Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY DOI: 10.1111/mpp.12470

Differential control and function of Arabidopsis *ProDH1* and *ProDH2* genes on infection with biotrophic and necrotrophic pathogens

YANINA SOLEDAD RIZZI, NICOLÁS MIGUEL CECCHINI, GEORGINA FABRO AND MARÍA ELENA ALVAREZ*

Centro de Investigaciones en Química Biológica de Córdoba, CIQUIBIC, CONICET, Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, Córdoba X5000HUA, Argentina

SUMMARY

Arabidopsis contains two proline dehydrogenase (ProDH) genes, *ProDH1* and *ProDH2*, encoding for homologous and functional isoenzymes. Although *ProDH1* has been studied extensively, especially under abiotic stress, *ProDH2* has only started to be analysed in recent years. These genes display distinctive expression patterns and show weak transcriptional co-regulation, but are both activated in pathogen-infected tissues. We have demonstrated previously that Arabidopsis plants with silenced *ProDH1/2* expression fail to trigger defences against the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato AvrRpm1* (*Pst-AvrRpm1*), and that *ProDH1* and *ProDH2* are differentially regulated by salicylic acid (SA). In the current work, we used *prodh1* and *prodh2* single-mutant plants to assess the particular contribution of each gene to resistance against *Pst-AvrRpm1* and the necrotrophic fungal pathogen *Botrytis cinerea*. In addition, we studied the sensitivity of *ProDH1* and *ProDH2* to the jasmonic acid (JA) defence pathway. We found that ProDH1 and ProDH2 are both necessary to achieve maximum resistance against *Pst-AvrRpm1* and *B. cinerea*. However, ProDH2 has a major effect on early restriction of *B. cinerea* growth. Interestingly, *ProDH1* is up-regulated by SA and JA, whereas *ProDH2* is only activated by JA, and both genes display transcriptional inter-regulation at basal and infection conditions. These studies provide the first evidence of the contribution of ProDH2 to disease resistance, and describe the differential regulation and non-redundant but complementary function of both enzyme isoforms in infected tissues, providing support for a fundamental role of ProDH in the control of biotrophic and necrotrophic pathogens.

Keywords: HopX1, jasmonate, *prodh1-3* and *prodh2-2*, proline dehydrogenase, *Pst-AvrRpm1*, *B. cinerea*, salicylic acid.

* *Correspondence*: Email: malena@mail.fcq.unc.edu.ar

INTRODUCTION

Proline dehydrogenase (ProDH) catalyses the first and rate-limiting step in the transformation of proline (Pro) into glutamic acid (Glu) that takes place at mitochondria. This enzyme oxidizes Pro into delta-1-pyrroline-5-carboxylate (P5C) using FAD as cofactor. Next, P5C is non-enzymatically transformed into glutamate semialdehyde (GSA), which is used to generate Glu and NADH by P5C dehydrogenase (P5CDH). In higher plants, ProDH is bound to the inner mitochondrial membrane and is suspected to transfer electrons to ubiquinone in the mitochondrial electron transport chain (mETC) (Elthon and Stewart, 1981, Kiyosue *et al.*, 1996; Liang *et al.*, 2013).

Arabidopsis contains two genes encoding for ProDH: *ProDH1* (*At3g30775*) and *ProDH2* (*At5g38710*) (Funck *et al.*, 2010; Kiyosue *et al.*, 1996). Their products are highly homologous (75% identical), but differ at their N-terminus and may have different subcellular localizations. Although ProDH1 and ProDH1-GFP fusion proteins are exclusively detected at mitochondria, ProDH2-GFP is found at mitochondria and plastids (Funck *et al.*, 2010; Kiyosue *et al.*, 1996; Van Aken *et al.*, 2009). ProDH1, also called ERD5 (Early Responsive to Dehydration 5), was the first to be described and is considered to be the predominant functional isoform under most studied conditions (Kiyosue *et al.*, 1996). ProDH2 has been recognized recently as an active enzyme, based on its ability to complement the Pro utilization defect of the Δ*put1* yeast strain, and the Pro hypersensitive phenotype of the *prodh1-1* Arabidopsis mutant (Funck *et al.*, 2010). Attempts to generate transgenic plants fully silenced in *ProDH1* and *ProDH2* were unsuccessful (Cecchini *et al.*, 2011a), but the first double-mutant plant (*prodh1-4*/*prodh2-2*) has been described recently (Cabassa-Hourton *et al.*, 2016).

ProDH1 and *ProDH2* genes have distinctive expression patterns and are controlled by different mechanisms. Although *ProDH1* is expressed in most tissues and developmental stages with greater intensity in stigma and pollen, *ProDH2* shows low expression, mostly at vascular tissues and senescent leaves (Funck *et al.*, 2010; GENEVESTIGATOR, https://genevestigator.com). In addition, transcripts of both genes are detected in root tissues and abscission zones (Funck *et al.*, 2010; GENEVESTIGATOR,

https://genevestigator.com; Nakashima *et al.*, 1998). The expression of *ProDH1* is modulated by S1 bZIP transcription factors (bZIP1 and bZIP53; Dietrich *et al.*, 2011) that heterodimerize with members of the C bZIP group (bZIP53/bZIP10; Weltmeier *et al.*, 2006), whereas *ProDH2* is a direct target of bZIP11 (Funck *et al.*, 2010; Hanson *et al.*, 2008). Both genes are repressed by sucrose and induced by exogenous Pro treatment, whereas *ProDH1* is repressed and *ProDH2* is induced by NaCl (Funck *et al.*, 2010; Hanson *et al.*, 2008; Satoh *et al.*, 2004). Microarray analysis (GENEVESTIGATOR, https://genevestigator.com) shows poor co-regulation of these genes, and recent studies have suggested that they play distinctive roles in plant development and stress adaptation (Funck *et al.*, 2010). Apparently, this may also occur with the *ProDH1* and *ProDH2* orthologues in *Nicotiana tabacum* and *Brassica napus* (*Bna*). Recent studies have reported the expression of six *BnaProDH1* and two *BnaProDH2* genes, showing that *ProDH1* is prevalent in pollen, *ProDH2* in senescent leaves and both are active in roots, as in Arabidopsis (Faës *et al.*, 2015; Ribarits *et al.*, 2007).

In recent years, ProDH has been implicated in defences against pathogens. Arabidopsis plants show enhanced gene expression and enzyme activity on elicitation of the hypersensitive response (HR) by recognition of *Pseudomonas syringae pv. tomato* (*Pst*) *AvrRpm1* (*Pst-AvrRpm1*) (Cecchini *et al.*, 2011a). *ProDH1/2* genes are also induced in *Nicotiana benthamiana* plants establishing a non-host interaction with *Pst* T1 (Senthil-Kumar and Mysore, 2012). In both cases, silencing of *ProDH* genes compromises the accumulation of reactive oxygen species (ROS), the generation of cell death and the establishment of disease resistance (Cecchini *et al.*, 2011a; Senthil-Kumar and Mysore, 2012). In addition, ProDH activation is believed to sustain the oxidative burst and to reduce cell viability in other kingdoms (Cecchini *et al.*, 2011b). However, the mechanism by which ProDH contributes to these responses is not understood. The consequences of enzyme activation have been analysed at the biochemical level for the Arabidopsis–*Pst-AvrRpm1* pathosystem. In this system, ProDH loses its coordination with P5CDH at the time of ROS increase, but this increases the level of the toxic metabolite P5C (Monteoliva *et al.*, 2014). Subsequently, Pro synthesis is induced in these tissues, suggesting that ProDH triggers long-term metabolic changes in HR (Rizzi *et al.*, 2015).

Currently, there are many basic questions about the regulation and function of ProDH1 and ProDH2 during the activation of plant defence. In Arabidopsis and *N. benthamiana*, ProDH1 apparently displays a predominant role in cell death-associated defences (Cecchini *et al.*, 2011a; Senthil-Kumar and Mysore, 2012). However, the role of ProDH2 in disease resistance has not been evaluated for any pathosystem. Moreover, the contribution of each isoform to resistance against pathogens with different lifestyles remains to be investigated. Defences against biotrophic and necrotrophic pathogens are mainly signalled by the salicylic acid (SA) and jasmonic acid (JA) pathways, respectively. These routes are inter-regulated by several negative crosstalks, but may have synergistic effects on particular genes at low concentrations (Caarls *et al.*, 2015; Mur *et al.*, 2006). The canonical SA pathway includes the action of the isochorismate synthase SID2 enzyme (SA induction-deficient 2), which is responsible for SA generation, as well as the SA receptor NPR1 (non-expressor of Pathogenesis-Related 1) (Wu *et al.*, 2012). Two major components of the JA pathway are JAR1 (JA responsive 1), which generates the active hormone form JA-isoleucine (JA-Ile), and COI1 (coronatine insensitive 1), sensing JA-Ile as part of the JA receptor (Katsir *et al.*, 2008; Staswick and Tiryaki, 2004). Some pathogen effectors alter the JA pathway to benefit the invader. For instance, *Pst* DC3000 secretes the JA-Ile analogue coronatine (COR) which down-regulates SA-dependent defences (Katsir *et al.*, 2008). In the same way, *Pseudomonas syringae* pv. *tabaci* generates HopX1, which binds to and degrades jasmonate ZIM-domain (JAZ) repressor proteins, therefore stimulating the JA route and, consequently, suppressing SA signalling (Gimenez-Ibanez *et al.*, 2014).

