suggests that the JA/ethylene signalling pathway plays a role in protein priming involved in the plant defence response to this necrotroph. Priming has been reported previously in the same pathosystem, based on the augmented accumulation of total and specific polyphenolic compounds in *Z. aethiopica* leaves following MJ treatment and challenge with *P. carotovorum* (Luzzatto *et al.*, 2007a). However, the quantitative outline of priming following MJ induction is shown here for the first time, revealing 88 proteins whose expression levels increased by more than two-fold relative to the control. Of the up-regulated proteins, the most abundant were 21 identified as nucleic acid binding proteins, implying the involvement of transcription factors, and 31 identified as ion binding proteins, with an addi-

tional 16 identified as cofactor binding proteins. These results are congruent with the high proportion of potentially *de novo* synthesized proteins (27 of 88) among those expressed in the primed state, suggesting an important role for transcriptional regulation activities in the priming process.

A recent study discussing the molecular aspects of priming assigned a key role for mitogen-activated protein kinases (MAPKs), also known as serine/threonine-specific protein kinases, in plant defence. These enzymes were found to function downstream of receptors and to transmit extracellular stimuli into intracellular responses, whilst amplifying transduction signals (Beckers *et al.*, 2009; Conrath, 2011). A protein from this group with 100% identity to several protein kinases from *O. sativa* and other monocots was identified in this study (protein ID 243, Table 1), and its level of expression was 2.5-fold higher in the primed state than in the control. MAPK was also reported to play a role in the ISR response of cucumber which is triggered by the beneficial fungus *Trichoderma* during the activation of the JA/ethylene signalling pathway (Shoresh *et al.*, 2006).

Another protein family with a well-established relationship to defence signalling is the transferases, specifically the methyl transferases. This family of proteins catalyses the formation of

small-molecule to volatile methyl esters, which are involved in the attraction of pollinators, as well as in defence against herbivores and pathogens (Kollner *et al.*, 2010).

We identified 17 primed transferases, including a methyl transferase (225) whose expression level increased nearly five-fold and another protein (224) that was expressed only in the treatment and below detection level in the control. Methyl esters act as interplant signals that are produced by infected plants and subsequently induce defence responses in their uninfected neighbours (Kollner *et al.*, 2010; Zubieta *et al.*, 2003).

Other notable induced proteins include: a 60-kDa chaperonin (208), which is essential for correct protein folding and sustained resistance to biotic stress; *S*-adenosylmethionine synthase (516), which is specifically regulated by fungal and bacterial elicitors and is involved in the plant's response to wounding; ribosomal proteins (38, 99, 198, 12, 27) required for protein translation; these might be associated with plant innate immunity, specifically the mass production of antimicrobial proteins; thioredoxin (515), which is involved in intracellular signalling and resistance to oxidative stress, as reported previously in the context of disease resistance of tomato, and which is significantly up-regulated in response to JA treatments (Afroz *et al.*, 2010; Mysore *et al.*, 2003); and, finally, oxidoreductases (123, 338, 134, 126). The expression levels of certain oxidoreductases increased more than two-fold (123, 338), whereas another increased more than five-fold (134) and one was defined as a *de novo* protein (126). Two of these proteins, 123 and 134, have been mentioned in relation to primary metabolism pathways involved in plant defence (Bolton, 2009).

The second noteworthy defence-related phenomenon involves the protein expression trends presented in clusters 2 and 3. These trends correspond to a pattern of repressed protein expression that may be seen as a trade-off effect. The priming of defence-related genes is often accompanied by the down-regulation of cellular processes or, in some cases, antagonistic pathways (Glazebrook, 2005; Pieterse *et al.*, 2001, 2009). The repression may be the result of the allocation of plant resources to the production of defence compounds or the detoxification of defence products during the induced resistance response, which carry costs for plant fitness and growth (Walters and Heil, 2007). Trade-offs observed between the rate of plant growth and disease resistance support the hypothesis that plant growth and defence are regulated by a network of interconnecting signalling pathways (Pieterse *et al.*, 2009). The consequence is expressed in the attenuation of functions, such as growth and reproduction (van Hulst *et al.*, 2006). Quantitative analysis of cluster 2 repressed proteins was based on a comparison of the expression levels in plants treated with MJ and then inoculated with *P. carotovorum* with the expression levels observed in the control. The threshold was set to $\geq 40\%$ reduced expression and up to 100% reduction. This analysis revealed that a total of 65 proteins were down-regulated following the MJ + pathogen treatment. Cluster 3 analysis (pathogen/

control) under the same conditions revealed 45 proteins whose expression was down-regulated following inoculation with the pathogen. An examination of the cluster 3 trend line showed that some of the same proteins were down-regulated in response to MJ treatment and following *P. carotovorum* infection, suggesting that MJ and the pathogen affect similar signalling pathways.

