Characterization of *Pectobacterium carotovorum* proteins differentially expressed during infection of *Zantedeschia elliotiana in vivo* and *in vitro* which are essential for virulence

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SUMMARY

The identification of phytopathogen proteins that are differentially expressed during the course of the establishment of an infection is important to better understand the infection process. *In vitro* approaches, using plant extracts added to culture medium, have been used to identify such proteins, but the biological relevance of these findings for *in planta* infection are often uncertain until confirmed by *in vivo* studies. Here, we compared the proteins of *Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1 differentially expressed in Luria–Bertani medium supplemented with extracts of the ornamental plant *Zantedeschia elliotiana* cultivar 'Black Magic' (*in vitro*) and in plant tissues (*in vivo*) by two-dimensional electrophoresis coupled with mass spectrometry. A total of 53 differentially expressed proteins (>1.5-fold) were identified (up-regulated or down-regulated *in vitro*, *in vivo* or both). Proteins that exhibited increased expression *in vivo* but not *in vitro*, or in both conditions, were identified, and deletions were made in a number of genes encoding these proteins, four of which (*clpP*, *mreB*, *flgK* and *eda*) led to a loss of virulence on *Z. elliotiana*, although *clpP* and *mreB* were later also shown to be reduced in growth in rich and minimal media. Although *clpP*, *flgK* and *mreB* have previously been reported as playing a role in virulence in plants, this is the first report of such a role for *eda*, which encodes 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, a key enzyme in Entner–Doudoroff metabolism. The results highlight the value of undertaking *in vivo* as well as *in vitro* approaches for the identification of new bacterial virulence factors.

Keywords: calla lily, Entner–Doudoroff, *in vivo*, *Pectobacterium*, proteomics, *Zantedeschia*.

INTRODUCTION

Summer-flowering calla lily (*Zantedeschia* spp.) cultivars play an important role in cut-flower and bulb production worldwide (Snijder *et al.*, 2004; Wright, 1998). Soft rot disease caused by *Pectobacterium carotovorum* ssp. *carotovorum* (Pcc; formerly *Erwinia carotovora* ssp. *carotovora*) leads to substantial economic losses in calla lily, as well as in other crops and ornamental monocots (Ma *et al.*, 2007; Mansfield *et al.*, 2012; Ni *et al.*, 2010; Snijder *et al.*, 2004; Toth *et al.*, 2003; Wright, 1998; Yishay *et al.*, 2008). As there are no effective measures to control the disease, it is a major limiting factor in horticultural production, although strict agronomic measures and the selection of resistant cultivars have alleviated disease losses to some extent (Luzzatto-Knaan *et al.*, 2014; Ni *et al.*, 2010).

The main pathogenicity determinants of Pcc are plant cell wall-degrading enzymes (PCWDEs), which include pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel) and protease (Prt) (Bortoli-German *et al.*, 1994; Heikinheimo *et al.*, 1995; Hinton *et al.*, 1990; Mae *et al.*, 1995; Pirhonen *et al.*, 1991; Toth *et al.*, 2003). The availability of genomic sequences for *Pectobacterium* strains (Bell *et al.*, 2004; Koskinen *et al.*, 2012; Park *et al.*, 2012) has facilitated detailed studies on these and other virulence determinants (Grinter *et al.*, 2012; Ma *et al.*, 2014; Yap *et al.*, 2004). However, the protein expression patterns of Pcc in a host plant during the establishment of infection remain poorly understood.

Proteomic approaches have been widely used in the study of plant–bacterial interactions. Examples include the identification and functional analysis of *Xanthomonas campestris* pv. *campestris* proteins expressed in culture medium (Chung *et al.*, 2007), *X. axonopodis* pv. *citri* proteins differentially expressed in response to different growth conditions (Mehta and Rosato, 2001), comparison of the secretomes of *Pectobacterium atrosepticum* induced by host extracts (Mattinen *et al.*, 2007) and impacts induced by host plant extracts or ions on *Dickeya dadantii* grown in minimal medium (MM) (Babujee *et al.*, 2007). Most of these studies have been performed with plant extracts to mimic *in planta* conditions. However, there is no evidence to show whether findings from these studies are directly relevant to what happens *in planta* during infection.

Mehta and Rosato (2003) developed a simple and rapid protocol for obtaining microorganisms directly from inoculated leaves. Five new *X. axonopodis* pv. *citri* proteins differentially regulated by the host plant *in vivo* were reported in this study. Using this protocol and the corresponding proteomics approaches, Andrade *et al.* (2008) analysed the *in vivo* proteome of *X. campestris* pv. *campestris* in different cultivars of infected *Brassica oleracea*. Despite such technical progress, comparison of data from plant–bacterial interactions *in vitro* and *in vivo* is in its infancy, hindering our understanding of pathogen responses to living plants in the establishment of infection.

Recently, the *in vitro* and *in vivo* profiles of outer membrane (OM) proteins in the rice pathogen *Acidovorax avenae* ssp. *avenae* have been reported (Ibrahim *et al.*, 2012). Combining liquid chromatography-tandem mass spectrometry (LC-MS/MS) and *in silico* OM proteome and *in silico* genome-wide analyses, it was anticipated that a high number of OM proteins were differentially expressed in the two conditions, with those expressed *in vivo* possibly indicating a role in virulence.

In this study, protein profiles from *in vitro* and *in vivo* conditions were generated for PccS1 following its interaction with the *Zantedeschia elliotiana* cultivar 'Black Magic'. With two-dimensional electrophoresis (2-DE) coupled with MS, we identified 53 proteins that revealed differential expression *in vitro*, *in vivo* or in both compared with the control. On testing mutant strains affected in genes encoding 14 of these proteins (ClpP, MreB, FlgK, KdgR, PrmA, AceE, Pgk, AspA, AtpD, HslO, Upp, NusA, SspA and Zwf), three (ClpP, MreB and FlgK) were found to be affected in virulence. In addition, following further analysis of a mutant in gene *zwf*, an additional protein, Eda, was further examined and found to be involved in virulence. Although ClpP, FlgK and MreB have previously been reported to play a role in the virulence of plant pathogens, to the best of our knowledge, this is the first report in which the Entner–Doudoroff pathway central enzyme Eda has been shown to play such a role.

RESULTS

Bacterial cells recovered from the plant-pathogenic bacterium infiltrated *in vivo*

An examination of bacterial populations in petioles of the potted plants (shown in Fig. S1, see Supporting Information) revealed that there was a clear increase in the population at 8 h after inoculation (HAI), and the initial symptoms of disease could be observed at this time. However, the rotting of plant tissue was not visualized clearly until 16 HAI, at which time there were sufficient quantities of proteins from extracts for 2-DE, and there was no bacterial protein in the negative controls (data not shown).

Comparative proteomic profiling analyses

The protein expression patterns of PccS1-LB [PccS1 cultured in Luria–Bertani broth (LB)], PccS1-LB-Ze (PccS1 cultured in LB + 0.4% petiole extract) and PccS1-V-Ze (PccS1 grown *in planta*) were analysed by 2-DE, and resulted in a total of 386, 366 and 369 protein spots, respectively, on the 2-DE maps (Fig. 1). When comparing the differential protein spots obtained from PccS1-LB-Ze or PccS1-V-Ze with the control (PccS1-LB), PccS1-LB-Ze showed 47 proteins with increased expression and 23 with decreased expression (70 proteins in total), whereas PccS1-V-Ze showed 50 proteins with increased expression and 45 with decreased expression (95 proteins in total) ($P \leq 0.05$, expression ratio ≥ 1.5). When comparing the 2-DE protein profiles between PccS1-V-Ze and PccS1-LB-Ze, there was a total of 133 proteins showing significant differential expression between PccS1-V-Ze and PccS1-LB-Ze, 68 showing increased and 65 showing decreased expression in PccS1-V-Ze relative to PccS1-LB-Ze.

Differentially expressed proteins analysed by MS

Of the 165 proteins showing differential expression in the *in vitro* condition [PccS1-LB-Ze (70 proteins)] and/or *in vivo* condition [PccS1-V-Ze (95 proteins)] compared with the control (PccS1-LB), 74 proteins were excised from silver-stained gels and their tryptic fragments were analysed by MS/MS. Of these 74 proteins, 53 were successfully identified and divided into eight groups based on functional annotation (Fig. 2), and the degrees of differential expression of the individual proteins within these groups were examined (Fig. 3). Table 1 shows details of the identification of differentially expressed proteins using MS analysis. Among the proteins identified, there were 37 proteins in PccS1-LB-Ze and 50 in PccS1-V-Ze showing differential expression when compared with the control (PccS1-LB). Of the 37 proteins in PccS1-LB-Ze, 23 were up-regulated and 14 were down-regulated, with nine of these proteins showing greater than a five-fold change in expression when compared with the control. In PccS1-V-Ze, 32 of the 50 proteins were up-regulated and 18 down-regulated, with 30 of the proteins showing greater than a five-fold change in expression when compared with the control.