Previous observations have suggested that *ProDH1* and *ProDH2* genes could be differentially regulated in infected tissues (Cecchini *et al.*, 2011a). Here, we evaluated the response of both genes to biotrophic and necrotrophic pathogens, and their contribution to disease resistance in the interaction of Arabidopsis with *Pst-AvrRpm1* and *Botrytis cinerea* B05.10. Our studies include the use of wild-type, *prodh1* and *prodh2* single-mutant plants to analyse pathogen growth, cytological defence markers and the regulation of *ProDH* genes at different infection conditions. We report a distinctive sensitivity of *ProDH1* and *ProDH2* to SA and JA, the requirement of both genes for resistance against both pathogens, and the predominant contribution of *ProDH2* in the early control of *B. cinerea* infection. In addition, we provide evidence for the inter-regulation between these genes at basal and infection conditions.

RESULTS

Responses of *prodh1* and *prodh2* mutants to *Pst-AvrRpm1*

Arabidopsis plants with silenced *ProDH1/2* expression show enhanced sensitivity to *Pst-AvrRpm1* (Cecchini *et al.*, 2011a). To assess how each gene individually contributes to this phenotype, we analysed pathogen growth in *prodh1* and *prodh2* single mutants. We used the previously characterized *prodh1-2* (SALK_081276; Col-0), *prodh1-4* (SALK_119334; Col-0) and *prodh2-1* (GT1788; Ler) alleles. In addition, we included the Col-0 mutant plants *prodh1-3* (GABI_308F08) and *prodh2-2* (GABI_328G05). As the last two mutants have not been characterized at the phenotypic level, we first evaluated two traits of ProDH deficiency: Pro accumulation and hypersensitivity to

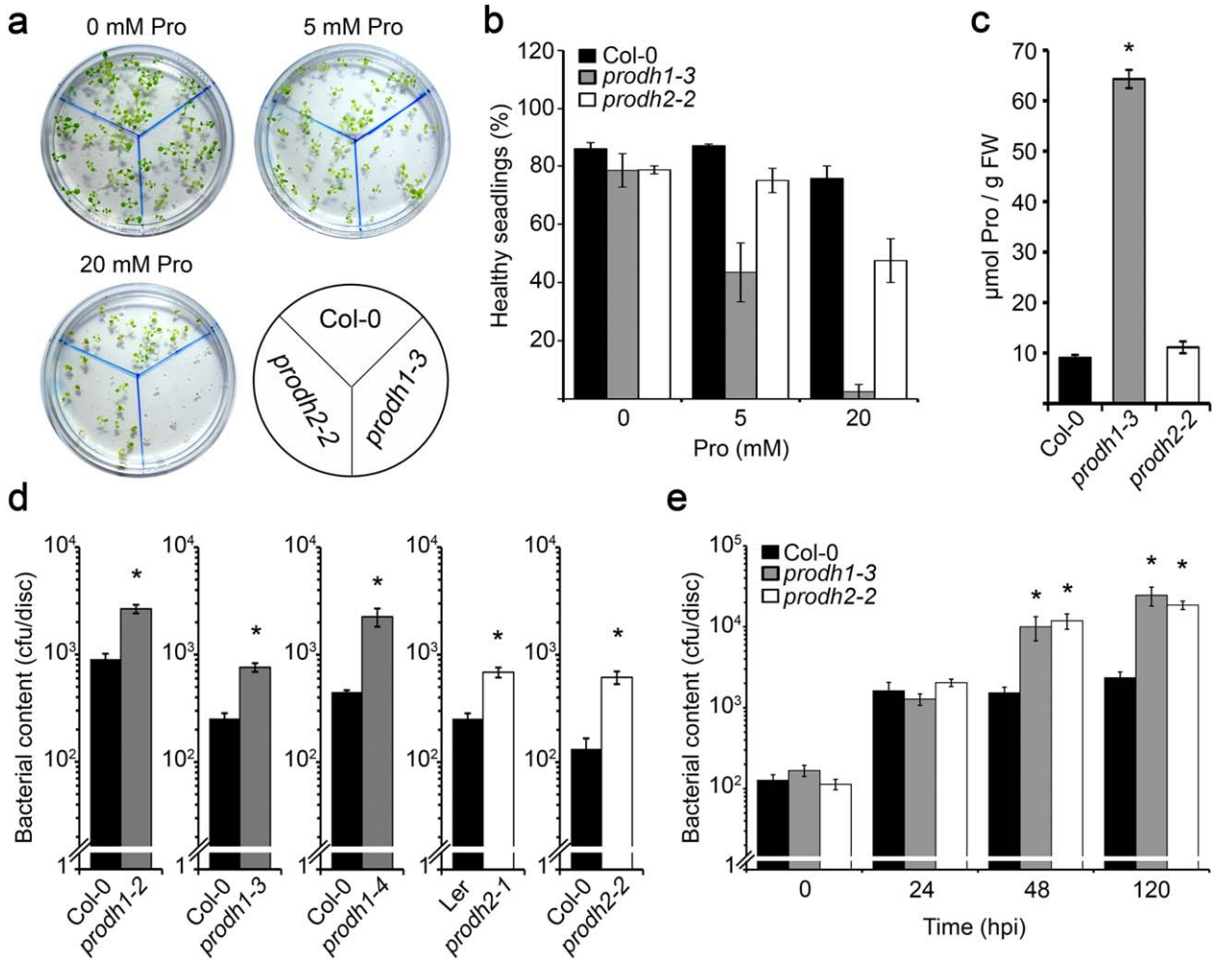

Fig. 1 Responses of *prodh1* and *prodh2* mutants to exogenous proline (Pro) and *Pseudomonas syringae* pv. *tomato AvrRpm1* (*Pst-AvrRpm1*). (a) Col-0, *prodh1-3* and *prodh2-2* seedlings were grown for 2 weeks in Gamborg's medium (GM) (1% sucrose) with 0, 5 or 20 mM Pro. (b) Percentage of healthy seedlings under the conditions described in (a). (c) Pro content in seedlings germinated in 5 mM Pro. FW, fresh weight. Values in (b) and (c) indicate mean ± standard error (SE) of two experiments. *Significant differences between mutant and wild-type plants ($P < 0.001$; *t*-test). (d) *Pst-AvrRpm1* content at 72 h post-inoculation (hpi) in Col-0, *prodh1-2*, *prodh1-3*, *prodh1-4*, Ler, *prodh2-1* and *prodh2-2* plants. (e) *Pst-AvrRpm1* content at 24, 48 and 120 hpi in Col-0, *prodh1-3* and *prodh2-2* plants. cfu, colony-forming unit. Six leaf discs from at least three plants were used to determine bacterial content in each sample; one representative of three independent assays is shown (d, e). *Significant differences between mutant and wild-type plants ($P < 0.05$; *t*-test).

exogenous Pro (Funck *et al.*, 2010; Sharma and Verslues, 2010). Mutant and control plants were germinated on 0, 5 and 20 mM Pro and analysed at the age of 2 weeks. Both *prodh* mutants contained less healthy seedlings than control plants, and this was more pronounced for *prodh1-3* (Fig. 1a,b). In addition, *prodh1-3*, but not *prodh2-2*, increased its Pro content (Fig. 1c). This indicates that the new mutants display similar phenotypes to other *prodh1* and *prodh2* alleles, confirming that ProDH1 is primarily responsible for the protection of plants against exogenous Pro (Funck *et al.*, 2010; Nanjo *et al.*, 2003).

