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RESEARCH PAPER

Role of Arginine decarboxylase (ADC) in *Arabidopsis thaliana* defence against the pathogenic bacterium *Pseudomonas viridiflava*

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ABSTRACT

Polyamine biosynthesis starts with putrescine production through the decarboxylation of arginine or ornithine. In *Arabidopsis thaliana*, putrescine is synthesised exclusively by arginine decarboxylase (ADC), which exists as two isoforms (*ADC1* and *2*) that are differentially regulated by abiotic stimuli, but their role in defence against pathogens has not been studied in depth. This work analysed the participation of ADC in *Arabidopsis* defence against *Pseudomonas viridiflava*. ADC activity and expression, polyamine levels and bacterial resistance were analysed in null mutants of each ADC isoform. In non-infected wild-type (WT) plants, *ADC2* expression was much higher than *ADC1*. Analysis of *adc* mutants demonstrated that ADC2 contributes to a much higher extent than ADC1 to basal ADC activity and putrescine biosynthesis. In addition, *adc2* mutants showed increased basal expression of salicylic acid- and jasmonic acid-dependent PR genes. Bacterial infection induced putrescine accumulation and *ADC1* expression in WT plants, but pathogen-induced putrescine accumulation was blocked in *adc1* mutants. Results suggest a specific participation of ADC1 in defence, although basal resistance was not decreased by dysfunction of either of the two *ADC* genes. In addition, and as opposed to WT plants, bacterial infection increased *ADC2* expression and ADC activity in *adc1* mutants, which could counterbalance the lack of ADC1. Results demonstrate a major contribution of ADC2 to total ADC activity and the specific induction of ADC1 in response to infection. A certain degree of functional redundancy between the two isoforms in relation to their contribution to basal resistance is also evident.

INTRODUCTION

The diamine putrescine (Put), the triamine spermidine (Spd) and the tetraamine spermine (Spm), collectively known as polyamines (PAs), are natural aliphatic polycations essential for prokaryotic and eukaryotic cells. At cellular pH, PAs are positively charged, thus interacting with anionic molecules such as nucleic acids, phospholipids and proteins. As a consequence, they modulate DNA–protein (Shah *et al.* 1999; D'Agostino *et al.* 2005) and protein–protein interactions (Thomas *et al.* 1999; Garufi *et al.* 2007) and RNA structure (Igarashi & Kashiwagi 2000). Thus, PAs act as regulatory molecules in many fundamental cellular processes, such as cell division, differentiation and proliferation, cell death, DNA and protein synthesis and gene expression (Igarashi & Kashiwagi 2000; Seiler & Raul 2005; Kusano *et al.* 2008).

In plants, PAs participate in many physiological processes, such as organogenesis, embryogenesis, floral initiation and development, leaf senescence and fruit development and ripening (Kumar *et al.* 1997; Kusano *et al.* 2008). In addition, PAs are involved in plant responses to diverse biotic and abiotic stresses (Bouchereau *et al.* 1999; Walters 2003a,b; Gill & Tuteja 2010; Bitrián *et al.* 2012; Gupta *et al.* 2013). Even though the physiological significance of stress-induced changes in PA levels has not always been clear, recent works have demonstrated that plant PAs can play a role as part of the complex signalling networks activated in response to stresses (Takahashi *et al.* 2003; Mitsuya *et al.* 2009; Moschou *et al.* 2009). Thus, understanding how PA levels are modulated in response to external stimuli can contribute to decipher their role in signalling networks. In this regard, it is noteworthy that PA levels are modulated through its biosynthesis and catabolism, as well as by conjugation to other molecules, epigenetic regulation of genes involved in PA metabolism and regulation of PA transport to different subcellular compartments (Biondi *et al.* 2001; Namy *et al.* 2008; Igarashi & Kashiwagi 2010).

The first step of the PA pathway is the biosynthesis of Put, the precursor for the production of higher PAs (Spd and Spm). Put can be synthesised directly from ornithine by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) or indirectly, *via* a series of intermediates following decarboxylation of arginine by arginine decarboxylase (ADC, EC 4.1.1.19). In turn, Spd and Spm are synthesised from Put by successive additions of aminopropyl groups provided by decarboxylated S-adenosylmethionine, a metabolite synthesised by the enzyme S-adenosylmethionine decarboxylase (EC 4.1.1.50). The aminopropyl

additions to Put are catalysed by the aminopropyltransferases spermidine (EC 2.5.1.16) and spermine (EC 2.5.1.22) synthases.