Almost one-half (25 proteins) of the 53 identified proteins belonged to groups I and III (Fig. 2): 'carbohydrate metabolism' and 'protein synthesis, folding and degradation', respectively. The majority of these proteins were from PccS1-V-Ze, which in almost all cases showed greater changes in expression than proteins from PccS1-LB-Ze. However, where changes in expression were observed, there was a good correlation with those exhibiting up- or down-regulation between the two conditions (Fig. 3). In the other groups, almost all of the proteins were significantly up-regulated in both PccS1-LB-Ze and PccS1-V-Ze, again with the majority of proteins from PccS1-V-Ze tending to exhibit greater changes in expression than those from PccS1-LB-Ze. All of the
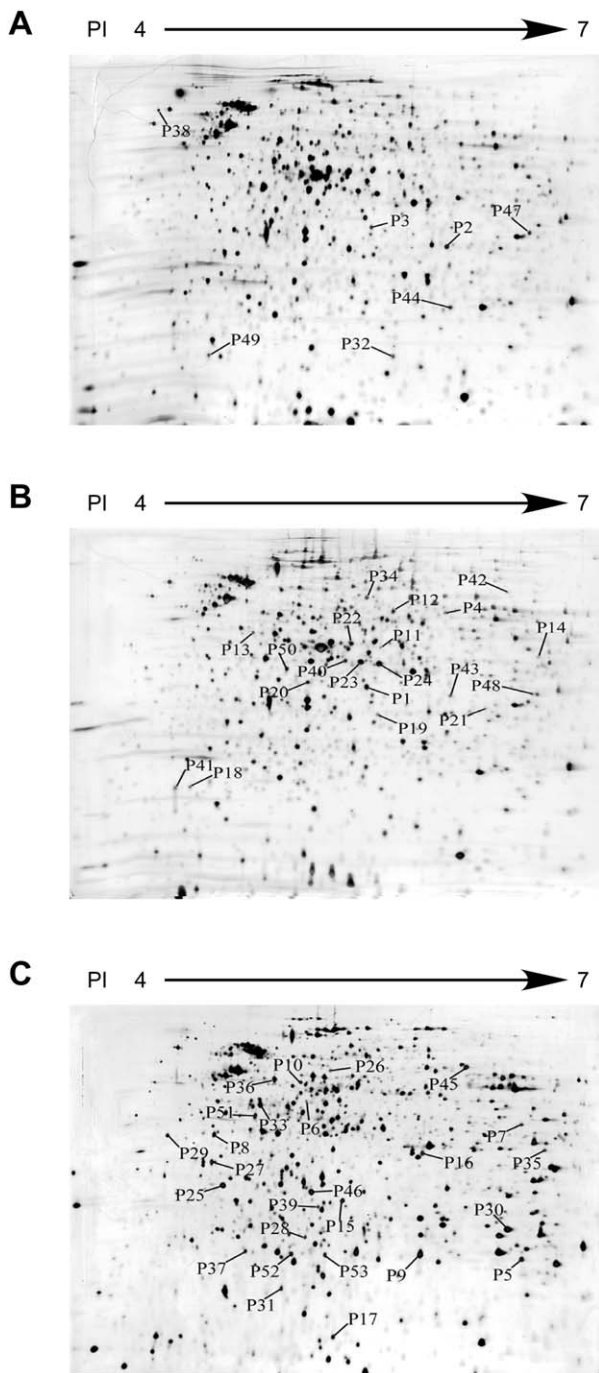
Fig. 1 Two-dimensional electrophoresis (2-DE) images of proteins extracted from the bacterial cells of *Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1 cultured in Luria–Bertani broth (LB) (PccS1-LB) (A), in LB supplemented with 0.4% petiole extract of *Zantedeschia elliotiana* (PccS1-LB-Ze, PccS1 interaction with the host plant *in vitro*) (B) and recovered from petioles of potted *Z. elliotiana* plants (PccS1-V-Ze, PccS1 interaction with the host *in vivo*) (C). The differentially expressed protein spots were numbered above the threshold ratio of 1.5. Protein spots identified by tandem mass spectrometry (MS/MS) are listed in Table 1.

proteins in groups IV and V, involved in 'nucleic acid metabolism' and 'transcription', were up-regulated in PccS1-V-Ze from at least 1.8- to over 10-fold. From all of the above proteins, 10 (PotD [P08], ClpP [P09], PotF [P16], RpsD [P28], PrmA [P29], KdgR [P39], AceE [P45], MreB [P50], FlgK [P51] and MotA [P52]) were selected for further study from PccS1-V-Ze, and showed expression levels over 10-fold up-regulated, but with little or no differential expression in PccS1-LB-Ze (when compared with the PccS1-LB control). In addition, eight proteins (Pgk [P05], Zwf [P07], AspA [P10], AtpD [P17], HslO [P27], Upp [P37], NusA [P38] and SspA [P53]) were selected showing up to 10-fold up-regulation in both PccS1-V-Ze and PccS1-LB-Ze compared with the control (in the majority of cases being more highly up-regulated in PccS1-V-Ze than in PccS1-LB-Ze).

Changes in the transcriptional levels of genes encoding significantly up-regulated proteins in the interaction of PccS1 with *Z. elliotiana in vivo*

To relate gene and protein expression levels, the transcription levels of genes encoding proteins with an eight- to more than 10-fold increase in expression level following the interaction of PccS1 with *Z. elliotiana in vivo* (PccS1-V-Ze) were assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. 4), and compared with that of PccS1 cultured in LB (PccS1-LB). All of these genes showed transcript levels up-regulated in PccS1-V-Ze with increased expression in their respective proteins following proteomics, e.g. P29 (*prmA*), P39 (*kdgR*), P45 (*aceE*) and P50 (*mreB*) had transcriptional levels up-regulated between four- and 11-fold compared with the control (Fig. 4). In all cases, the transcript levels of these genes in PccS1-LB-Ze were similar to those of the control.

Pathogenicity assay of mutant strains

Experiments were set up to investigate the role of the 10 differentially expressed proteins from PccS1-V-Ze in the virulence of PccS1. These experiments involved biparental mating to produce single gene deletion mutants of *clpP* [P09], *prmA* [P29], *kdgR* [P39], *aceE* [P45], *mreB* [P50] and *flgK* [P51]. Diagnostic PCR for individual genes confirmed that they were disrupted in the mutant strains (data not shown). No deletion mutants were obtained for *potD* [P08], *potF* [P16], *rpsD* [P28] and *motA* [P52], and these were not investigated further.

Pathogenicity tests of the six mutant strains compared with that of the wild-type were performed by inoculation into detached leaves from whole plants of *Z. elliotiana* (Fig. 5A). The maceration assay revealed that there was no significant difference in virulence between the *prmA*, *kdgR* and *aceE* mutants and the wild-type strain, whereas *clpP*, *mreB* and *flgK* mutants exhibited reduced virulence and a number of other phenotypic effects. Details on changes in virulence and other phenotypes,
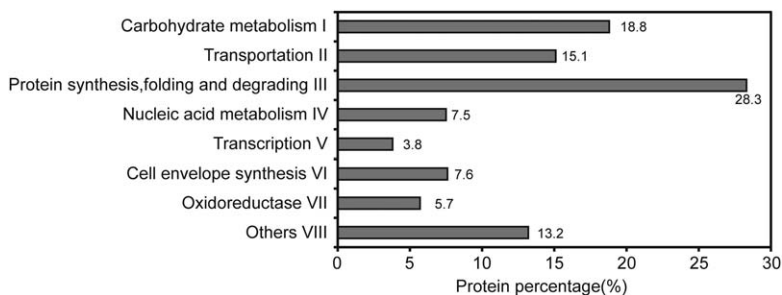
Fig. 2 Distribution of 53 differentially expressed proteins following proteomic analyses *in vivo* and *in vitro* based on functional annotation according to the COG (Clusters of Orthologous Groups) database of the National Center for Biotechnology Information (NCBI).

together with the complementation of mutants *clpP*, *flgK* and *mreB*, have been reported elsewhere (Deng *et al.*, 2014; Li *et al.*, 2013; Yang *et al.*, 2012). It should be noted that both *clpP* and *mreB* mutants were also affected in growth in both rich and minimal media, which appears to explain their reduced virulence phenotype. ClpP and FlgK have been reported previously to play a role in the virulence of plant pathogens, including FlgK in *Xanthomonas axonopodis* pv. *glycines* on soybean (Athinuwat *et al.*, 2009) and ClpP in *D. dadantii* on Chinese cabbage (Li *et al.*, 2010). The roles of MreB and FlgK in virulence carried out as part of this study have also been reported previously (Deng *et al.*, 2014; Yang *et al.*, 2012).