To test the involvement of ProDH1 and ProDH2 in resistance against *Pst-AvrRpm1*, we quantified the bacterial content in the five *prodh* mutants. At late infection stage [72 h post-inoculation (hpi)], all mutants contained higher pathogen titres than did wild-type plants (three to eight times higher) (Fig. 1d). However, no significant differences were observed between *prodh1* and *prodh2* alleles. Next, we assessed *Pst-AvrRpm1* infection progression on one mutant of each type. *prodh1-3* and *prodh2-2* were selected for this purpose as they lack native transcripts (see below) and share the Col-0 background, well characterized in the interaction with *Pst*. The *Pst-AvrRpm1* content was quantified at 24, 48 and 120 hpi (Fig. 1e). *prodh1-3* and *prodh2-2* contained similar pathogen concentrations at all the analysed stages and manifested their susceptibility by 48 hpi. Therefore, loss of either ProDH1 or

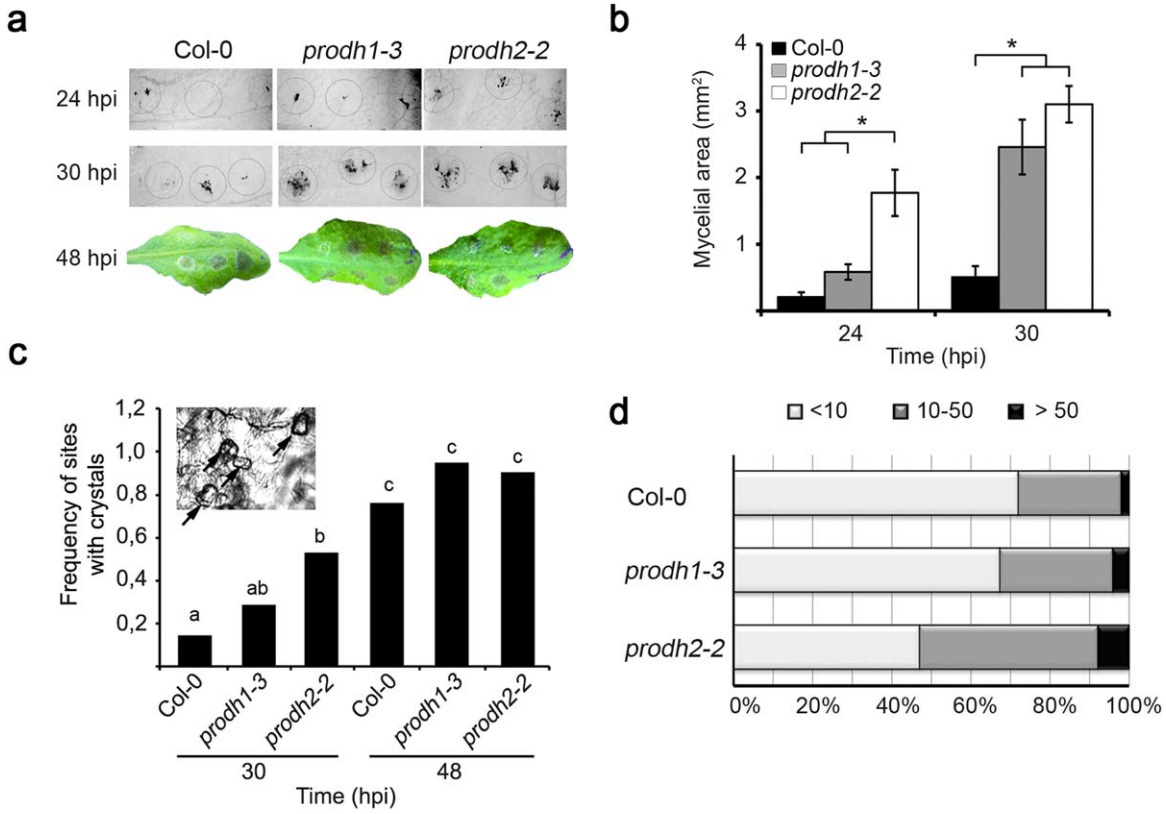

Fig. 2 Growth of *Botrytis cinerea* in *prodh1-3* and *prodh2-2* mutants. (a) Leaves inoculated at different points (circles) with conidial suspension (5 μL, 10^4/mL) were stained with trypan blue to detect fungal mycelium at 24 or 30 h post-inoculation (hpi). Macroscopic lesions developed at 48 hpi with 10^3 (top half) or 10^4 (botton half) conidia/mL. (b) Leaf area occupied by mycelia at 24 and 30 hpi with 10^4 conidia/mL (ImageJ program) based on images obtained as in (a). In (a) and (b), one representative of four independent experiments is shown (nine spots from three leaves from three plants were analysed in each case). Values indicate mean ± standard error. *Significant differences between plants ($P < 0.05$; *t*-test). (c) Frequency of infection sites containing at least one crystal at 30 and 48 hpi in leaves treated with 10^3 conidia/mL. Different letters indicate significant differences among samples ($P < 0.05$; chi-squared test). One representative of four independent assays is shown. (d) Number of crystals per site at 48 hpi with 10^4 conidia/mL. Intervals with less than 10, 10–50 or 50–100 crystals are shown, based on the analysis of four independent experiments (18–21 spots from seven leaves from at least three plants per sample). *prodh2-2* values are significantly different from those of the other two plants ($P < 0.05$; chi-squared test).

ProDH2 reduces resistance to *Pst-AvrRpm1* in a similar manner, indicating that both isoforms help to generate defences against this hemibiotrophic pathogen.

Responses of *prodh1-3* and *prodh2-2* mutants to *B. cinerea*

Next, we analysed the susceptibility of *prodh1-3* and *prodh2-2* plants to *B. cinerea*. Conidial suspensions (10^3 or 10^4 conidia/mL) were deposited on the adaxial surface of the leaves (three spots per side) and samples were taken before (24 and 30 hpi) and after (48 hpi) the observation of necrotic lesions. Fungal hyphae were detected by trypan blue staining of infected leaves (Fabro *et al.*, 2008) (Fig. 2a), and mycelial development was determined by the quantification of stained areas (ImageJ program) in these samples (Fig. 2b). At 24 hpi, *prodh2-2* showed greater mycelium expansion than *prodh1-3* or control plants (Fig. 2a,b). Later (30 hpi), hyphal growth was higher in both mutants than in control plants, with a tendency of *prodh2-2* to be more susceptible than *prodh1-3* (Fig. 2b). At 48 hpi, necrotic lesions were manifested in all plants without significant differences among genotypes (Fig. 2a, bottom).

Several necrotrophic fungal pathogens secret oxalic acid (OA) during plant invasion, and this appears to be critical for *B. cinerea* infection, as strains unable to produce OA cannot colonize Arabidopsis (Kunz *et al.*, 2006; Prins *et al.*, 2000). The amount of OA crystals increases during infection and is indicative of disease progression (Prins *et al.*, 2000; Uloth *et al.*, 2015). Thus, we used this marker to further evaluate the response of ProDH-deficient plants to this fungus. Crystals (Fig. 2c, inset) were quantified at 30 and 48 hpi in wild-type and mutant plants, and differences were detected among genotypes. At 30 hpi, the frequency of sites containing at least one crystal was higher for *prodh2-2* (9/17, 5/17 and 2/17 sites