As a general rule, ODC is the sole pathway for Put production in mammalian and fungal cells. In contrast, both the ADC and ODC pathways are present in many higher plants and bacteria (Fuell *et al.* 2010). However, the absence of an *ODC* gene in the *A. thaliana* genome, as well as other experimental evidence, suggest that this species lacks a functional ODC pathway and PA metabolism. Thus, PA biosynthesis seems to have evolved differently in some plant species (Hanfrey *et al.* 2001). In this way, *A. thaliana* is the only vascular plant so far described that depends solely on ADC for Put biosynthesis, similar to the moss *Physcomitrella patens*, another species that lost the ODC pathway (Fuell *et al.* 2010). As a consequence, modulation of ADC activity is of key importance for regulation of the whole PA biosynthesis pathway in response to external stimuli in *A. thaliana*. This species harbours two *ADC* genes (*ADC1* and *2*), which show different expression patterns. *ADC2* expression is strongly associated with seed germination, root and leaf development, whereas *ADC1* promoter activity is low during vegetative development (Hummel *et al.* 2004). High osmolarity, drought, salinity and wounding induce *ADC2* expression, while low temperatures and freezing conditions have diverse effects on expression of each *AtADC* gene (Soyka & Heyer 1999; Perez-Amador *et al.* 2002; Urano *et al.* 2003; Hummel *et al.* 2004; Alcázar *et al.* 2006, 2010; Cuevas *et al.* 2008). Simultaneous knockout of both *AtADC* genes leads to a lethal phenotype, thus confirming that *A. thaliana* strongly depends on ADC for Put biosynthesis, and reinforces the suggestion of the lack of an active ODC pathway in this species (Urano *et al.* 2005).

Although involvement of the two different *A. thaliana ADC* genes in responses to the above-mentioned abiotic stresses has been analysed, their participation in defence responses to pathogen infection has not been addressed in depth so far. In this regard, a recent paper focused on the role of Put in the regulation of MAPK cascades reported an increase in *AtADC2* expression during *A. thaliana* response to *Pseudomonas syringae* pv. *tomato* DC3000 infection, as well as increased susceptibility to this bacterium in an *adc2* mutant (Kim *et al.* 2013). Previously, the expression of both ADC isoforms was reported to increase during the hypersensitive response triggered by *A. thaliana* infection with avirulent cucumber mosaic virus (Mitsuya *et al.* 2009). Similarly, infection of *A. thaliana* roots by the cyst nematode *Heterodera schachtii* increased expression of both *ADC* genes, along with other genes involved in PA biosynthesis (Hewezi *et al.* 2010). However, a comprehensive analysis of the induction of both ADC isoforms in response to infection, and the impact of disruption of each ADC isoform on PA metabolism and *A. thaliana* response to pathogen infection has not been performed so far.

As part of the multiple responses triggered in response to pathogen infection, changes in PA metabolism have been described in numerous reports (Walters 2003b). In recent years, significant progress has been made in understanding the physiological significance of pathogen-induced changes in PA metabolism. In this sense, PA accumulation and further oxidation by polyaminooxidases were found to play a key role in plant defence against viruses, bacterial and fungal pathogens, as demonstrated in several pathosystems (Marina *et al.* 2008; Mitsuya *et al.* 2009; Moschou *et al.* 2009; Sagor *et al.* 2009; Gonzalez *et al.* 2011). The tetraamine Spm was found to induce acidic pathogenesis-related protein expression in tobacco mosaic virus (TMV)-infected tobacco plants (Yamakawa *et al.* 1998), and to activate a subset of hypersensitive response (HR)-specific genes as a consequence of a mitochondrial dysfunction induced *via* a signalling pathway that stimulates MAPK (Takahashi *et al.* 2003, 2004). More recently, Spm was demonstrated to induce the expression of a number of *A. thaliana* genes in common to cucumber mosaic virus (CMV) infection and was proposed to play a role in signalling defence responses of *A. thaliana* against this pathogen (Mitsuya *et al.* 2009; Sagor *et al.* 2009). A role for Spm in modulating global changes in the expression of defence-related genes in *A. thaliana* has also been described, and it was demonstrated that manipulation of Spm levels through modifications of spermine synthase gene expression can modulate *A. thaliana* resistance to the bacterial pathogen *Pseudomonas viridiflava* (Gonzalez *et al.* 2011). The above-mentioned studies stress the importance of understanding the regulation of *A. thaliana* PA biosynthesis in response to pathogen attack, which would contribute to further understand the role of these metabolites in plant–pathogen interactions. Taking into account the absolute dependence of *A. thaliana* on ADC for the initial step in PA biosynthesis, and the scarce information about the role of *ADC* genes in defence of this model plant species against pathogens, the present work aimed to evaluate the participation of *AtADCs* in defence responses and their contribution to basal resistance to the phytopathogenic bacterium *P. viridiflava*.