The *in vivo* assay conducted uncovered proteins involved in virulence that were not identified in the *in vitro* screen, suggesting that a combination of both *in vitro* and *in vivo* analyses adds value to the search for virulence. However, the proteins identified from the *in vivo* screen have previously been associated with reduced virulence (see above). We therefore extended the study to include an additional set of proteins (Pgk [P05], Zwf [P07], AspA [P10], AtpD [P17], HslO [P27], Upp [P37], NusA [P38] and SspA [P53]), which showed increased expression both *in vivo* and *in vitro*, in an attempt to identify novel virulence determinants. To accelerate virulence testing for this group of proteins, we first generated insertion mutants and tested their virulence (Fig. 5B). Of these mutants, only *zwf*(i), affected in *zwf* and encoding an enzyme in the pentose phosphate pathway involved in metabolism, was reduced in virulence, and was taken forward for deletion mutagenesis in line with the mutants produced following the *in vivo* screen. However, when the *zwf* deletion mutant (Δ*zwf*) was tested *in planta*, no reduction in virulence was observed.

In *D. dadantii* 3937, *zwf* lies upstream of gene *eda* in a two-gene operon (Conway *et al.*, 1991; Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1994). *eda* encodes 2-keto-3-deoxy-6-phosphogluconate aldolase, an enzyme in the Entner–Doudoroff pathway involved in the catalysis of glucose to pyruvic acid as an alternative pathway to glycolysis in the production of ATP (Conway *et al.*, 1991). Based on the incomplete draft genome sequence of PccS1, a similar operon structure was also observed in this strain (Fig. 6A). PCR, using primers *zwf*-F and *eda*-R on cDNA, confirmed that *zwf* and *eda* are co-transcribed in PccS1

(Fig. 6A,B). To determine whether the insertion mutant in *zwf* affected virulence through its actions on the expression of *eda*, a deletion mutant in *eda* was generated and was shown to be reduced in virulence, whereas complementation of the *eda* mutant restored virulence (Fig. 6C). The mutation had no effect on growth in both LB and MM compared with the wild-type and complemented strains (Fig. S2, see Supporting Information). An alignment of amino acids from Eda using BioEdit software (Hall, 1999) against six other Eda proteins, including *D. dadantii* 3937 KdgA, showed 88% identity with *Escherichia coli* K-12 and 89% identity with *D. dadantii* 3937 (Fig. 7A). In line with the findings in *D. dadantii* 3937, which suggest that KdgA (an equivalent to Eda) is involved in the utilization of pectin ((Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1994), growth of the PccS1 *eda* mutant on pectinase agar was reduced compared with that of the wild-type strain (Fig. 7B), whereas complementation of the mutant restored the wild-type phenotype.

DISCUSSION

Recently, functional genomic strategies, including proteomics and transcriptomics, have contributed to our understanding of plant–pathogen interactions (Delaunois *et al.*, 2014; Mark *et al.*, 2005). Proteomics has improved dramatically to assist in the large-scale functional assignment of candidate proteins involved in pathogenicity (Mehta *et al.*, 2008). Such studies include the identification of important proteins involved in pathogenesis, e.g. Svx, a quorum-sensing-controlled virulence factor secreted via the type II secretion pathway (Corbett *et al.*, 2005), and the porin OmpA, involved in binding to specific host cell receptor molecules (Babujee *et al.*, 2007). As a result of the difficulties of extracting bacterial nucleic acids and proteins from plant material, most studies are performed by the growth of bacteria in medium supplemented with plant extracts to mimic *in planta* conditions, and this has revealed many important bacterial proteins involved in both pathogenic and symbiotic interactions with plants (Cheng *et al.*, 2010). However, the major drawback of this approach is the lack of a true interaction between the pathogen and its host (Mark *et al.*, 2005). Although an increasing number of researchers are studying the differential expression of nucleic acids in plants during both pathogenic and beneficial interactions, only a very few have
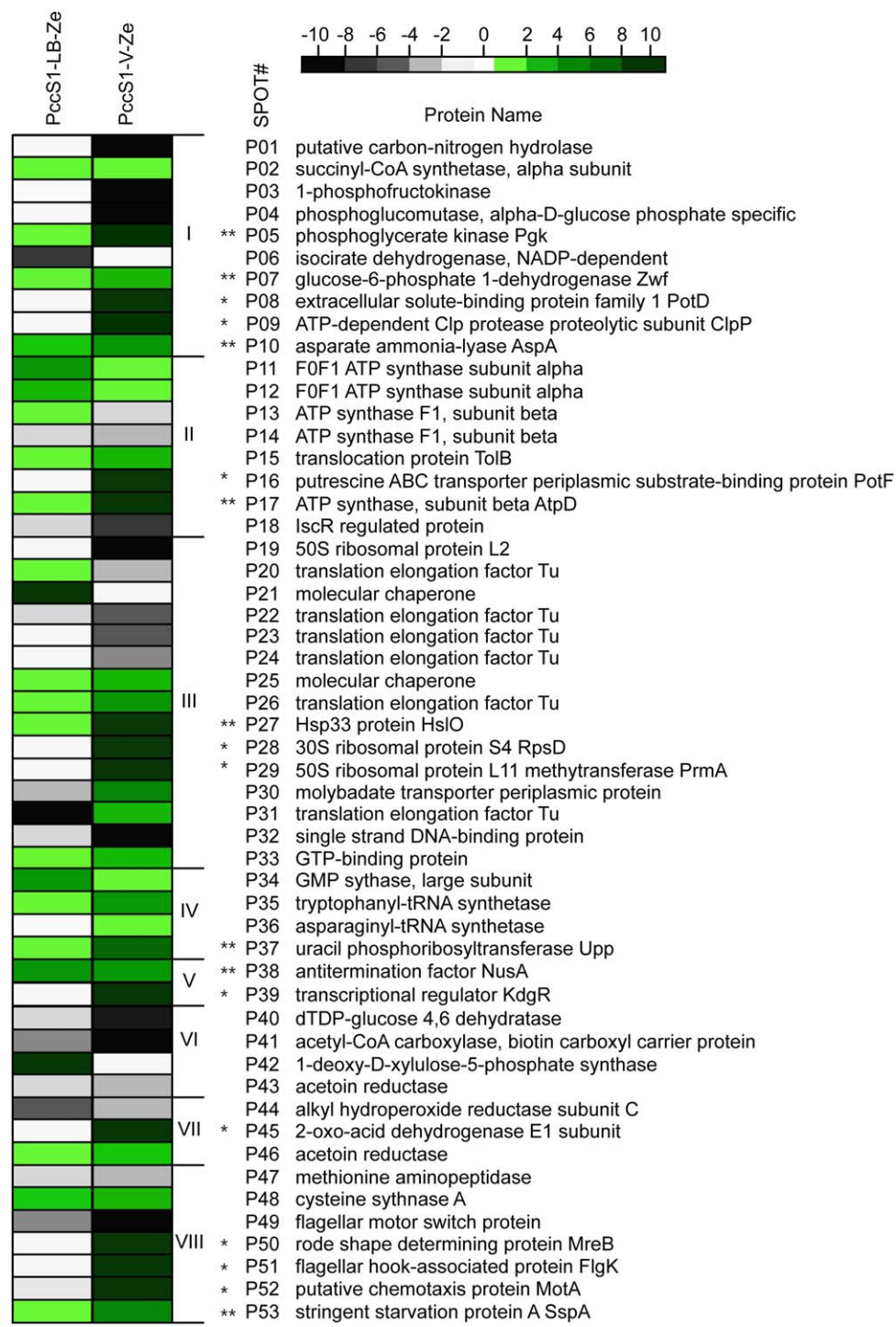
Fig. 3 Differentially expressed protein profiles of PccS1-LB-Ze [*Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1 cultured in Luria–Bertani broth (LB) supplemented with 0.4% petiole extract of *Zantedeschia elliotiana*] (left column) and PccS1-V-Ze (recovered from petioles of potted *Z. elliotiana* plants) (right column) compared with those of PccS1-LB (PccS1 cultured in LB); the degrees of differential expression are presented according to the colour scale at the top left; the green boxes indicate up-regulation, the black boxes indicate down-regulation and the white boxes represent proteins with no significant difference from those of PccS1-LB. *Proteins significantly up-regulated only *in vivo*. **Proteins significantly up-regulated both *in vitro* and *in vivo*.