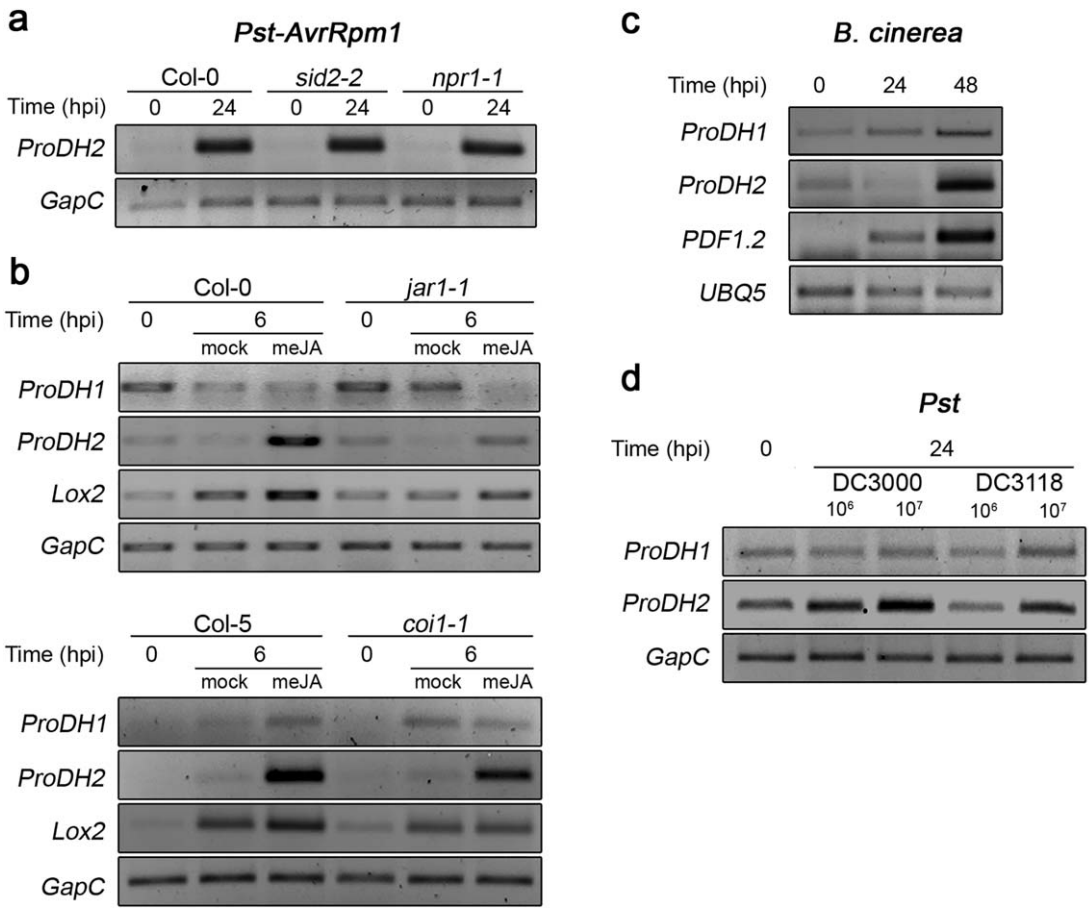

Fig. 3 Sensitivity of *ProDH1* and *ProDH2* to the salicylic acid (SA) and jasmonic acid (JA) pathways. (a) *ProDH2* expression in Col-0, *sid2-2* and *npr1-1* plants at 0 and 24 h post-inoculation (hpi) with *Pseudomonas syringae* pv. *tomato* (*Pst*)-*AvrRpm1*. (b) *ProDH1* and *ProDH2* transcripts in Col-0, *jar1-1*, Col-5 and *coi1-1* plants exposed to methyl jasmonate (meJA) (0.84 μM) or mock solution at 0 and 6 h post-treatment (hpt). *Lox2* was used as a JA-responsive gene. (c) *ProDH1* and *ProDH2* expression in Col-0 plants infected with *Botrytis cinerea* (six spots of 5 μL, 10^5 conidia/mL) at 0, 24 and 48 hpi. *PDF1.2* was used as a marker of the JA pathway. (d) *ProDH1* and *ProDH2* transcripts in Col-0 plants infected with *Pst* DC3000 or coronatine (COR)-deficient *Pst* DC3118 [10^6 or 10^7 colony-forming units (cfu)/mL] at 24 hpi. Gene expression was determined by semi-quantitative polymerase chain reaction using *GapC* or *UBQ5* as endogenous controls, under the conditions described in Table S2 (see Supporting Information). One representative of three biological replicates is shown.

for *prodh2-2*, *prodh1-3* and Col-0, respectively) (Fig. 2c). At 48 hpi, most sites contained at least one crystal in all plants (19/21, 20/21 and 16/21 sites for *prodh2-2*, *prodh1-3* and Col-0, respectively) (Fig. 2c), but the number of crystals per site was also higher for *prodh2-2*. Under the latter condition, sites with more than 10 crystals represented 53% of the total in *prodh2-2*, and 28% or 32% of the total in wild-type and *prodh1-3*, respectively (Fig. 2d). Thus, although ProDH1 and ProDH2 are both required for full resistance against *B. cinerea*, ProDH2 shows a predominant role in the early control of fungal infection.

Regulation of *ProDH1* and *ProDH2* genes by SA and JA

We have shown that *ProDH1* and *ProDH2* are induced by *Pst-AvrRpm1* infection, but only the former is activated by exogenous SA (Cecchini *et al.*, 2011a). To learn more about the response of these genes to biotic stresses, we analysed their sensitivity to other pathogens and hormones. To set up these experiments, we took into account that *ProDH1* and *ProDH2* have different expression levels (Fig. S1a, see Supporting Information) and regulation by light (Fig. S1b). Therefore, treatments were started 2 h after light phase onset and samples were taken 6, 24 or 48 h later in order to avoid the major effects of light (Fig. S1b).

Initially, we analysed the behaviour of *ProDH2*. The finding of SA-sensitive elements in its promoter region (Table S1, see Supporting Information) prompted us to test whether the SA pathway mediates its induction on interaction with *Pst-AvrRpm1* (Cecchini *et al.*, 2011a). With this purpose, the pathogen was inoculated in plants lacking SID2 or NPR1. Neither *sid2-2* nor *npr1-1* plants reduced *ProDH2* activation by *Pst-AvrRpm1* (Fig. 3a), indicating

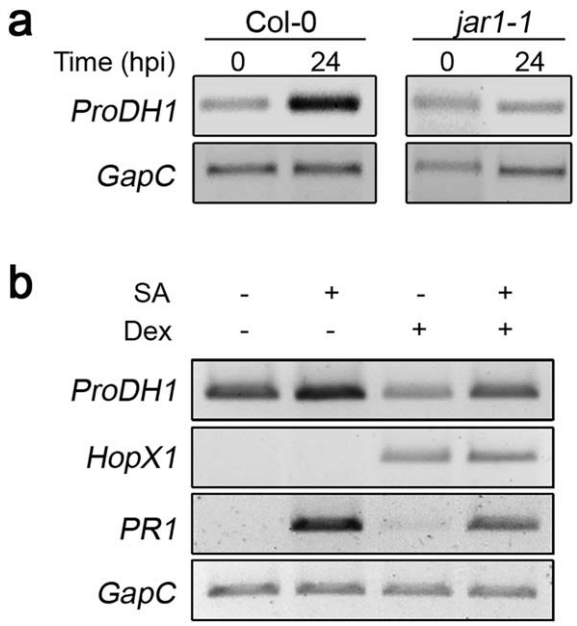

Fig. 4 Regulation of *ProDH1* expression by JAR1 and HopX1. (a) *ProDH1* transcripts in Col-0 and *jar1-1* plants infected with *Pseudomonas syringae* pv. *tomato* (*Pst*)-*AvrRpm1* at 24 h post-inoculation (hpi). (b) Effect of HopX1 on *ProDH1* induction by salicylic acid (SA). Transgenic plants *Dex:HopX1* were initially exposed to dimethylsulfoxide (DMSO) (–) or dexamethasone (Dex) (5 μM) for 24 h, and then treated with H_2O (–) or SA (1 mM) for 24 h. *PR1* was used as SA-sensitive gene and *GapC* as housekeeping gene. One representative from three independent assays is shown. Semi-quantitative polymerase chain reaction conditions are described in Table S2 (see Supporting Information).

that the canonical SA route is dispensable for this response. Arabidopsis also accumulates JA in tissues that are infected with avirulent *Pst* (De Vos *et al.*, 2005). In addition, the *ProDH2* promoter also contained JA-responsive elements (Table S1). Therefore, we assessed whether *ProDH2* may respond to the JA route. First, we analysed the effect of exogenous methyl jasmonate (meJA) (0.84 μM). Wild-type plants (Col-0, Col-5) induced *ProDH2* expression in response to this treatment (Fig. 3b). In contrast, *jar1-1* (Col-0) mutants strongly reduced such a response (Fig. 3b, top). In turn, *coi1-1* plants induced the gene at lower level than control (Col-5) plants (Fig. 3b). Therefore, in wild-type plants, *ProDH2* seems to be up-regulated by JA-Ile partially via COI1, but also through another pathway.

To gain an insight into the regulation of *ProDH2* by endogenous activation of the JA pathway, we evaluated its expression under infection conditions that increased the hormone levels. Plants challenged with *B. cinerea* accumulate high levels of JA (Liu *et al.*, 2015). Interestingly, this treatment induced *ProDH2*, but triggered the maximum expression of the JA-responsive gene marker *PDF1.2* (Fig. 3c). In addition, we took advantage of the fact that *Pst* DC3000 synthesizes the JA-Ile analogue COR and that the *Pst* DC3118 strain is deficient in this capacity (Katsir *et al.*, 2008). Thus, we compared the responses of both strains to test the effect of this JA-Ile analogue on *ProDH2* expression. *Pst* DC3118 produced lower gene induction than *Pst* DC3000, and this was reproduced at different pathogen concentrations (Fig. 3d), indicating that COR may activate *ProDH2* on infection with *Pst* DC3000.