MATERIAL AND METHODS

Plant material and growth conditions

The Columbia Col-0 ecotype of *A. thaliana* was used as the WT. T-DNA insertion mutants of the *ADC1* (*adc1-3*) and *ADC2* (*adc2-3*) genes were kindly provided by J.C. Cuevas (Facultad de Farmacia y Bioquímica, Universidad de Barcelona, Spain). These mutants were previously characterised by Cuevas *et al.* (2008), and are available at The Arabidopsis Information Resource (www.arabidopsis.org), with stock numbers CS9657 and CS9659, respectively. Seeds were surface-disinfected with 75% (v/v) ethanol for 1.5 min followed by 5% (v/v) bleach for 15 min and thorough rinsing with sterile distilled water. Disinfected seeds were plated on Murashige and Skoog medium supplemented with 3% (w/v) sucrose, and stratified at 4 °C for 2 days in the dark. Plates were subsequently incubated for 2 weeks in a growth chamber with a 16-h light/8-h dark photoperiod at 25/20 °C, 55/65% relative humidity (day/night) and a photon flux density of 200 $\mu mol \cdot m^2 \cdot s^{-1}$ provided by cool-white and Grolux fluorescent lamps.

Bacterial strain, plant inoculation and disease evaluation

Pseudomonas viridiflava strain Pvalb8 (Alippi *et al.* 2003) was kindly provided by Dr. A. Alippi (Facultad de Agronomía, Universidad Nacional de La Plata, Argentina). This bacterium, which is virulent on *A. thaliana* (Gonzalez *et al.* 2011), was routinely maintained at −80 °C in Luria-Bertani (LB) medium amended with 20% (v/v) glycerol. Plant infections were

performed as described in Marina *et al.* (2013). Briefly, *P. viridiflava* inocula were prepared by streaking bacteria from −80 °C on to LB agar plates, incubating plates at 28 °C for 24 h, then scraping bacterial cells off plates into 10 mM $MgCl_2$, pH 7.0, to yield 5×10^8 CFU·ml^{-1}. An aliquot of 5 µl bacterial suspension was placed on each plant (four to six rosette leaves) and an identical volume of 10 mM $MgCl_2$, pH 7.0, was applied to controls. Plants were incubated in the growth chamber and sampled at different times after inoculation for analysis of bacterial propagation. Prior to evaluating *in planta* bacterial growth, inoculated plants were surface disinfected with 70% (v/v) ethanol for 2 min and washed several times with sterile water. Plants were then homogenised in 10 mM $MgCl_2$, pH 7.0, and serial dilutions of the extracts thus obtained were plated on King's B agar medium for 24 h at 28 °C in order to determine the number of CFU per plant.

Disease incidence, defined as the percentage of diseased plants, was evaluated at different times after inoculation by determining the number of diseased plants among a total of 20 to 25 inoculated plants. Disease severity, defined as the level of damage in diseased plants, was evaluated by estimating the percentage of diseased tissue in the inoculated leaves that showed symptoms. The percentage of diseased tissue was quantified after image acquisition with a Nikon SMZ binocular microscope (Nikon, Melville, NY, USA) coupled to a digital camera and further image analysis with Image-Pro®Plus version 4.1 software (Media Cybernetics, Rockville, MD, USA). On the basis of the percentage of the total leaf surface that exhibited symptoms, a severity rank between 1 and 5 was assigned to each diseased plant. Ranks were as follows: 1, <10%; 2, 11 to 25%; 3, 26 to 50%; 4, 51 to 75%; 5, 76% to completely necrotic leaf and lesions spreading to the rest of the plant (Fig. S1).