SPOT#	Protein Name
P01	putative carbon-nitrogen hydrolase
P02	succinyl-CoA synthetase, alpha subunit
P03	1-phosphofructokinase
P04	phosphoglucomutase, alpha-D-glucose phosphate specific
** P05	phosphoglycerate kinase Pgk
P06	isocitrate dehydrogenase, NADP-dependent
** P07	glucose-6-phosphate 1-dehydrogenase Zwf
* P08	extracellular solute-binding protein family 1 PotD
* P09	ATP-dependent Clp protease proteolytic subunit ClpP
** P10	asparate ammonia-lyase AspA
P11	F0F1 ATP synthase subunit alpha
P12	F0F1 ATP synthase subunit alpha
P13	ATP synthase F1, subunit beta
P14	ATP synthase F1, subunit beta
P15	translocation protein TolB
* P16	putrescine ABC transporter periplasmic substrate-binding protein PotF
** P17	ATP synthase, subunit beta AtpD
P18	IscR regulated protein
P19	50S ribosomal protein L2
P20	translation elongation factor Tu
P21	molecular chaperone
P22	translation elongation factor Tu
P23	translation elongation factor Tu
P24	translation elongation factor Tu
P25	molecular chaperone
P26	translation elongation factor Tu
** P27	Hsp33 protein HslO
* P28	30S ribosomal protein S4 RpsD
* P29	50S ribosomal protein L11 methytransferase PrmA
P30	molybadate transporter periplasmic protein
P31	translation elongation factor Tu
P32	single strand DNA-binding protein
P33	GTP-binding protein
P34	GMP sythase, large subunit
P35	tryptophanyl-tRNA synthetase
P36	asparaginyl-tRNA synthetase
** P37	uracil phosphoribosyltransferase Upp
** P38	antitermination factor NusA
* P39	transcriptional regulator KdgR
P40	dTDP-glucose 4,6 dehydratase
P41	acetyl-CoA carboxylase, biotin carboxyl carrier protein
P42	1-deoxy-D-xylulose-5-phosphate synthase
P43	acetoin reductase
P44	alkyl hydroperoxide reductase subunit C
* P45	2-oxo-acid dehydrogenase E1 subunit
P46	acetoin reductase
P47	methionine aminopeptidase
P48	cysteine sythnase A
P49	flagellar motor switch protein
* P50	rode shape determining protein MreB
* P51	flagellar hook-associated protein FlgK
* P52	putative chemotaxis protein MotA
** P53	stringent starvation protein A SspA

addressed changes in the bacterial proteome during these interactions, e.g. proteomic analysis of *Xanthomonas campestris* pv. *campestris* during its interaction with the host plant *in vivo* (Andrade *et al.*, 2008; Villeth *et al.*, 2009). It is clear that differences are certain to occur between bacterial interactions with the host plant *in vitro* and *in vivo*, with many processes occurring *in vivo* that would not occur *in vitro*, e.g. the onset and targeting of host defences and the way in which a pathogen responds to these defences, such as altering one or more of its metabolic pathways or switching ion channels. Such complex interactions could not be replicated using plant extracts alone (Haider and Pal, 2013; Nie *et al.*, 2007).

Table 1 Differentially expressed proteins identified by tandem mass spectrometry.

| Spot	Accession number	Organism	Score	M_r (cal)	p_i (cal)	Sequence coverage (%)	No. of peptides matched
Carbohydrate metabolism							
P01	gi|50123194	*Pectobacterium atrosepticum* SCRI1043	228	32.8	5.53	18	4
P02	gi|253687633	*P. carotovorum* PC1	268	30.1	5.56	23	6
P03	gi|50121652	*P. atrosepticum* SCRI1043	342	33.8	5.46	29	7
P04	gi|253687606	*P. carotovorum* PC1	459	58.9	5.70	28	14
P05	gi|50122833	*P. atrosepticum* SCRI1043	107	41.3	5.24	8	3
P06	gi|253688259	*P. carotovorum* PC1	543	46.2	5.09	45	18
P07	gi|253688219	*P. carotovorum* PC1	503	56.2	6.07	38	16
P08	gi|253688246	*P. carotovorum* PC1	102	38.7	5.01	16	3
P09	gi|227328849	*P. carotovorum* WPP14	339	23.2	5.57	19	4
P10	gi|253686801	*P. carotovorum* PC1	306	53.3	5.13	16	7
Transportation							
P11	gi|50123430	*P. atrosepticum* SCRI1043	103	55.4	5.49	21	8
P12	gi|227113925	*P. brasiliensis* PBR1692	524	55.4	5.49	34	11
P13	gi|253690617	*P. carotovorum* PC1	531	50.3	4.96	36	11
P14	gi|227327556	*P. carotovorum* WPP14	309	45.8	6.97	28	8
P15	gi|254421831	*Synechococcus* sp. PCC 7335	274	52.3	4.56	12	6
P16	gi| 253688080	*P. carotovorum* PC1	240	44.3	5.72	20	7
P17	gi|112791384	*P. odoriferum*	77	46.2	4.71	14	2
P18	gi|253690287	*P. carotovorum* PC1	286	21.4	4.63	29	6
Protein synthesis, folding and degradation							
P19	gi|224585232	*Salmonella enterica* ssp. *enterica* serovar Paratyphi C str. RKS4594	375	27.9	10.82	39	12
P20	gi|253686604	*P. carotovorum* PC1	329	43.4	5.23	32	9
P21	gi|50122803	*P. atrosepticum* SCRI1043	293	68.9	4.83	10	4
P22	gi|253686604	*P. carotovorum* PC1	486	43.4	5.29	47	17
P23	gi|253686604	*P. carotovorum* PC1	531	43.4	5.29	48	19
P24	gi|253686604	*P. carotovorum* PC1	117	43.4	5.23	36	10
P25	gi|50122803	*P. atrosepticum* SCRI1043	421	68.9	4.83	16	7
P26	gi|253686604	*P. carotovorum* PC1	259	43.4	5.23	31	11
P27	gi|253690256	*P. carotovorum* PC1	413	32.7	4.68	21	6
P28	gi|50122927	*P. atrosepticum* SCRI1043	240	23.5	10.08	59	12
P29	gi|253686645	*P. carotovorum* PC1	504	32.3	4.40	44	11
P30	gi|253687663	*P. carotovorum* PC1	160	27.8	6.12	7	2
P31	gi|253686604	*P. carotovorum* PC1	113	43.4	5.23	13	5
P32	gi|227326029	*P. carotovorum* WPP14	476	19.2	5.46	29	7
P33	gi|253686962	*P. carotovorum* PC1	140	43.1	4.79	16	5
Nucleic acid metabolism							
P34	gi|253689372	*P. carotovorum* PC1	269	58.9	5.37	20	9
P35	gi|253690238	*P. carotovorum* PC1	339	37.6	5.94	20	11
P36	gi|253688172	*P. carotovorum* PC1	369	52.6	5.00	29	14
P37	gi|253687524	*P. carotovorum* PC1	148	22.6	5.74	29	5
Transcription							
P38	gi|253686985	*P. carotovorum* PC1	313	56.5	4.53	27	15
P39	gi|253688272	*P. carotovorum* PC1	218	29.7	5.25	52	11
Cell envelope synthesis							
P40	gi|145298428	*Aeromonas salmonicida* ssp. *salmonicida* A449	133	41.4	5.78	19	4
P41	gi|253686650	*P. carotovorum* PC1	230	16.6	4.71	20	3
P42	gi|50120070	*P. atrosepticum* SCRI1043	338	67.9	6.04	19	10
P43	gi|253689156	*P. carotovorum* PC1	323	43.9	5.74	24	8
Oxidoreductase							
P44	gi|123443378	*Yersinia enterocolitica* ssp. *enterocolitica* 8081	179	22.4	5.80	18	3
P45	gi| 251756507	*P. carotovorum PC1*	135	99.8	5.46	11	11
P46	gi|253686701	*P. carotovorum* PC1	463	27.1	5.14	37	12
Others							
P47	gi|253687336	*P. carotovorum* PC1	239	29.6	5.88	43	10
P48	gi|50119836	*P. atrosepticum* SCRI1043	546	34.5	5.51	34	9
P49	gi|253688960	*P. carotovorum* PC1	117	14.9	4.62	29	3
P50	gi|253686657	*P. carotovorum* PC1	254	37.1	5.06	32	9
P51	gi| 253688966	*P. carotovorum* PC1	101	60.1	4.74	3	6
P52	gi|148254924	*Bradyrhizobium* sp. BTAiI	79	28.2	5.17	3	4
P53	gi| 49609794	*P. atrosepticum* SCRI1043	52	24.5	5.11	9	2
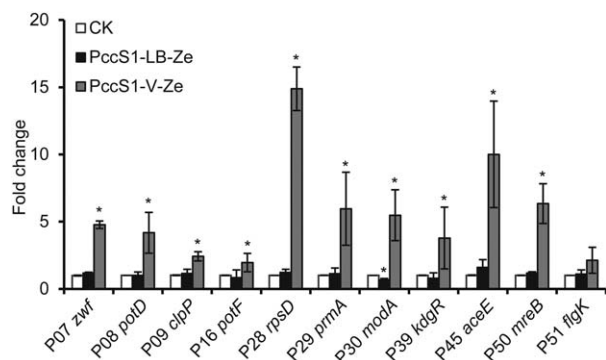
Fig. 4 Relative mRNA levels of genes in *Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1 in different conditions determined by quantitative reverse transcription-polymerase chain reaction. The data were analysed using 16s rDNA as an endogenous control. The white bar represents the relative mRNA levels of genes from bacteria (PccS1-LB) grown to stationary phase in Luria–Bertani broth (LB) used as a control, the black bar the relative mRNA levels of genes from bacteria (PccS1-LB-Ze) grown to stationary phase in LB + 0.4% petiole extract of *Zantedeschia elliotiana*, and the grey bar the relative mRNA levels of genes from bacteria following interaction with *Z. elliotiana* in vivo (PccS1-V-Ze) for 16 h. *Significant difference in mRNA level of the bacterial cells compared with that cultured in LB ($P < 0.05$, Student's *t*-test).