Next, we analysed the sensitivity of *ProDH1* to SA and JA. Responsive elements for both hormones were detected in the promoter of this gene (Table S1). As described, the SA pathway mediates early (6 hpi), but not late (24 hpi), *ProDH1* activation on interaction with *Pst-AvrRpm1* (Cecchini *et al.*, 2011a). Thus, we tested whether the latter response involved the JA pathway. *ProDH1* expression was evaluated at 1 day post-infection in control and *jar1-1* mutant tissues. *jar1-1* plants did not activate the gene under this condition (Fig. 4a), implicating JA signalling in *ProDH1* regulation. JA-Ile triggers *ProDH1* induction in wild-type tissues infected with *Pst-AvrRpm1*. This was certainly surprising, as *ProDH1* did not respond to exogenous meJA (Fig. 3b).

ProDH1 activation can be triggered by either SA (Cecchini *et al.*, 2011b) or JA (Fig. 4a) pathways, suggesting that the balance between the two routes may affect the gene expression levels. To investigate this, we used transgenic plants expressing the bacterial effector HopX1 under the control of dexamethasone (Dex). As expected (Gimenez-Ibanez *et al.*, 2014), HopX1 suppresses SA signalling as it reduces the capacity of SA to induce the *PR1* gene marker (Fig. 4b). Interestingly, HopX1 also reduces the induction of *ProDH1* by SA. Therefore, non-infected tissues expressing HopX1 override the *ProDH1* activation by SA (Fig. 4b). Taken together, these results suggest that JA displays different effects on *ProDH1* expression depending on whether it is exogenously applied as meJA or is generated by infected tissues. This suggests that JA does not act alone in the induction of *ProDH1* by *Pst-AvrRpm1*.

Inter-regulation between *ProDH1* and *ProDH2*

Finally, we evaluated whether *ProDH1* and *ProDH2* display some type of inter-regulation. For this, we quantified gene expression in single-mutant plants. As shown in Fig. 5, we confirmed that *prodh1-3* and *prodh2-2* are null mutants, as no transcripts were detected at basal or infection conditions for the mutant genes. In uninfected tissues, *ProDH1* showed higher activation in *prodh2-2* than in control plants (4.5-fold difference), whereas *ProDH2* showed similar expression in *prodh1-3* and wild-type plants (Fig. 5a). Thus, *ProDH1* expression seems to be sensitive to *ProDH2* deficiency, but not vice versa. Then, we analysed *Pst-AvrRpm1*- and *B. cinerea*-infected tissues using untreated samples as control. After bacterial infection, *ProDH1* induction was marginally affected by *ProDH2* deficiency, as its transcripts were increased 960 and 1200 times in *prodh2-2* and Col-0 plants, respectively

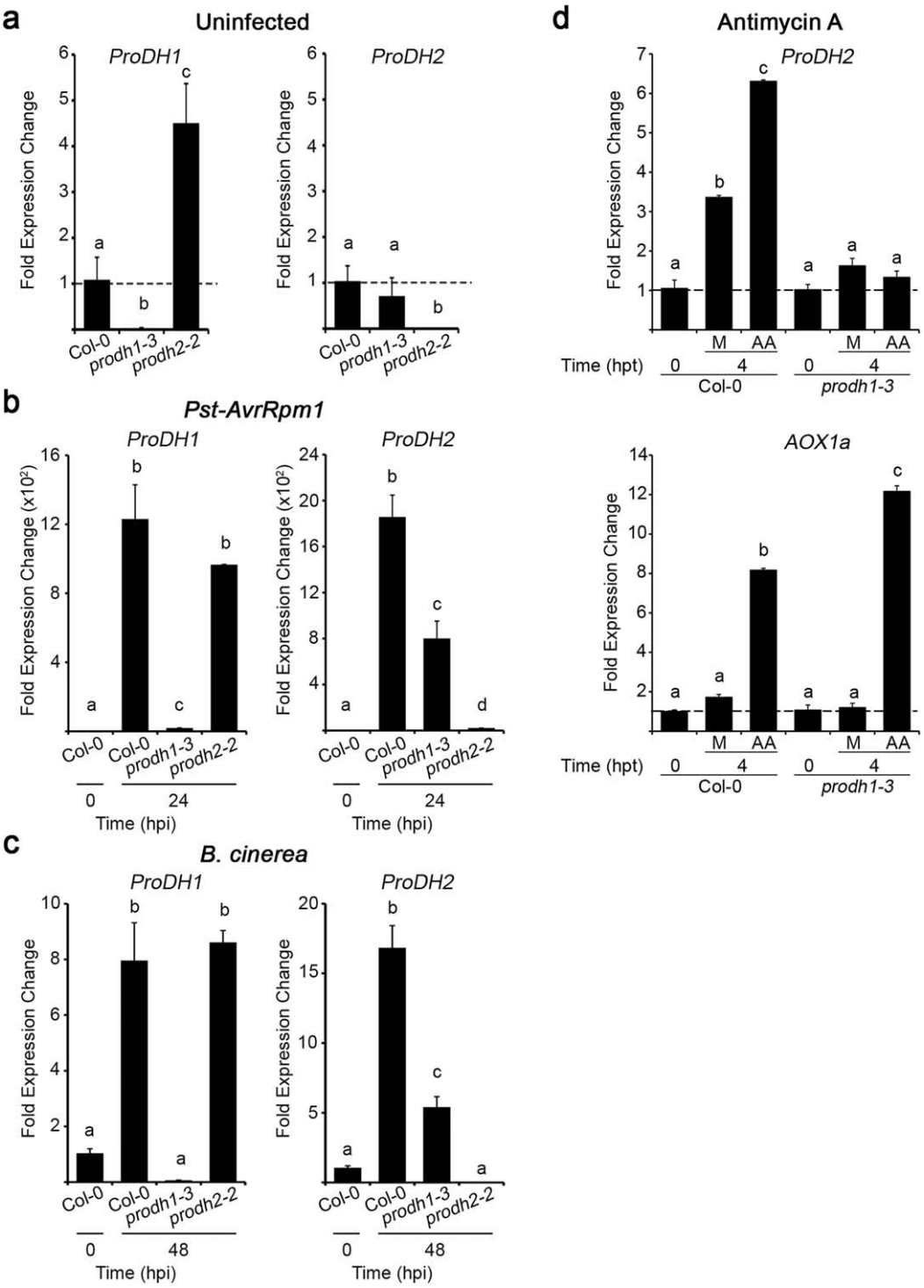

Fig. 5 Inter-regulation of *ProDH1* and *ProDH2* expression. Transcripts were quantified in Col-0, *prodh1-3* and *prodh2-2* leaves at basal (uninfected) conditions (a), at 24 h post-inoculation (hpi) with *Pseudomonas syringae* pv. *tomato* (*Pst*)-*AvrRpm1* [10^7 colony-forming units (cfu)/mL] (b) or 48 hpi with *Botrytis cinerea* (six spots of 5 μL with 10^5 conidia/mL per leaf) (c). *ProDH2* and AOX1a (alternative oxidase) expression in Col-0 and *prodh1-3* tissues treated with antimycin A (AA, 10 μM) for 4 h (hpt, h post-treatment) (d). Gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), applying the ΔΔCt method relative to each gene transcript level in Col-0 at 0 hpi (a–c) or Col-0 or *prodh1-3* at 0 hpi (d). Bars represent average ± standard deviation of three replicates. *UBQ5* was used as a housekeeping gene. In each case, one representative of three independent experiments is shown. Different letters indicate significant differences among samples ($P < 0.05$ in a–c and $P < 0.01$ in d; *t*-test).

(Fig. 5b). In contrast, bacterial-mediated *ProDH2* activation was strongly reduced in *prodh1-3* plants (800- and 1800-fold increase in *prodh1-3* and control plants, respectively) (Fig. 5b). *Botrytis cinerea* triggered lower *ProDH1* and *ProDH2* induction than did *Pst-AvrRpm1* treatment. However, *ProDH* genes displayed similar responses under both infection conditions as fungal infection generated similar *ProDH1* activation in wild-type and *prodh2-2* plants (7.9- and 8.6-fold increase, respectively), and lower *ProDH2* activation in *prodh1-3* plants (16.8- and 5.4-fold increase in control and mutant, respectively) (Fig. 5c). Therefore, regardless of the gene induction level achieved under these infection conditions, in both cases, the absence of *ProDH1* reduces *ProDH2* activation, but not vice versa. This set of results reveals the inter-regulation of the two genes, indicating that, although hyper-activation of *ProDH1* partially compensates for the absence of *ProDH2* at basal conditions, such compensation is not observed in infected tissues, where *ProDH1* deficiency limits *ProDH2* activation.