Polyamine analysis

Polyamines were extracted by grinding 200 mg fresh plant material in 0.6 ml 5% (v/v) perchloric acid with a pestle and incubating extracts at 4 °C. Extracts thus obtained were split in two fractions, which were used for free and soluble conjugated PA analysis. Fractions used for free PA analysis were kept at −20 °C until further derivatisation. Fractions used for soluble conjugated PA analysis were amended with HCl (6 M final concentration) and incubated at 120 °C for 18 h. Free and soluble conjugated PA extracts were then dried under vacuum and subsequently dansylated using 20 µl 0.1 mM 1,7-heptanediamine (HTD) per sample as internal standard, as previously described (Marina *et al.* 2008). Dansylated PAs were dissolved in 100 µl acetonitrile and analysed with reversed phase HPLC using a Waters 1525 Binary HPLC Pump and a 2475 Multi λ Fluorescence Detector, as described previously (Marcé *et al.* 1995).

Quantitative real-time PCR (qRT-PCR)

Plants were frozen in liquid nitrogen and total RNA extracted with TRI reagent (Sigma Chemicals) according to the manufacturer's instructions. First-strand cDNAs were synthesised using *Moloney Murine Leukemia Virus* Reverse Transcriptase (MMLV-RT) (Promega, www.promega.com). For quantification of mRNA levels by qRT-PCR, 1 µl synthesised first-strand cDNA (1:5 dilution) was further diluted to 7.5 µl with water, and the same volume of FastStart Universal SYBR Green Master (Rox) was added to a final volume of 15 µl. Primers used in these reactions are listed in Table S1. Reactions were performed in an Mx3005P qPCR system with the aid of MxPro qPCR software 4.0 (Stratagene, La Jolla, CA, USA). Relative quantification was performed by the comparative cycle threshold method with the ubiquitin-encoding gene *UBQ10* as endogenous control. For comparative purposes, relative gene expression in control plants was defined as 1. The REST® software tool was used to calculate the relative expression ratios on the basis of group means for target gene transcripts *versus* the reference gene transcript (Pfaffl *et al.* 2002). Raw data of gene expression analysis are provided in Table S2.

Arginine decarboxylase activity

Arginine decarboxylase activity was measured by quantifying the $^{14}CO_2$ released, employing [U-^{14}C]-L-arginine as substrate according to the method described in Flores & Galston (1984) with some modifications. Immediately after harvest, *Arabidopsis* seedlings were homogenised with a mortar and pestle in liquid-N_2 and 200–250 mg of powder were subsequently suspended in 0.25 ml extraction buffer (15 mM KH_2PO_4, 20 mM sodium ascorbate, 0.5 mM EDTA, 1 mM PMSF, 10 mM DTT, 1 mM PLP, 85 mM Na_2HPO_4, pH 7.5). After centrifugation for 20 min at 12,000 *g* and 4 °C, 190 µl of supernatant were added to a reaction mixture containing 10 mM L-arginine, 1 mM urea and 50 nCi [U-^{14}C]-L-arginine (Amersham, UK; 346 nCi·nmol^{-1}) in a final volume of 200 µl. After 45 min at 37 °C, the reaction was stopped with 0.2 ml 10% (v/v) perchloric acid and the $^{14}CO_2$ released was trapped in absorbent paper embedded in 2 M KOH. Radioactive emission was measured in a Perkin Elmer Tri-Carb 2810 TR scintillation counter. Protein content was determined with the Bradford's method, using bovine serum albumin as the reference standard.

Statistics

Each experiment was independently conducted at least twice with similar results. Results from representative experiments are shown as means ± SD. Bacterial propagation, PA levels, ADC activity and qRT-PCR data correspond to the mean of four to five replicates, each consisting of pools of two plants for the analysis of bacterial growth and 20–30 plants for the other parameters. ADC activity and levels of polyamine data were analysed by one-way ANOVA and Tukey's multiple comparison test. qRT-PCR results were analysed with the REST software, version 2.0.7 (Pfaffl *et al.* 2002). Disease incidence and frequency distribution of disease severity data were analysed with a chi-square test on 20–25 plants.