It is highly likely that the proteins found in our 2-DE maps do not represent all of the proteins expressed during the infection process, but rather result in only a partial view of this aspect of the PccS1 proteome. The gel electrophoresis and subsequent MS identification steps are both biased largely towards highly abundant proteins (Lubec and Afjehi-Sadat, 2007; Mehta *et al.*, 2008). However, even with this proviso, there were still a large number of differentially expressed proteins (133 proteins) between the *in vivo* (PccS1-V-Ze) and *in vitro* (PccS1-LB-Ze) conditions, and between these conditions and the control PccS1-LB (165 proteins). Among the 53 proteins successfully identified from these 165 proteins, the expression levels of 16 proteins in PccS1-LB-Ze were similar to those in PccS1-LB, but only three proteins in PccS1-V-Ze showed the same expression level as in PccS1-LB, suggesting that a more diverse range of proteins is differentially expressed in the *in vivo* condition (Fig. 3).

The 53 proteins were successfully identified and divided into eight groups depending on their functional annotation (Fig. 2). The most abundant proteins present in the total fraction were involved in protein synthesis, folding, degradation and carbohydrate metabolism. Many of the proteins that were up-regulated in PccS1-LB-Ze in response to petiole exudates were involved in the utilization of nutrients. This observation is consistent with a transcriptomic study of bacterial responses to plant root exudates (Mark *et al.*, 2005). However, in PccS1-V-Ze, expression of these proteins showed some differences with PccS1-LB-Ze, i.e. proteins including P09 (ClpP), P16 (PotF), P29 (PrmA), P39 (KdgR), P45 (AceE), P50 (MreB) and P51 (FlgK) were highly up-regulated,
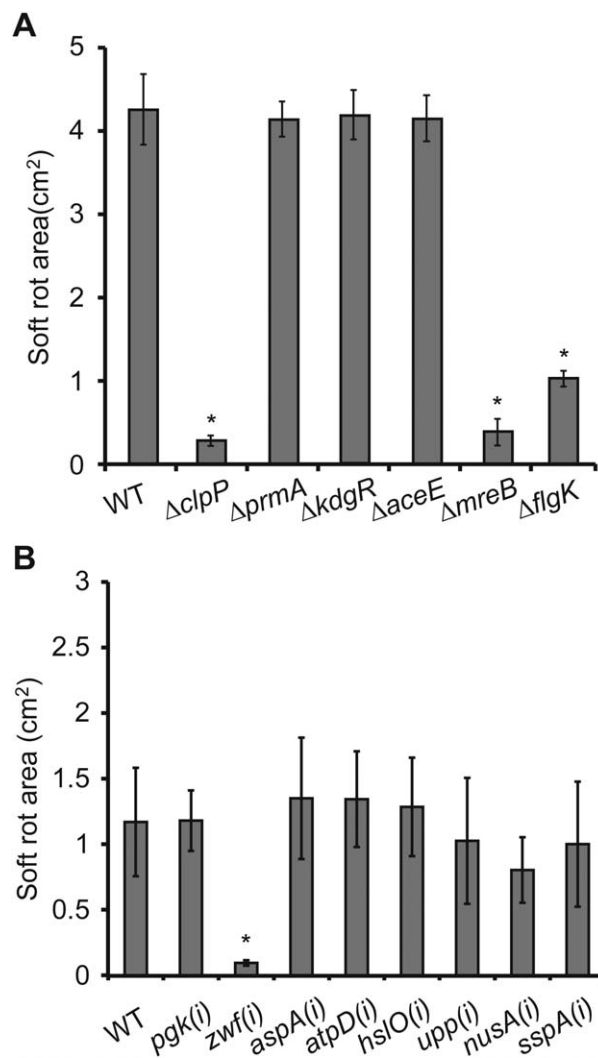

Fig. 5 Disease lesions caused by *Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1 (wild-type) and deletion mutants Δ*clpP*, Δ*mreB*, Δ*flgK*, Δ*prmA*, Δ*kdgR* and Δ*aceE* in leaves of *Zantedeschia elliotiana* (A) and PccS1 (wild-type) and insertion mutants *pgk*(i), *zwf*(i), *aspA*(i), *atpD*(i), *hslO*(i), *upp*(i), *nusA*(i) and *sspA*(i) in Chinese cabbage leaves (B). The leaves were inoculated at small wound sites with a 10-μL bacterial suspension at an optical density at 600 nm (OD$_{600}$) of 1.2. Three replicates were used for each treatment, and the experiment was repeated three times. Vertical bars represent standard errors. *Significant difference between the wild-type strain and mutant ($P < 0.05$; *t*-test).

whereas others, including P1 (Cpa), P3 (FruK) and P4 (Pgm), were highly down-regulated. When the bacteria were cultured in medium supplemented with plant extracts, the metabolic pathways of the bacteria were affected, and the expression of nutrient-related genes involved in pathogenesis was induced differentially. However, when the pathogen infects plants, these plants will produce defence factors, e.g. signal molecules, proteins and hormones, to defend against invasion by the pathogen, and
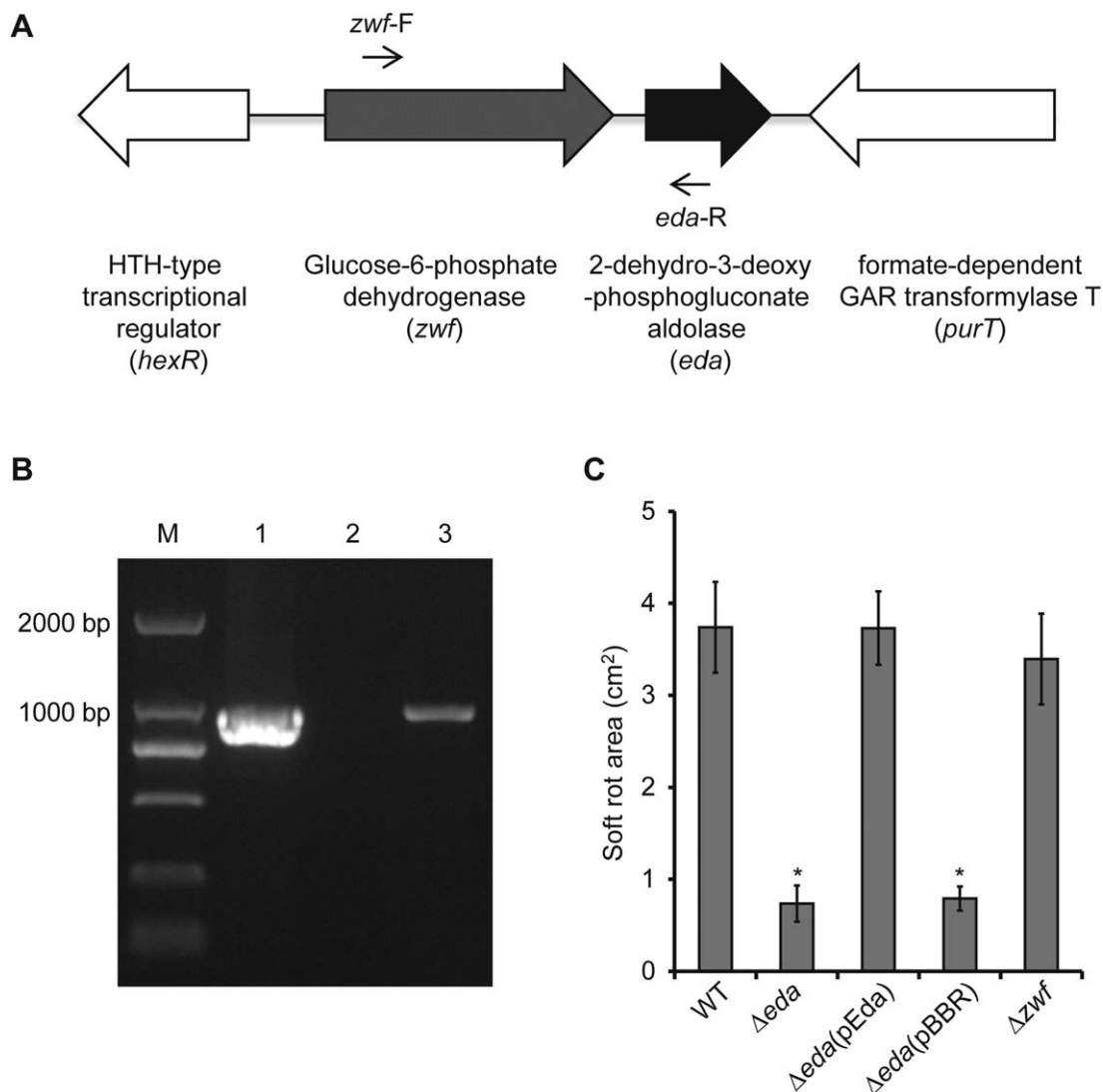
Fig. 6 (A) Genetic structure of the *zwf-eda* operon in the draft genome sequence of *Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1. (B) Polymerase chain reaction (PCR) analysis showing the co-transcription of *zwf-eda* using the primers zwf-F and eda-R which span across both genes: 1, genomic DNA; 2, RNA (no reverse transcript); 3, cDNA. The product size is 1000 bp. (C) Pathogenicity assay on *Zantedeschia elliotiana* leaves showing the reduction in virulence of the Δ*eda* deletion mutant and its subsequent complementation, but no reduction in virulence for Δ*zwf*. *Significant difference between the wild-type strain and mutant ($P < 0.05$; *t*-test).

pathogenic bacteria will also change their gene expression to influence the plant accordingly, complex reactions that cannot take place when using plant extracts alone. Perhaps for this reason, proteins involved in protein synthesis, folding and degradation from the *in vivo* conditions accounted for 28.3% of the eight protein functions. Another group of proteins that were up-regulated belonged to nucleic acid metabolism and transportation, including ATP synthase, translocation protein and GTP-binding protein, which have all been shown previously to be required during the interaction of bacteria with their plant hosts (Caldelari *et al.*, 2006).