Finally, we tested whether *ProDH1* affects *ProDH2* expression after the mitochondrial ROS burst. For this purpose, we treated wild-type and *prodh1-3* leaf tissues with antimycin A (AA), an inhibitor of mETC that increases mitochondrial ROS levels (Fabro *et al.*, 2016). *ProDH2* was induced by AA in wild-type, but not mutant, plants, revealing an inter-regulation of these genes that is sensitive to ROS signalling (Fig. 5d). Mock treatments produced a lower *ProDH2* activation, which was also dependent on *ProDH1*, suggesting that mechanical stress and/or light phase conditions affect this response.

DISCUSSION

This work evaluates the contribution of ProDH1 and ProDH2 to defences against adapted biotrophic and necrotrophic pathogens in Arabidopsis. Both enzyme isoforms were found to be necessary to establish full resistance against *Pst-AvrRpm1* (Fig. 1d,e). This was expected for ProDH1, whose transcriptional activation requires SID2 and NPR1, which signal resistance against this biotrophic pathogen (Cecchini *et al.*, 2011a). In contrast, *ProDH2* induction does not involve this pathway (Fig. 3a), but still supports plant immunity, as *prodh2* plants show enhanced susceptibility to the bacteria (Fig. 1d,e). As discussed below, *ProDH2* is up-regulated by JA. Similarly, other genes associated with defences against biotrophic pathogens are induced by COR. These include anthocyanin, phenylpropanoid, terpenoid and shikimate synthesis genes (CSH, PAL1, DHS1, etc.), as well as *ELI3* (At4g37990), considered to be a marker of RPM1-dependent resistance (Thilmony *et al.*, 2006). Another example is the activation of JA gene markers occurring in the *cpr22* (Yoshioka *et al.*, 2001) and *hrl1* (Devadas *et al.*, 2002) mutants, which show enhanced resistance to biotrophic agents. Furthermore, in the interaction with avirulent *Pst*, SA and JA increase (De Vos *et al.*, 2005), but SA signalling cannot completely suppress the JA pathway (Spoel *et al.*, 2007). Thus, activation of *ProDH2* by the JA route may persist under this condition.

The *ProDH1* and *ProDH2* genes show different expression levels and sensitivity to light, although their differences are small in adult leaves at the light phase stages analysed here (Fig. S1). *ProDH2* maintains lower expression than *ProDH1* in most conditions, but reaches maximal induction in infected tissues (GENEVESTIGATOR). These genes display distinctive responses under biotic stress. *ProDH2* is up-regulated by exogenous meJA (Fig. 3b) and COR derived from *Pst* DC3000 (Fig. 3d), consistent with transcriptomic analysis data of *ProDH* and other genes associated with amino acid metabolism (Thilmony *et al.*, 2006). Curiously, *coi1-1* plants did not lose the ability to induce *ProDH2* by meJA treatment (Fig. 3b), indicating that a COI1-independent pathway signals gene activation. Indeed, nearly 26% of meJA-responsive genes maintain their regulation in the absence of COI1 (Devoto *et al.*, 2005), and components of COI1 alternative pathways have begun to be studied (Geng *et al.*, 2014), and so the routes leading to *ProDH2* induction in infected tissues could be identified soon.

ProDH1 was up-regulated by exogenous SA, requiring SID2 and NPR1 for early stimulation by *Pst-AvrRpm1* (Cecchini *et al.*, 2011a). Curiously, this gene is not induced by meJA (Fig. 3b), but depends on JAR1 for late activation by *Pst-AvrRpm1* (Fig. 4a). Thus, unlike *ProDH2*, *ProDH1* is sensitive to SA and JA, and is apparently affected by the balance between these hormones, as its activation by SA is reduced by HopX1 (Fig. 4b). JA induces *ProDH1* in *Pst-AvrRpm1* tissues that accumulate SA, but suppresses its up-regulation by SA in uninfected tissues expressing HopX1. In addition, *ProDH1* is induced by *B. cinerea* infection (Fig. 5c), where the contents of SA and JA are increased (Liu *et al.*, 2015). Therefore, variations in SA/JA levels or hormone combinations might differently affect *ProDH1* expression, as suggested for other genes sensitive to SA and JA (Mur *et al.*, 2006).

This is the first report of the contribution of ProDH to resistance against necrotrophic pathogens. The *B. cinerea* strain used here is adapted to suppress host defences. Nevertheless, the *prodh* mutants displayed enhanced susceptibility to the fungus. At early infection stages, mycelial expansion was faster in *prodh2-2* than in wild-type plants, and this was also observed, albeit less noticeable, in the *prodh1-3* mutant. Therefore, ProDH and, mostly, ProDH2 seem to act early, probably counteracting fungal germination, penetration, hyphal development or other initial infection events. We did not evaluate whether the enzyme also strengthen late defences, as this should be tested with a less virulent fungal strain. Under the analysed conditions, lack of ProDH had no obvious effects on the development of necrotic lesions.

A key finding of this study was that *ProDH1* and *ProDH2* provided resistance against pathogens with different lifestyles, suggesting their effect on a primary or fundamental process required to cope with infection. Consistently, both isoforms contributed to

generate the oxidative burst after the perception of flagellin (Fabro *et al.*, 2016). Similarly, in animal cells, ProDH plays protective roles in different adverse conditions, such as genotoxic processes, inflammation or metabolic stress (Phang and Liu, 2011).

ProDH is associated with the inner mitochondrial membrane and has the capacity to charge electrons into the respiratory chain at ubiquinone. It is expected that this enzyme affects respiration, energy production and the redox balance (Hancock *et al.*, 2015; Schertl and Braun, 2014). Its coordination with P5CDH yields 30 moles of ATP per mole of Pro. Its coupling with P5CR has been suggested to activate the Pro/P5C cycle, which may either increase mitochondrial ROS or enhance reducing power at mitochondria (Ben Rejeb *et al.*, 2014). However, there is no conclusive evidence that this cycle operates in plants at present. Currently, the exact consequences of ProDH activation in different infection conditions are unknown. The same applies for other mitochondrial enzymes controlling basic cellular functions that support defences against biotrophs and necrotrophs. For instance, mutations impairing the activity of mitochondrial succinate dehydrogenase (complex II) slow respiration and reduce ROS, weakening resistance against *Pst*, *Rhizoctonia solani* and *Alternaria brassicicola* (Gleason *et al.*, 2011). Deficiency in hydroxymethyltransferase serine (SHMT1), involved in photorespiration, generates redox alterations and increases susceptibility to *Pst-AvrRpm1*, *Alternaria brassicicola* and *B. cinerea* (Moreno *et al.*, 2005).

It was interesting that in infected tissues the healthy gene present in the *prodh* single mutants did not compensate for the lack of the second gene. This suggests that *ProDH1* and *ProDH2* provide non-redundant functions in these tissues. These studies were conducted with *prodh1-3* and *prodh2-2* plants which were found to be null mutants (Fig. 5). Both plants were hypersusceptible to exogenous Pro (Fig. 1a,b), something new for *prodh2* alleles, as *prodh2-1* plants previously analysed in this sense were in the Ler background which is Pro sensitive (Funck *et al.*, 2010). At present, we do not know how ProDH1 and ProDH2 are coordinated in infected tissues. One possibility is that both isoenzymes work in different cells or tissues, and their combination leads to full resistance. This is consistent with the induction of both genes in tissues infected with *Pst-AvrRpm1* or *B. cinerea*. Prior knowledge of these genes suggests a ubiquitous function for ProDH1 and a predominant role of ProDH2 in perivascular tissues (Faës *et al.*, 2015; Funck *et al.*, 2010; GENEVESTIGATOR; https://genevestigator.com). Alternatively, both isoforms may coexist in the same cells either in the same or different organelles (mitochondria and chloroplast; Funck *et al.*, 2010; Van Aken *et al.*, 2009). Adding complexity to this issue, *ProDH1* and *ProDH2* are inter-regulated. Lack of *ProDH2* increases *ProDH1* expression in uninfected, but not infected, tissues (Fig. 5). Probably, Pro-treated *prodh2-2* plants do not accumulate Pro as a result of ProDH1 compensation (Fig. 1c). In turn, lack of *ProDH1* prevents maximum *ProDH2* activation on infection, having no effect on basal gene expression. The induction of *ProDH2* also requires *ProDH1* in tissues treated with AA, an inducer of the mitochondrial ROS burst (Fabro *et al.*, 2016), suggesting that *ProDH1* contributes to *ProDH2* transcriptional control under oxidative stress conditions.