RESULTS

Expression of ADC isoforms in WT Col-0 *A. thaliana* and *adc* mutants in response to infection with *P. viridiflava*

As an initial approach to analyse the participation of the two *A. thaliana* ADC isoforms in defence against pathogen infection, the expression levels of *ADC1* and *ADC2* were analysed by qRT-PCR, using specific primers for each gene. In

non-inoculated WT plants, *ADC2* expression was approximately 400-fold higher than *ADC1* (Fig. 1). In non-inoculated *adc* mutants, the lack of each ADC isoform had no effect on expression of the other isoform. In this way, *ADC1* expression in *adc2-3* was similar to WT plants, and *ADC2* expression in *adc1-3* was also similar to WT plants (Fig. 1). *P. viridiflava* infection of WT plants caused a five- to six-fold increase in *ADC1* expression, while *ADC2* expression remained unaffected, as evaluated at both 24 and 48 h post-inoculation (HPI; Fig. 2A).

Infection of the *adc2-3* mutant also caused a several fold increase in *ADC1* expression at both times after inoculation (Fig. 2B). In addition, infection of the *adc1-3* mutant also increased *ADC2* expression, although in this case the increase was moderate (Fig. 2B). Nevertheless, it should be kept in mind that basal levels of *ADC2* expression in non-inoculated *adc1-3* mutants are considerably higher than basal *ADC1* expression in the *adc2-3* mutant (Fig. 1).

Effect of *P. viridiflava* infection on ADC activity and polyamine levels of WT Col-0 *A. thaliana* and *adc* mutants

In non-inoculated plants, ADC activity of *adc1-3* mutants was similar to WT Col-0, while *adc2-3* mutants exhibited an 83% reduction in ADC activity (Fig. 3). *P. viridiflava* infection did not affect ADC activity of WT plants, but caused a 50% decrease in ADC activity of *adc1-3* mutants (Fig. 3). In contrast, *P. viridiflava* infection enhanced ADC activity of *adc2-3* 1.3-fold (Fig. 3).

In order to evaluate the contribution of each ADC isoform to Put accumulation under control conditions, as well as in response to *P. viridiflava* infection, both free and conjugated PA levels were analysed. For non-inoculated plants, free and conjugated Put levels were lower in *adc2-3* mutants than in

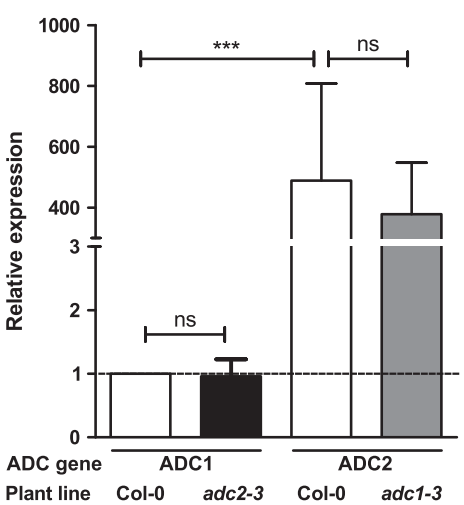

Fig. 1. Expression of *ADC* genes in WT Col-0 *A. thaliana* plants and *adc* mutants under control conditions. qRT-PCR was used to analyse the abundance of *ADC1* and *ADC2* transcripts in 15-day-old WT Col-0 and homozygous mutant lines for *ADC1* and *ADC2* genes (*adc1-3* and *adc2-3*, respectively). mRNA levels of both *ADC* genes were analysed in WT plants (white bars), while *ADC1* mRNA was quantified in the *adc2-3* mutant (black bar) and *ADC2* mRNA was quantified in the *adc1-3* mutant (grey bar). Results are expressed relative to *UBQ10* and normalised with respect to *ADC1* level in the WT. Results presented are means ± SD of five replicates and statistically significant differences in gene expression, as analysed with the REST software, are shown as: ns, not significant and $***P \leq 0.001$.