Among the genes encoding the 10 proteins with expression levels over 10-fold in PccS1 when interacting with *Z. elliotiana in vivo* compared with *in vitro*, six genes were successfully deleted in PccS1 to generate mutants. The remaining four could not be deleted, and may therefore be necessary for PccS1 viability. The pathogenicity of the six mutants was tested on *Z. elliotiana*, and the results revealed that mutants Δ*prmA*, Δ*aceE* and Δ*kdgR* showed no significant difference in virulence compared with the wild-type strain, and were therefore not studied further. However, mutants Δ*clpP*, Δ*flgK* and Δ*mreB* showed a significant reduction in virulence on the leaves or petioles of *Z. elliotiana*. ClpP is a

A
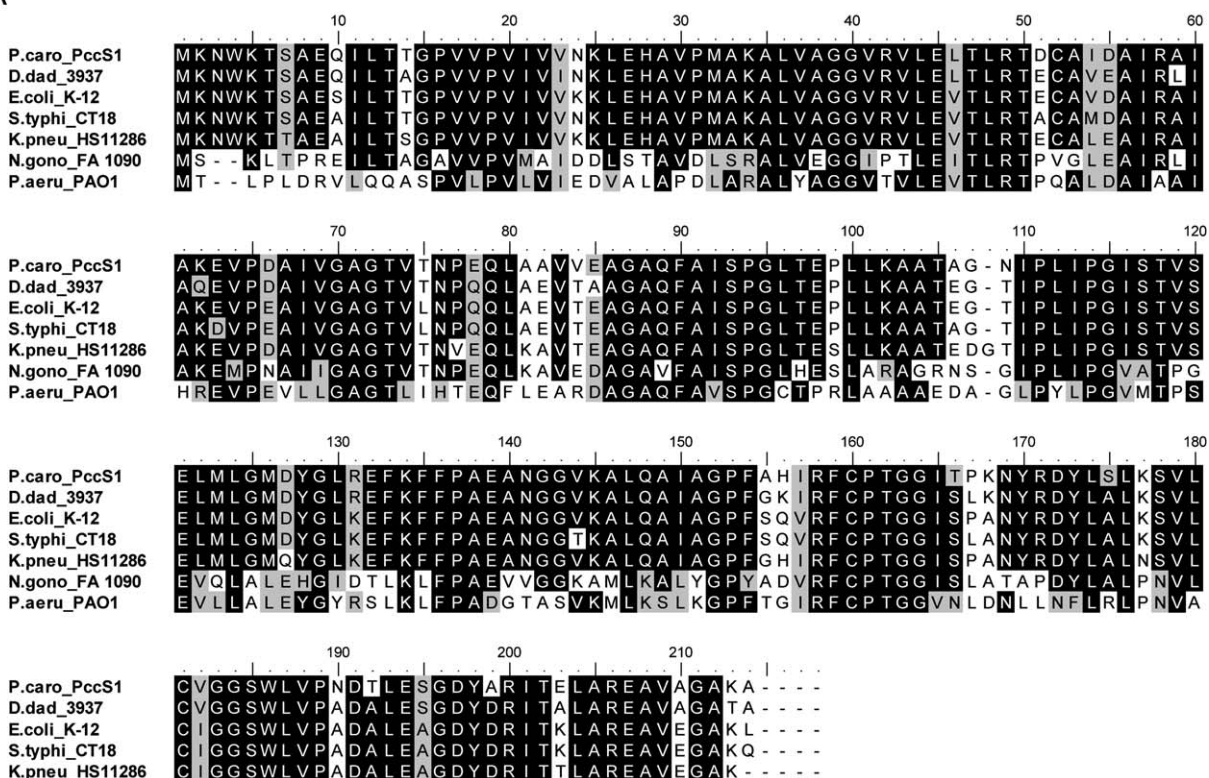
B
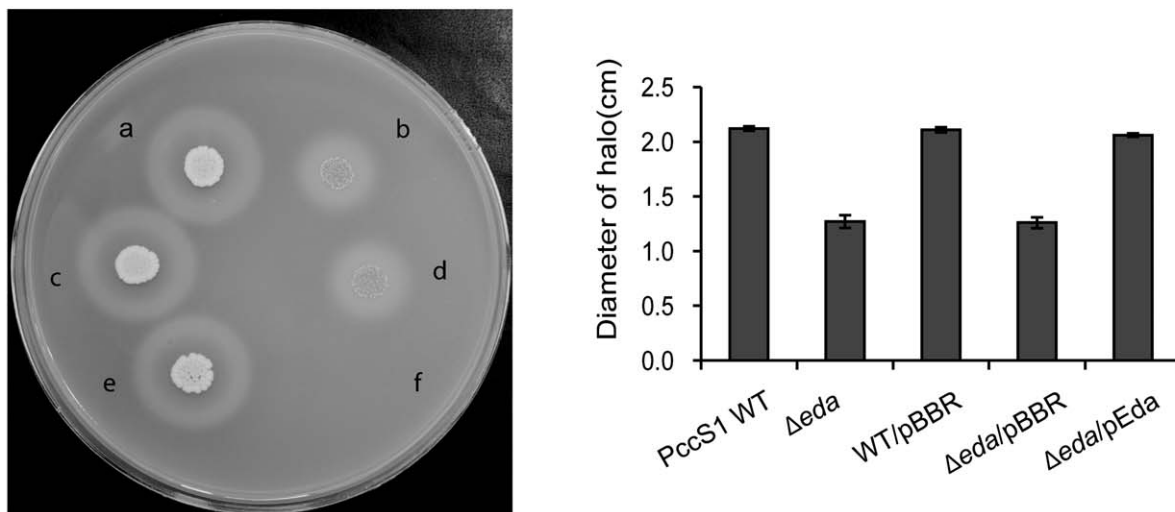

Fig. 7 Protein alignment of Eda and growth of the eda mutant on pectinase agar. (A) Alignment of deduced amino acid sequences of *Pectobacterium carotovorum* PccS1 protein Eda with homologues from other bacteria. Identical residues are shaded in black, conservative substitutions (similarity > 70%) are shaded in grey and non-conservative substitutions are shaded in white. The multiple sequence alignment was generated using BioEdit (Version 7.2.5) and the alignment output was produced using Adobe Ilustrator (CS5). Sequence IDs are abbreviated as follows: *Pectobacterium carotovorum* ssp. *carotovorum* PccS1, 'P.caro_PccS1'; *Dickeya dadantii* 3937, 'D.dad_3937'; *Escherichia coli* K-12 substr. MG1655 (NC_000913.3), 'E.coli_K12'; *Salmonella enterica* ssp. *enterica* serovar Typhi str. CT18 (NC_003198.1), 'S.typhi_CT18'; *Klebsiella pneumoniae* ssp. *pneumoniae* (NC_016845.1), 'K.pneu_HS11286'; *Neisseria gonorrhoeae* FA 1090 (NC_002946.2), 'N. gono_FA 1090'; *Pseudomonas aeruginosa* PAO1 (NC_002516.2), 'P.aeru_PAO1'. (B) Growth of PccS1 strains on pectinase agar at 27°C for 72 h followed by flooding with 7.5% (w/v) copper acetate for 1–2 h: a, PccS1 WT; b, Δ*eda*; c, WT/pBBR; d, Δ*eda*/pBBR; e, Δ*eda*/pEda (complemented strain); f, MgSO$_4$ buffer control.

serine-type Prt and is required for many important functions, including growth at high temperature, motility, synthesis of degradative enzymes and sporulation in *E. coli* (Porankiewicz *et al*., 1999), the rapid adaptive response of intracellular pathogens, such as *Listeria monocytogenes*, during their infectious process (Gaillot *et al*., 2000), and the mediation of biofilm formation on implanted medical devices in *Staphylococcus epidermidis* (Wang *et al*., 2007). ClpP proteolytic activity is essential for the virulence of many animal-pathogenic bacteria, including *Salmonella enterica* serovar *typhimurium*, *Streptococcus pneumoniae*, *L. monocytogenes*, *E. coli* and *Staphylococcus aureus* (Frees *et al*., 2012; Gaillot *et al*., 2000; Iyoda and Watanabe, 2005; Knudsen *et al*., 2013; Michel *et al*., 2006; Park *et al*., 2010; Tomoyasu *et al*., 2005), but there are fewer studies on ClpP associated with the virulence of plant-pathogenic bacteria, e.g. only in *D. dadantii* 3937 (Li *et al*., 2010). FlgK is a component of the hook-filament junction of flagella in bacteria, which is an organelle used for motility in liquids and on surfaces. Bacterial motility has been described as a pathogenicity determinant in pectobacteria (Perombelon, 2002). MreB is a rod shape-determining and cytoskeletal protein, and has been implicated in peptidoglycan biosynthesis and cell stabilization (Garner *et al*., 2011). The results from these single gene deletion mutants of PccS1 suggest that all three genes are important to Pcc infection of the living host.