The results described herein provide novel findings on ProDH action under biotic stress which are useful for the further evaluation of the circuits responsible for the inter-regulation and balance of both *ProDH* genes, the pathways regulating their expression, and the subcellular and tissue locations of each enzyme isoform under different infection conditions.

EXPERIMENTAL PROCEDURES

Plant material

The Arabidopsis (*Arabidopsis thaliana*) Col-0 plants used in this study include *sid2-2*, *npr1-1* and *jar1-1* (Arabidopsis Biological Resource Center, Columbus, OH, USA), as well as *prodh1-2* (SALK_081276), *prodh1-3* (GABI_308F08), *prodh1-4* (SALK_119334) and *prodh2-2* (GABI_328G05). *prodh2-1* (GT1788), *coi1-1* (Katsir *et al.*, 2008) and the *HopX1* transgenic line (Gimenez-Ibanez *et al.*, 2014) are Ler, Col-5 and Aa-0 plants, respectively. Seeds were germinated on Gamborg's medium (GM) plates for 10 days and transferred to soil to be grown under an 8-h light/16-h dark cycle at 22 °C. For infection studies, plants were used at the age of 6 weeks. Sensitivity to Pro (5, 10 or 20 mM) was assayed on GM plates with 1% sucrose using 2-week-old plants. The Pro content was determined according to Bates *et al.* (1973).

Treatments with pathogens, JA and AA

Pst DC3000 (virulent), *Pst-AvrRpm1* (avirulent) and *Pst* DC3118 (COR-deficient mutant; Thilmony *et al.*, 2006) were grown on King's B medium supplemented with antibiotics. Pathogens were infiltrated into leaf tissues at 5×10^5 colony-forming units (cfu)/mL for bacterial growth curves and 10^6 or 10^7 cfu/mL for expression analyses (Pavet *et al.*, 2005). *Botrytis cinerea* B05.10 was grown in potato dextrose agar (PDA) for 10 days, exposed to black light for 2 days and maintained under normal growth conditions for 4 additional days. At this time, conidia were harvested, washed once in 0.1% Tween 20, twice in water, resuspended and maintained for 2 h in 10 mM K3PO4 with 10 mM sucrose, and deposited onto the adaxial side of the leaf using 5-μL aliquots. Fungal hyphae were detected by trypan blue staining (Fabro *et al.*, 2008) and infection areas were quantified with the ImageJ program. meJA (95%; Sigma Aldrich Buenos Aires, Argentina) was applied at 0.84 μM concentration as described previously (Fabro *et al.*, 2008). Samples contained a set of three leaves collected from different plants. Treatments with AA (10 μM; Sigma Aldrich, Buenos Aires, Argentina, A8674) were performed as described previously (Fabro *et al.*, 2016) using leaf discs incubated overnight in water and then exposed to water (mock) or AA for 4 h.

In silico gene promoters and gene expression analysis

Gene promoter sequences were analysed with PLACE (Higo *et al.*, 1999), Agris (Davuluri *et al.*, 2003) and Plant CARE (Lescot *et al.*, 2002) programs after defining the promoter region according to Agris (2481 and 2562 bp

for *ProDH1* and *ProDH2* promoters, respectively). Elements recognized by at least two programs were selected. Gene expression was determined by semi-quantitative (Monteoliva *et al.*, 2014) or quantitative (Fabro and Alvarez, 2012) reverse transcription-polymerase chain reaction (RT-PCR) using *GapC* or *UBQ5* as control genes. The RT-PCR conditions are described in Table S2 (see Supporting Information). For quantitative RT-PCR, the gene expression values were determined by the ΔΔCt method using *UQB5* as housekeeping gene, and primers at 200 nM, except for *ProDH2* Rv, which was applied at 300 nM.

ACKNOWLEDGEMENTS

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT 2012-2117; PICT 2014-3255) and Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba to M.E.A. Y.S.R. is a CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) fellow. G.F. and M.E.A. are senior Career Investigators of CONICET. We thank Drs A. Savouré (UPMC, Université Paris 06, Jussieu, France), D. Funck (University of Konstanz, Germany) and S. Gimenez-Ibañez (CNB-CSIC, Madrid, Spain) for providing seeds, and Dr F. Pieckenstain (IIB-INTECH, Argentina) for the *Botrytis cinerea* B05.10 strain.

REFERENCES

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant Soil*, **39**, 205–207.

Ben Rejeb, K., Abdelly, C. and Savouré, A. (2014) How reactive oxygen species and proline face stress together. *Plant Physiol. Biochem.* **80**, 278–284.

Caarls, L., Pieterse, C.M. and Van Wees, S.C. (2015) How salicylic acid takes transcriptional control over jasmonic acid signaling. *Front. Plant Sci.* **6**, 170.

Cabassa-Hourton, C., Schertl, P., Bordenave-Jacquemin, M., Saadallah, K., Guivarc'h, A., Lebreton, S., Planchais, S., Klodmann, J., Eubel, H., Crilat, E., Lefebvre-De Vos, D., Ghelis, T., Richard, L., Abdelly, C., Hans-Peter Braun, P. and Savoure, A. (2016) Proteomic and functional analysis of ProDH1 link proline catabolism to mitochondrial electron transport in Arabidopsis thaliana. *Biochem. J.* **473**, 2623–2634.

Cecchini, N.M., Monteoliva, M.I. and Alvarez, M.E. (2011a) Proline dehydrogenase contributes to pathogen defense in Arabidopsis. *Plant Physiol.* **155**, 1947–1959.

Cecchini, N.M., Monteoliva, M.I. and Alvarez, M.E. (2011b) Proline dehydrogenase is a positive regulator of cell death in different kingdoms. *Plant Signal. Behav.* **6**, 1195–1197.

Davuluri, R.V., Sun, H., Palaniswamy, S.K., Matthews, N., Molina, C., Kurtz, M. and Grotewold, E. (2003) AGRIS: Arabidopsis gene regulatory information server, an information resource of Arabidopsis *cis*-regulatory elements and transcription factors. *BMC Bioinformatics*, **4**, 25.

Devadas, S.K., Enyedi, A. and Raina, R. (2002) The Arabidopsis hrl1 mutation reveals novel overlapping roles for salicylic acid, jasmonic acid and ethylene signalling in cell death and defence against pathogens. *Plant J.* **30**, 467–480.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Metraux, J.P., Van Loon, L.C., Dicke, M. and Pieterse, C.M. (2005) Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. *Mol. Plant–Microbe Interact.* **18**, 923–937.

Devoto, A., Ellis, C., Magusin, A., Chang, H.S., Chilcott, C., Zhu, T. and Turner, J.G. (2005) Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. *Plant Mol. Biol.* **58**, 497–513.

Dietrich, K., Weltmeier, F., Ehlert, A., Weiste, C., Stahl, M., Harter, K. and Droge-Laser, W. (2011) Heterodimers of the Arabidopsis transcription factors bZIP1 and bZIP53 reprogram amino acid metabolism during low energy stress. *Plant Cell*, **23**, 381–395.

Elthon, T.E. and Stewart, C.R. (1981) Submitochondrial location and electron transport characteristics of enzymes involved in proline oxidation. *Plant Physiol.* **67**, 780–784.

Fabro, G. and Alvarez, M.E. (2012) Loss of compatibility might explain resistance of the *Arabidopsis thaliana* accession Te-0 to *Golovinomyces cichoracearum*. *BMC Plant Biol.* **12**, 143.

Fabro, G., Di Rienzo, J.A., Voigt, C.A., Savchenko, T., Dehesh, K., Somerville, S. and Alvarez, M.E. (2008) Genome-wide expression profiling of Arabidopsis at the stage of *Golovinomyces cichoracearum* haustorium formation. *Plant Physiol.* **146**, 1421–1439.

Fabro, G., Rizzi, Y.S. and Alvarez, M.E. (2016) Arabidopsis proline dehydrogenase contributes to flagellin-mediated PAMP-triggered immunity by affecting RBOHD. *Mol. Plant–Microbe Interact.* **29**, 620–628.

Faës, P., Deleu, C., Ainouche, A., Le Caherec, F., Montes, E., Clouet, V., Gouraud, A.M., Albert, B., Orsel, M., Lassalle, G., Leport, L., Bouchereau, A. and Niogret, M.F. (2015) Molecular evolution and transcriptional regulation of the oilseed rape proline dehydrogenase genes suggest distinct roles of proline catabolism during development. *Planta*, **241**, 403–419.