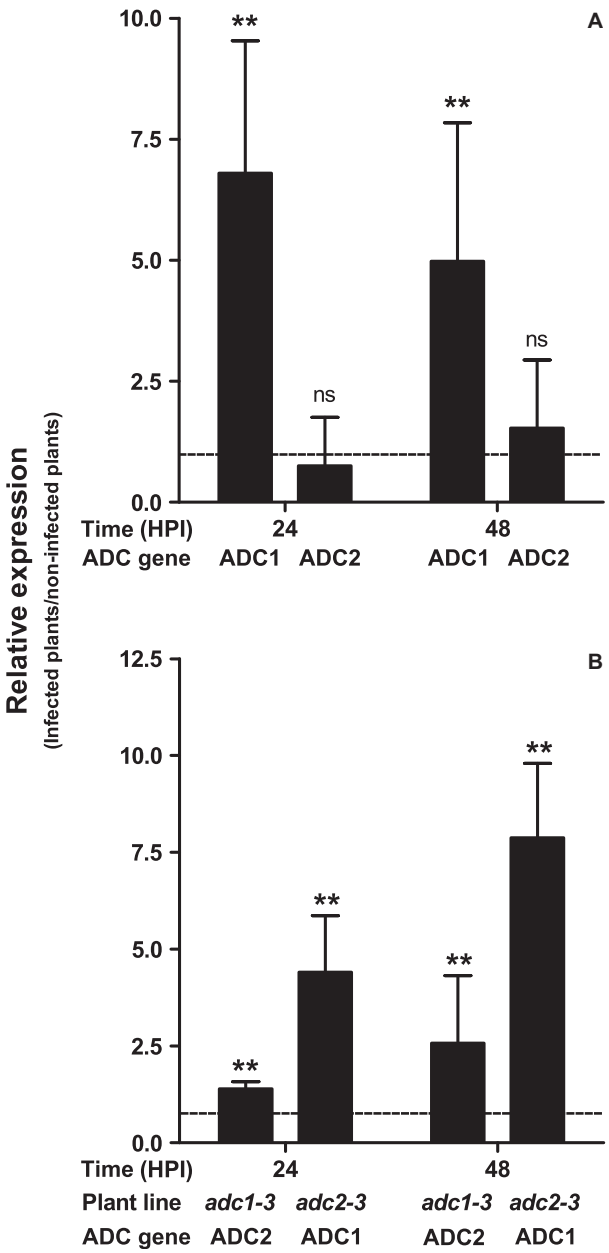

Fig. 2. Expression of *ADC* genes in WT Col-0 *A. thaliana* plants and *adc* mutants in response to *P. viridiflava* infection. qRT-PCR was used to analyse the abundance of *ADC1* and *ADC2* transcripts in 15-day-old WT Col-0 and homozygous mutant lines for *ADC1* and *ADC2* genes (*adc1-3* and *adc2-3*, respectively). (A) mRNA levels of *ADC1* and *ADC2* in WT Col-0 plants infected with *P. viridiflava* at 24 and 48 HPI. (B) Expression of *ADC2* in *adc1-3* and *ADC1* in *adc2-3*, at 24 and 48 HPI with *P. viridiflava*. In panels (A) and (B), results are expressed relative to *UBQ10* and normalised with respect to mock-inoculated plants, which were assigned to 1 and are represented by a horizontal dotted line. Results presented in both panels are means ± SD of five replicates and statistically significant differences in gene expression, as analysed with the REST software, are shown as: ns, not significant) and $**P \leq 0.01$. Comparisons in panels (A) and (B) were performed between infected and mock-inoculated plants.

Col-0. In contrast, free and conjugated Put levels in non-inoculated *adc1-3* mutants did not differ from those of Col-0 (Fig. 4A and B). Free and conjugated Spd levels of non-inoculated *adc* mutants were similar to those in WT plants (Fig. 4C and D), while free and conjugated Spm levels of the *adc2-3* mutant were, respectively, 40 and 36% higher than those of WT plants (Fig. 4E and F).

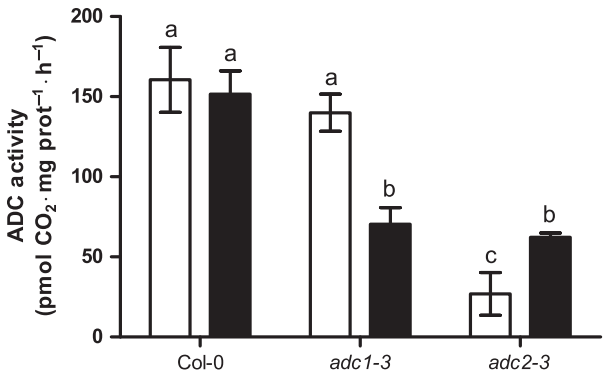

Fig. 3. Changes in ADC activity of WT Col-0 *A. thaliana* and *adc* mutants in response to *P. viridiflava* infection. Fifteen-day-old WT Col-0 and *adc1-3* and *adc2-3* mutants were inoculated with *P. viridiflava*. Plants were harvested at 48 HPI and immediately used for quantification of ADC activity, which was measured on the basis of the release of $^{14}CO_2$ using L-[U-^{14}C]arginine as substrate. White and black bars represent mock-inoculated and inoculated plants, respectively. Results are means of five replicates ± SD and different letters indicate statistically significant differences ($P \leq 0.05$) according to one-way ANOVA and Tukey's multiple comparison test.