Several studies have reported trade-offs related to antagonism between the JA/ethylene and SA signalling pathways (Glazebrook, 2005; Pieterse *et al.*, 2001, 2009; Proietti *et al.*, 2013), and such trade-offs are also evident in the down-regulation of proteins observed in this study. Both clusters 2 and 3 present trade-offs associated with the down-regulation of catalase isoenzymes (89 in Table 2 and 362 in Table 3, EC 1.11.1.6) in response to the activation of the JA signalling pathway by MJ or the pathogen. Catalase protects the cell from the toxic effects of H_2O_2 generated as a by-product of cell metabolism by catalysing its decomposition. There have been several recent reports of reduced catalase activity following the application of MJ to *Ricinus communis* leaves and *Arabidopsis thaliana* roots (Loyola-Vargas *et al.*, 2012; Soares *et al.*, 2010).

The protein expression pattern of cluster 4 demonstrates a cross-talk between the two most recognized defence pathways, SAR and ISR (Heil and Bostock, 2002; Pieterse *et al.*, 2009; Proietti *et al.*, 2013; Thaler *et al.*, 2012). It clearly reveals that proteins that are elevated following the application of BTH reveal low levels of expression following treatment with MJ. In addition, subsequent inoculation with *P. carotovorum* (which activates the JA signalling pathway) represses BTH protein expression, revealing an antagonistic interaction between the SA signalling pathway (activated by BTH) and the JA signalling pathway (activated by the pathogen). Antagonism between the two signalling pathways has been widely described and is often accompanied by trade-offs in gene expression (Glazebrook, 2005; Pieterse *et al.*, 2001, 2009; Proietti *et al.*, 2013). This antagonism has been documented for *P. carotovorum*, which was found to suppress the SA-mediated SAR response of *A. thaliana* during an early stage of that plant-pathogen interaction (Vidal *et al.*, 1997). Twenty-three proteins were expressed at higher levels following treatment with BTH. This increased expression was slightly repressed following inoculation with *P. carotovorum* (BTH + pathogen); only five of these proteins displayed more than two-fold increased expression, two of which were detected in the treatment, but were below the detection level in the control.

A consideration of the proteins involved in the priming of defence processes in *Z. aethiopica* suggests an important role for oxidoreductases, which account for 20 of the GO function classifications supported by protein annotations. As these enzymes are related to biotic and abiotic stress, further effort was devoted to the study of the activities and localization of defence-related members of the group. Oxidoreductase activities have been shown earlier to increase following JA treatment and in correlation with

the accumulation of secondary metabolites (Constabel and Ryan, 1998; Thipyapong and Steffens, 1997). Moreover, the involvement of PODs and PPOs in the JA-dependent signalling observed in response to pathogens and herbivores has led, in some cases, to the use of these enzymes as indicators for these responses (Baker *et al.*, 1997; Cosio and Dunand, 2009; Halfeld-Vieira *et al.*, 2006; Li and Steffens, 2002). PODs, which have been localized to a variety of cellular locations, catalyse the oxidation of phenolic compounds and other substrates through the associated reduction of H₂O₂ (Cosio and Dunand, 2009; Pourcel *et al.*, 2007). PPOs are nuclear-encoded proteins that catalyse the oxygen-dependent oxidation of polyphenols to quinones and are thought to be involved in plant defence activities (Koussevitzky *et al.*, 2004; Li and Steffens, 2002; Mayer, 2006; Pourcel *et al.*, 2007).

The *in vitro* activity observed in this study clearly demonstrates a role for oxidoreductases, such as PODs and PPOs, in the priming response of *Z. aethiopica* following treatment with MJ, but not BTH. POD activity in *Z. aethiopica* leaves was activated following *P. carotovorum* infection, as well as following MJ treatment. No POD or PPO activity was observed in the control treatment. According to the priming model, these activities were further augmented following challenge with *P. carotovorum*. POD activity gels showed parallel induced bands for the *P. carotovorum*-only treatment, the MJ-only treatment (but not BTH treatment) and the MJ + pathogen treatment, again supporting the hypothesis of a common signalling pathway for MJ and *P. carotovorum*.