Although this study identified three proteins ($\Delta clpP$, $\Delta flgK$ and $\Delta mreB$) from the *in vivo*-only condition that play a role in virulence, only MreB was identified for the first time as part of this study, and this finding has been published elsewhere (Deng *et al*., 2014). We therefore investigated proteins that were increased in expression in both *in vivo* and *in vitro* conditions in order to identify further virulence-related proteins. Of the eight further genes tested, insertion mutagenesis identified only *zwf* as a potential virulence candidate. However, after further investigation, a mutation in the *eda* gene, which is adjacent to and downstream of *zwf* in the PccS1 genome, was found to be responsible for the reduced virulence phenotype. Although *zwf* is part of the pentose phosphate pathway that operates alongside glycolysis in primary bacterial metabolism, *eda* is part of the alternative Entner–Doudoroff pathway (Conway *et al*., 1991). In *D. dadantii* strain 3937 (formerly *Erwinia chrysanthemi*), the KDPG adolase enzyme encoded by *kdgA* (which is an alternative name used for the *eda* gene in this bacterium) is involved in the catabolism of pectins following breakdown of 2-keto-3-deoxygluconate (KDG) to pyruvate and glyceraldehyde phosphate (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1994). This is in line with our findings that the *eda* gene in PccS1 is highly similar to that of *D. dadantii* 3937 (Fig. 7A), and that the mutant is less able to grow on pectinase medium than the wild-type and complemented strains (Fig. 7B). The role of *eda*, a key enzyme in the Entner–Doudoroff pathway, in virulence may thus be associated with the breakdown of pectin during the infection process.

Although this study was limited to the capture of proteins inside the cells of PccS1 only, and at only a single time point during the establishment of infection, we identified a number of proteins that showed increased expression *in vivo* compared with *in vitro* conditions, as well as *in vivo* and *in vitro* compared with the control. Based on these expression profiles, 14 mutants were produced in a number of selected genes, with four of these demonstrating a role in virulence of PccS1. Further studies are now required to use this and other approaches to identify further proteins/genes involved in the virulence of PccS1 on *Z. elliotiana*, and to further elucidate the role of Eda in the virulence of *Pectobacterium*.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this work are listed in Table S1 (see Supporting Information). All bacterial strains were cultured in Luria–Bertani broth (LB) or on LB agar plates (LA) (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar for LA plate, w/v). The growth temperature for PccS1 and its mutant strains was 28°C and for *E. coli* strains was 37°C. When required, antibiotics were added to the medium at the following final concentrations: rifampicin (Rif), 100 μg/mL; kanamycin (Kan), 50 μg/mL; gentamicin (Gm), 25 μg/mL.

Plant extract preparation and bacterial growth

Plant extracts were prepared according to Mattinen *et al*. (2007) and Mehta and Rosato (2001). Briefly, 100 g of fresh petioles from *Z. elliotiana* 'Black Magic', following 45 days of growth in the glasshouse, were ground with 1000 mL of water in a blender. The mixture was then filtered through micro-cloth and centrifuged at 4 000 *g* for 20 min. The supernatant was passed through a 0.2-μm filter, followed by ultrafiltration (Millipore, Red Bank, NJ, USA) twice to eliminate proteins larger than 5 kDa. Finally, the extract was filter sterilized again and stored at −20°C.

Bacteria were grown in LB supplemented with 0.4% petiole extracts of *Z. elliotiana* (hereafter, PccS1-LB-Ze (*in vitro*)] until the optical density at 600 nm (OD$_{600}$) reached 1.2, and were then used for protein extraction. Protein extracted from the bacteria grown to stationary phase in LB was used as a control (PccS1-LB).

Infiltration and recovery of bacterial cells from the host plant

To determine the optimum period for maximum bacterial growth and cell recovery from inoculated plants, to enable sufficient total protein for analysis, cells were extracted just prior to collapse of the cell walls at the surface of necrosis in the petioles. Testing of the bacterial population dynamics in the host after inoculation was performed according to Andrade *et al*. (2008). Briefly, a single colony of PccS1 was inoculated into 20 mL of LB and grown at 28°C until stationary phase (OD$_{600}$ = 1.2). After centrifugation at 3 000 *g* for 10 min at 4°C, the cell pellets were washed three times with distilled water and finally suspended in distilled water at a cell density of OD$_{600}$ = 1.2. Potted plants of *Z. elliotiana* 'Black Magic'

were grown in the glasshouse, and 45-day-old petioles were chosen for inoculation with 100 μL of PccS1 suspension. Water-infiltrated petioles were used as controls. The infiltrated petioles were collected at 0, 4, 8, 12, 16 and 20 HAI, sterilized with 70% alcohol and then cut into smaller pieces with a sterile scalpel and maintained for 1 h in sterile glass plates containing 20 mL of distilled water to allow the bacteria to move from the petioles into the water. After this time, 100 μL of the water was plated onto LB and incubated at 28°C for 16 h, and the number of resulting colonies was counted. The tests were repeated three times with three to five replicate petioles at each time point, and the results were then subjected to a *t*-test.

Pathogen cells taken directly from plant material [PccS1-V-Ze (*in vivo*)] were recovered at 16 HAI (population dynamics and protein concentration tests confirmed that this was the optimum period for cell recovery) and harvested by centrifugation at 4 000 *g* for 20 min. The cell pellets were used for protein extraction and total RNA isolation.

Extraction of proteins

Proteins were extracted from three replicate bacterial cell pellets of PccS1-V-Ze, PccS1-LB and PccS1-LB-Ze for each condition. Briefly, the cell pellets were washed twice with 20 mL of washing buffer (50 M Tris-HCl, pH 7.2), centrifuged at 14 000 *g* for 30 min at 4°C, and then disrupted by an Qproteome Bacterial Protein Prep Kit (Qiagen, Hilden, Germany) according to the *Qproteome Bacterial Protein Preparation Handbook*. The cell pellets were thawed on ice for 15 min and resuspended in 10 mL of native lysis buffer (with lysozyme and nuclease added). The samples were incubated on ice for 30 min and then gently mixed two or three times by swirling the solution before centrifugation at 14 000 *g* for 30 min at 4°C to pellet the cellular debris. The cell lysate supernatant was retained and then 100 mL of acetone were added and the samples were precipitated for 12 h at −20°C. The proteins were pelleted by centrifugation at 14 000 *g* for 20 min and the pellet was resuspended in rehydration buffer (RB). Protein samples were stored at −20°C and protein quantification was performed according to Bradford (1976). The concentration of the extracted protein was detected using a 2-D Quant Kit (GE Healthcare, Chicago, IL, USA).

2-DE, gel staining and image generation

Isoelectric focusing (IEF, first dimension) was performed with an IPGphor 3 system a gel system of immobilized pH gradients (GE Healthcare). Dry IPG strips (24 cm, pH 4–7, GE Healthcare) containing 1.2 mg of protein were rehydrated for 12 h at 20°C in rehydration buffer. IEF was performed in five steps: 300 V for 0.5 h, 700 V for 0.5 h, 1500 V for 1.5 h, linear gradient increase to 9000 V for 3 h and 9000 V for 4 h. The focused strips were equilibrated first in sodium dodecylsulfate (SDS) equilibration buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue) containing 1% w/v dithiothreitol for 15 min, and then in equilibration solution with 4% w/v iodoacetamide for 15 min. For the second dimension, proteins were separated according to their molecular weights on 12.5% polyacrylamide gels. After being stained with colloidal Coomassie blue G-250 (CBB), the wet gels were scanned using an ImageScanner (UMAX Powerlook1100, Taiwan, China). Gel images were analysed with ImageMaster 2D platinum 5.0 software (GE Healthcare). At least three gels were analysed for each of the different conditions. Proteins were

considered to be differentially expressed when the average values exceeded the threshold of a two-fold difference ($P < 0.05$) (Cheng *et al.*, 2009; Zhao *et al.*, 2011).