Funck, D., Eckard, S. and Muller, G. (2010) Non-redundant functions of two proline dehydrogenase isoforms in Arabidopsis. *BMC Plant Biol.* **10**, 70.

Geng, X., Jin, L., Shimada, M., Kim, M.G. and Mackey, D. (2014) The phytotoxin coronatine is a multifunctional component of the virulence armament of *Pseudomonas syringae*. *Planta*, **240**, 1149–1165.

Gimenez-Ibanez, S., Boter, M., Fernandez-Barbero, G., Chini, A., Rathjen, J.P. and Solano, R. (2014) The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in Arabidopsis. *PLoS Biol.* **12**, e1001792.

Gleason, C., Huang, S., Thatcher, L.F., Foley, R.C., Anderson, C.R., Carroll, A.J., Millar, A.H. and Singh, K.B. (2011) Mitochondrial complex II has a key role in mitochondrial-derived reactive oxygen species influence on plant stress gene regulation and defense. *Proc. Natl. Acad. Sci. USA*, **108**, 10768–10773.

Hancock, C.N., Liu, W., Alvord, W.G. and Phang, J.M. (2015) Co-regulation of mitochondrial respiration by proline dehydrogenase/oxidase and succinate. *Amino Acids*, **48**, 859–872.

Hanson, J., Hanssen, M., Wiese, A., Hendriks, M.M. and Smeekens, S. (2008) The sucrose regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of ASPARAGINE SYNTHETASE1 and PROLINE DEHYDROGENASE2. *Plant J.* **53**, 935–949.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* **27**, 297–300.

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y. and Howe, G.A. (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci. USA*, **105**, 7100–7105.

Kiyosue, T., Yoshiba, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1996) A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in Arabidopsis. *Plant Cell*, **8**, 1323–1335.

Kunz, C., Vandelle, E., Rolland, S.P., Poinssot, B., Bruel, C., Cimerman, A.S., Zotti, C., Moreau, E., Vedel, R., Pugin, A. and Boccara, M. (2006) Characterization of a new, nonpathogenic mutant of *Botrytis cinerea* with impaired plant colonization capacity. *New Phytol.* **170**, 537–550.

Lescot, M., Dehais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouze, P. and Rombauts, S. (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **30**, 325–327.

Liang, X., Zhang, L., Natarajan, S.K. and Becker, D.F. (2013) Proline mechanisms of stress survival. *Antioxid. Redox Signal.* **19**, 998–1011.

Liu, S., Kracher, B., Ziegler, J., Birkenbihl, R.P. and Somssich, I.E. (2015) Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards *Botrytis cinerea* 2100. *Elife*, **4**, e07295.

Monteoliva, M.I., Rizzi, Y.S., Cecchini, N.M., Hajirezaei, M.R. and Alvarez, M.E. (2014) Context of action of proline dehydrogenase (ProDH) in the Hypersensitive Response of Arabidopsis. *BMC Plant Biol.* **14**, 21.

Moreno, J.I., Martin, R. and Castresana, C. (2005) Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. *Plant J.* **41**, 451–463.

Mur, L.A., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C. (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol.* **140**, 249–262.

Nakashima, K., Satoh, R., Kiyosue, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) A gene encoding proline dehydrogenase is not only induced by proline

and hypoosmolarity, but is also developmentally regulated in the reproductive organs of Arabidopsis. *Plant Physiol.* **118**, 1233–1241.

Nanjo, T., Fujita, M., Seki, M., Kato, T., Tabata, S. and Shinozaki, K. (2003) Toxicity of free proline revealed in an arabidopsis T-DNA-tagged mutant deficient in proline dehydrogenase. *Plant Cell Physiol.* **44**, 541–548.

Pavet, V., Olmos, E., Kiddle, G., Mowla, S., Kumar, S., Antoniw, J., Alvarez, M.E. and Foyer, C.H. (2005) Ascorbic acid deficiency activates cell death and disease resistance responses in Arabidopsis. *Plant Physiol.* **139**, 1291–1303.

Phang, J.M. and Liu, W. (2011) Proline metabolism and cancer. *Front. Biosci.* **17**, 1835–1845.

Prins, T.W., Tudzynski, P., von Tiedemann, A., Tudzynski, B., Ten Have, A., Hansen, M.E., Tenberge, K. and van Kan, J.A.L. (2000) Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. In: *Fungal Pathology* (Krostand, J. W., ed.), pp. 33–64. XXXX: Springer.

Ribarits, A., Abdullaev, A., Tashpulatov, A., Richter, A., Heberle-Bors, E. and Touraev, A. (2007) Two tobacco proline dehydrogenases are differentially regulated and play a role in early plant development. *Planta*, **225**, 1313–1324.

Rizzi, Y.S., Monteoliva, M.I., Fabro, G., Grosso, C.L., Larovere, L.E. and Alvarez, M.E. (2015) P5CDH affects the pathways contributing to Pro synthesis after ProDH activation by biotic and abiotic stress conditions. *Front. Plant Sci.* **6**, 572.

Satoh, R., Fujita, Y., Nakashima, K., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2004) A novel subgroup of bZIP proteins functions as transcriptional activators in hypoosmolarity-responsive expression of the ProDH gene in Arabidopsis. *Plant Cell Physiol.* **45**, 309–317.

Schertl, P. and Braun, H.P. (2014) Respiratory electron transfer pathways in plant mitochondria. *Front. Plant Sci.* **5**, 163.

Senthil-Kumar, M. and Mysore, K.S. (2012) Ornithine-delta-aminotransferase and proline dehydrogenase genes play a role in non-host disease resistance by regulating pyrroline-5-carboxylate metabolism-induced hypersensitive response. *Plant Cell Environ.* **35**, 1329–1343.

Sharma, S. and Verslues, P.E. (2010) Mechanisms independent of abscisic acid (ABA) or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. *Plant Cell Environ.* **33**, 1838–1851.

Spoel, S.H., Johnson, J.S. and Dong, X. (2007) Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl. Acad. Sci. USA*, **104**, 18 842–18 847.

Staswick, P.E. and Tiryaki, I. (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. *Plant Cell*, **16**, 2117–2127.

Thilmony, R., Underwood, W. and He, S.Y. (2006) Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* **46**, 34–53.

Uloth, M.B., Clode, P.L., You, M.P. and Barbetti, M.J. (2015) Calcium oxalate crystals: an integral component of the *Sclerotinia sclerotiorum*/*Brassica carinata* pathosystem. *PLoS One*, **10**, e0122362.

Van Aken, O., Zhang, B., Carrie, C., Uggalla, V., Paynter, E., Giraud, E. and Whelan, J. (2009) Defining the mitochondrial stress response in *Arabidopsis thaliana*. *Mol. Plant.* **2**, 1310–1324.

Weltmeier, F., Ehlert, A., Mayer, C.S., Dietrich, K., Wang, X., Schutze, K., Alonso, R., Harter, K., Vicente-Carbajosa, J. and Droge-Laser, W. (2006) Combinatorial control of Arabidopsis proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors. *EMBO J.* **25**, 3133–3143.

Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I.D., De Luca, V. and Despres, C. (2012) The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* **1**, 639–647.

Yoshioka, K., Kachroo, P., Tsui, F., Sharma, S.B., Shah, J. and Klessig, D.F. (2001) Environmentally sensitive, SA-dependent defense responses in the cpr22 mutant of Arabidopsis. *Plant J.* **26**, 447–459.

SUPPORTING INFORMATION

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

Fig. S1 Expression of *ProDH1* and *ProDH2* at different stages of development (a) and during the light/dark cycle (b). (a) Comparison of *ProDH1* (red), *ProDH2* (blue) and *GapC* (green) expression using GENEVESTIGATOR tools. (b) Gene expression levels of *ProDH1* and *ProDH2* in 7-day-old seedlings grown under the light/dark cycle used in this study (8 h light, white bar; 16 h dark, black bar), according to data informed by the DIURNAL website (http://diurnal.mocklerlab.org/). Black arrows indicate the time points at which samples were analysed [0, 6 and 24 h post-treatment (hpt)]. Differences in *ProDH1* and *ProDH2* expression are low in adult leaves (a) at the light phase stages selected for this study (b).

Table S1 *cis*-regulatory elements in the *ProDH1* and *ProDH2* gene promoters.

Table S2 Primers and conditions used in reverse transcription-polymerase chain reaction (RT-PCR) assays.