Afroz *et al.* (2010) have reported recently that, in a proteomic analysis of tomato cultivars, PPO was significantly up-regulated in plants treated with JA, but not SA. Over-expression of PPO in transgenic tomato was found to enhance bacterial disease resistance, and the down-regulation of constitutive and induced expression of PPO resulted in the hypersusceptibility to pathogens (Li and Steffens, 2002; Thipyapong and Steffens, 1997).

In order to localize *in situ* the POD and PPO activities that were observed *in vitro*, these activities were visualized in leaf tissues. Our results show that MJ treatment increased POD and PPO activity following infection with *P. carotovorum*, implying the direct involvement of these enzymes in plant induced-defence responses and priming. No polymerized dark product accumulated around the penetration site in the control or BTH treatment. Treatment with BTH, which acts through the SA signalling pathway, did not appear to increase the expression of these oxidizing enzymes, whereas subsequent infection with *P. carotovorum* apparently had an antagonistic effect.

In summary, a series of induced resistance treatments was applied to *Z. aethiopica* leaves, which were further inoculated with the soft rot bacterium *P. carotovorum*, and proteomic techniques were used to elucidate the defence processes involved in this pathosystem. This approach shed light on the nature of the response and the pathways involved. This is the first time that priming has been clearly demonstrated to play a key role in the

defence response of a monocot geophyte to the soft rot bacterium *P. carotovorum*.

EXPERIMENTAL PROCEDURES

Chemicals

All of the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise. Chemicals for electrophoresis were purchased from Bio-Rad (Hercules, CA, USA). BTH (Bion; 50% active ingredient) was kindly provided by Syngenta (Basel, Switzerland). Chemicals used to prepare samples and all solvents used for high-performance liquid chromatography (HPLC) and LC-MS/MS were HPLC- and LC-MS/MS-grade materials purchased from BDH (Poole, Dorset, UK).

Plant material

Zantedeschia aethiopica plants were grown in pots in the glasshouse for two seasons (25/10 °C maximum/minimum, natural daylight). The youngest fully spread leaf was cut at the base of the petiole and suspended (25 °C) in water (control) or BTH (5 µg/mL) in water or sprayed with MJ (10 mg/mL). After 24 h, leaves were treated with ddw or *P. carotovorum* [10⁴ colony-forming units (cfu)/mL], applied to four spots on each leaf (challenge inoculation), and incubated for an additional 24 h. There were three biological replicates of each treatment.

Extraction of proteins

Plant material was harvested 24 h after the induction treatment and frozen in liquid nitrogen. Each sample was then placed in a 2-mL tube and ground in a bead beater (Retsch, Haan, Germany) with two 3-mm tungsten beads at 23 Hz/s for 2 min. Leaf tissue was homogenized with 10 mM sodium acetate buffer (pH 5.6) and centrifuged at 10 000 *g* for 15 min at 4 °C. The supernatant was used as a crude extract. Protein concentration was determined according to the Bradford assay with bovine serum albumin used as a standard.

Proteolysis

The proteins were mixed with 8 M urea and 100 mM ammonium bicarbonate, and reduced with 2.8 mM dithiothreitol (DTT) (60 °C for 30 min), modified with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark at room temperature for 30 min) and then digested overnight at 37 °C with modified trypsin (Promega, Madison, WI, USA) in a solution of 2 M urea and 25 mM ammonium bicarbonate at a 1 : 50 enzyme to substrate ratio. A second digestion took 4 h.

MS analysis

The peptides were desalted using C18 tips (Harvard, Holliston, MA, USA), dried and resuspended in 0.1% formic acid. The peptides were then resolved by reversed-phase chromatography on 0.075 × 200-mm² fused silica capillaries (J&W) packed with Reprosil reversed-phase material (Dr Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The peptides were

eluted with linear 214-min gradients of 7%–40% for 8 min in 95% acetonitrile with a 0.1% aqueous formic acid solution at a flow rate of 0.25 µL/min. MS was performed using an ion-trap mass spectrometer (Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) in a positive mode, with repetitively full MS scans, followed by collision-induced dissociation (CID) of the seven most dominant ions selected from the first MS scan.