Trypsin digestion and MS analysis

The protein profile of PccS1 interaction with *Z. elliotiana* [PccS1-V-Ze (*in vivo*)] was compared with that from the cells of PccS1 cultured in LB [PccS1-LB (control)] or LB + 0.4% petiole extract of *Z. elliotiana* [PccS1-LB-Ze (*in vitro*)]. Protein spots of interest were excised from the 2-D gels and destained in 120 μL of 50% methanol. The pieces of gel were shrunk with 100 μL acetonitrile and then rehydrated with 50 μL of 100 mM NH$_4$HCO$_3$ three times, and the samples were dried by vacuum centrifugation in a vacuum heater. Trypsin solution was added to the dried gel spots and incubated at 4°C for 30 min. In-gel tryptic digestion was performed overnight at 37°C. Samples were frozen at −20°C before spotting on a matrix-assisted laser desorption/ionization (MALDI) plate. Aliquots of each hydrolysed sample were mixed with 1.5 μL of matrix solution of 0.5% α-cyano-4-hydroxycinnamic acid (CHCA, w/v) in 70% acetonitrile and 30% trifluoroacetic acid (0.1% TFA, w/v), 800 μL of which were immediately spotted and air dried before MS analysis (Mattinen *et al.*, 2007; Zhao *et al.*, 2011). The samples were analysed on a 4700 Proteomics Analyzer MALDI-TOF/TOF system (ABI, Carlsbad, CA, USA). All spectra were obtained in positive reflector mode, using an accelerating voltage of about 20 kV for the MS mode. Molecular ions displaying sufficient signal were submitted to MS/MS analyses, carried out in the positive precursor ion fragmentation mode at a laser frequency of 200 Hz. The MS/MS spectra were acquired in the reflector-positive mode after collision-induced dissociation (CID) or LIFT^{TM} fragmentation with external calibration. The MS/MS spectra were interpreted automatically and manually (*de novo* sequencing) using Mascot distiller software. The peptide masses obtained were employed for identification with the MASCOT program (Matrix Science, London, UK) using the National Center for Biotechnology Information (NCBI) and Pfam (http://pfam.sanger.ac.uk/), and only the matches to *Pectobacterium* species were considered as positive identifications. Peptide sequences obtained by *de novo* sequencing were analysed using BLAST (Mattinen *et al.*, 2007; Shevchenko *et al.*, 1996; Villeth *et al.*, 2009).

RNA extraction and qRT-PCR analysis

To confirm the differential expression of proteins in PccS1 *in vitro* and following interaction with the host plant (*in vivo*), the transcriptional levels of the selected genes in PccS1 were measured by qRT-PCR. Bacterial total RNA was extracted using an OMEGA Kit (OMEGA, Norcross, GA, USA) according to the manufacturer's instructions. The purity and concentration of RNA were determined with a microspectrophotometer (Nanodrop 1000, Thermo TaKaRa, Dalian, China) and gel electrophoresis. Two micrograms of RNA were used to synthesize cDNA according to the manufacturer's instructions for the PrimeScript^{TM} RT Reagent (Perfect Real Time) Kit (TaKaRa). The 16S rDNA was used as a normalized control. The primers (Table S2, see Supporting Information) used for qRT-PCR were designed using primer 3.0 (http://primer3.ut.ee/). qRT-PCR was performed with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using SYBR Premix Ex Taq^{TM} (Perfect Real Time) (TaKaRa). Primer specificity was assessed using the dissociation curve protocol. The conditions for PCR amplification were as follows: initial denaturation of

95°C for 15 s, followed by 40 cycles of 95°C for 5 s and 64°C for 34 s. The data were analysed by 7500 software (v.2.0.6) and standard deviations were calculated. The expression of the genes was calculated by the comparative C_T method described by Schmittgen and Livak (2008) using the following formula: fold change $= 2^{-\Delta\Delta CT}$, where $\Delta\Delta C_{Ti}$ for gene of interest $i = (C_{Tgenei} - C_{T16SrDNA})_{mutant}/(C_{Tgenei} - C_{T16SrDNA})_{wild-type}$. For the analysis of co-transcribed genes, PCR with primers (Table S3, see Supporting Information) flanking *zwf* (*zwf*-F) and *eda* (*eda*-R) was conducted as described previously (Pacheco *et al.*, 2012). Two micrograms of RNA (no reverse transcript) and cDNA were used as PCR templates, with genomic DNA used as the positive control. There were three independent biological experiments with sampling carried out in duplicate.

Gene modification in PccS1

Both gene insertion and gene deletion mutants were used in this study. Gene insertion mutants were constructed according to Yu *et al.* (2007). In short, the internal fragments from the target genes were amplified by the gene-specific primers (Table S3), and released by *Kpn*I and *Hin*dIII digestion from the pMD19-T (simple) subclones. These fragments were cloned into the same sites of pEX18 (Table S1) to produce gene insertion constructs. The constructs were conjugally transferred to PccS1 by *E. coli* S17-1 (Table S1). The conjugant cells were spread onto LB plates supplemented with Rif and Gm. The plates were incubated at 28°C for 24–48 h for selection. The colonies that developed on the selection medium were candidates for target gene insertion mutants. To confirm the insertion mutants, in addition to the above gene-specific primers, Gm-specific primers and PccS1 strain-specific primers were also used.

The gene deletion mutants were constructed and complemented according to Li *et al.* (2010). Briefly, the upstream and downstream regions of the target genes were amplified by PCR using the relevant primers (Table S3). A Kan cassette, amplified from pET30 (Table S1), was ligated with these two fragments and cloned into pMD19-T, verified by sequencing, excised with appropriate restriction enzymes (as indicated in Table S3) and cloned into pEX18 (Table S1) to yield a gene insertion plasmid vector. This vector was transferred into PccS1 by conjugation using *E. coli* S17-1 (Table S1). To select strains with chromosomal deletions, transconjugants were plated on LB containing 10% sucrose (Solarbio, Beijing, China) and Kan. Colonies having resistance to sucrose and Kan were isolated and confirmed by PCR using primers outside the gene of interest. To construct plasmids for complementation, target genes and their promoter region were amplified and cloned into pBBR1MCS5 (Table S1). Complementation vectors (Table S1) were introduced into the mutants by conjugation, as described above, and complemented strains were selected by resistance to Gm. All of the constructs and mutants were verified by PCR and DNA sequencing.

Pathogenicity assay

Pathogenicity assays were performed as described previously (Gu *et al.*, 2009; Murata *et al.*, 1991). PccS1 and the mutant strains were grown in LB at 28°C overnight. Cells were harvested and resuspended in distilled water and adjusted to an OD_{600} of 1.2 for inoculation. For all deletion mutants, petioles of leaves detached from approximately 45-day-old *Z. elliotiana* plants were used for the pathogenicity assays. For insertion mutants, as a screen prior to retesting of deletion mutants on *Z. elliotiana*,

leaves of Chinese cabbage were used. Before inoculation, the leaves were washed thoroughly with distilled water. For each site, the surface of the leaves was punctured with a sterile toothpick and 10 µL of the wild-type or mutant strains of PccS1 were inoculated in parallel into the tissue. Distilled water was inoculated into leaves as a control. The leaves were placed in a moist container and incubated in a moist chamber at 28°C. The area (cm²) of maceration was measured at 16 HAI. The pathogenicity assays were repeated three times with three replicates for each treatment. Growth of the *eda* mutant in both LB and MM (with 0.2% glucose) at 28°C was compared with that of the wild-type and complemented strains over a 12-h period following inoculation of a 100-fold dilution of overnight culture of the same medium. Where relevant, the growth of other mutants in rich and minimal media was tested and published elsewhere.

Exo-enzyme activity assays

To examine the role of the PccS1 *eda* gene in pectin utilization, the exo-enzyme activity of the wild-type and mutant strains was assayed. Briefly, 10 µL of an overnight culture (normalized to 10^4 cells/mL in 10 mM MgSO$_4$) were spotted onto pectinase plates as described in Andro *et al.* (1984) and incubated at 27°C for 72 h, followed by flooding with 7.5% (w/v) copper acetate for 1–2 h. Colonies positive for Pel activity produce creamy double haloes on a translucent light blue background. Three repetitions were performed for each assay.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Bacterial population dynamics curve during the establishment of infection. A *Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1 double-distilled water suspension (100 μL) [optical density at 600 nm (OD_{600}) = 1.2] was inoculated in 45-day-old petioles of potted plants of *Zantedeschia elliotiana*. The infiltrated petioles were collected at 0, 4, 8, 12, 16 and 20 h after inoculation (HAI), and the number of colonies were counted. The tests were repeated three times with three to five plants each time and the results were subjected to *t*-test.

Fig. S2 Growth of *Pectobacterium carotovorum* ssp. *carotovorum* strain PccS1 wild-type (WT), Δeda, WT with empty vector pBBR, Δeda with empty vector pBBR and Δeda with complementing vector pBBR (pEda). Luria–Bertani broth (LB) (A) and minimal medium (MM) plus 0.2% glucose (B) over a 12-h period at 28°C.

Table S1 Bacterial strains and plasmids used in this study.

Table S2 Primers used in this study for quantitative real-time polymerase chain reaction.

Table S3 Oligonucleotide sequences for gene modification in this study.