Five wash runs were made between the samples. For the blank, 0.1% formic acid was injected using a 34-min gradient of 7%–40%, followed by 9 min of 95% acetonitrile with an aqueous 0.1% formic acid solution. The MS data were analysed using MaxQuant 1.2.2.5 software (M. Mann's group, Max Planck Institute) searching against the *Oryza* part of the NCBI nr database with 1% false discovery rate (FDR). The data were quantified by label-free analysis using the same software by calculating the elution peak volume of the identified peptides. The protein intensities were normalized by comparing the summed intensities of each sample with that of the control. Ratios were calculated from the normalized intensities of the proteins and were loaded to Perseus software (Mattias Mann's laboratory), log₂ transformed and the significance A and B values (which are the *P* values for the detection of significant outlier ratios) were calculated as described by Cox and Mann (2008).

Functional and clustering analysis

GenInfo identifier (GI) numbers were converted to UniProt accessions using the DAVID gene ID conversion tool (<http://david.abcc.ncifcrf.gov/conversion.jsp>; da Huang *et al.*, 2008). The Gene Ontology Annotation (UniProt-GOA) Database (<http://www.ebi.ac.uk/GOA/>) was used to classify each UniProt-identified item by GO term. The GO term distribution was extracted using BLAST2GO (<http://www.blast2go.com/b2gohome>). GO enrichment analysis was performed using the Singular Enrichment Analysis (SEA) tool in agriGO, and statistical significance was determined using the hypergeometric test ($P < 0.05$) (<http://bioinfo.cau.edu.cn/agriGO/index.php>; Du *et al.*, 2010). Proteins were sorted into clusters based on their expression intensities, using the CLICK algorithm and Expander 6 software (Ulitsky *et al.*, 2010).

Spectrophotometric assay of POD and PPO activity *in vitro* and *in situ*

POD activity was assayed using 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H₂O₂ as the POD substrate. PPO activity was assayed using dopa as a substrate. The reaction was performed at 25 °C in 10 mM sodium acetate buffer (pH 5.6) with 5 mM dopa in a total volume of 200 µL using 96-well microtitre plates. Enzyme activity was monitored at 475 nm with a spectrophotometer plate reader. All experiments were carried out with at least three biological replications. Statistical comparisons were made using one-way analysis of variance (ANOVA) by PRISM 5.00 software (GraphPad, San Diego, CA, USA). Where ANOVA yielded significance ($P < 0.01$), *post hoc* analysis was performed using the Tukey–Kramer multiple comparisons test. Data presented are the means ± standard error (SE).

Protein gel electrophoresis and in-gel activity

Proteins were separated using 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein samples were

mixed with sample buffer consisting of 0.5 M Tris-HCl (pH 6.8), 10% glycerol and bromophenol blue as the tracking dye. Proteins were separated using SDS-PAGE (10% running gel, pH 8.8 and 5% stacking gel, pH 6.8) at room temperature in Tris-glycine buffer (pH 10.8). Electrophoretic separation was performed at 100 V for stacking and 130 V for separation. A prestained protein molecular weight marker (#SM0441; Fermentas, Pittsburgh, PA, USA) was used as a standard. After electrophoresis, the gels were treated with reaction buffer (10 mM sodium acetate, pH 5.6) for 10 min and then rinsed with ddw for 10 min. The gel was incubated for 10 min with 25 mL of reaction buffer containing 0.1 mg/mL TMB, and then 3 mM H₂O₂ was added to assay POD activity. For the analysis of PPO activity, the gel was incubated with 25 mM dopa in 25 mL of reaction buffer for 30 min, and then photographed.

POD and PPO activity *in situ*

Following induction treatments, discs (20 mm in diameter) were excised from *Z. aethiopica* leaves and inoculated with 10 µL of *P. carotovorum* (10⁶ cfu/mL) using a 10-µL tip, as described previously (Luzzatto *et al.*, 2007b). The leaf discs were then transferred to 24-well microtitre plates, in which they were immersed in 1 mL DAB (1 mg/mL) or 1 mL dopa (25 mM) in reaction buffer. The plates were then incubated on a shaker at room temperature for 10 h. To visualize the *in situ* accumulation of the enzymatic reaction product, the leaf discs were bleached with boiling ethanol (96%) and viewed under a Leica MZFLIII stereomicroscope (Solms, Germany) equipped with a DS-Fi1 camera (Nikon, Melville, NY, USA) and NIS-Elements (Nikon) software (ver. 3.06).

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