Pseudomonas viridiflava infection caused a 90% increase in free Put in Col-0 plants. Free Put levels of *adc2-3* were also increased (five-fold) by infection, but in this case the levels reached for this diamine were lower than those detected in infected WT plants. In contrast, free and conjugated Put concentration of *adc1-3* was not affected by *P. viridiflava* infection (Fig. 4A and B). Except for a slight decrease in free Spd levels of *adc1-3*, *P. viridiflava* infection had no effect on free and conjugated forms of this triamine. Free and conjugated Spm levels were not affected in any plant line at the times after inoculation analysed in the present work (Fig. 4C–F).

Resistance of *adc* mutants to *P. viridiflava* infection and defence-related gene expression

Colonisation of WT plants by *P. viridiflava* was evident at 24 HPI, and further increased at 48 and 72 HPI. A similar level of *P. viridiflava* propagation was detected in *adc1-3* and *adc2-3* mutants, and in Col-0 plants at all times after inoculation (Fig. 5A). Thus, the lack of a functional *ADC1* or *ADC2* gene did not affect *P. viridiflava* propagation in *A. thaliana* plants. Disease symptoms were evidenced as water-soaked translucent spots, which later developed into chlorotic and necrotic lesions (Fig. S1). Disease incidence reached 65% for Col-0 plants at 48 HPI (Fig. 5B) and progressively increased up to 100% at 144 HPI (data not shown). When *adc* mutants were analysed,

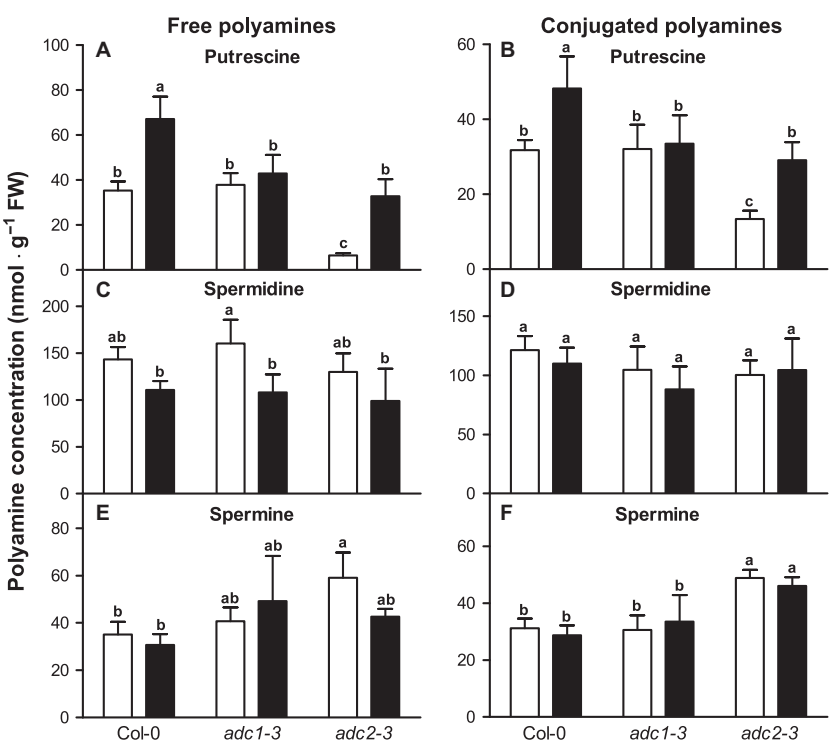

Fig. 4. Free and conjugated PA levels of WT Col-0 *A. thaliana* and *adc* mutants in response to *P. viridiflava* infection. Polyamines were extracted from mock- (white bars) and *P. viridiflava*-inoculated (black bars) WT Col-0 and *adc* mutants at 48 HPI. Free and conjugated putrescine (panels A and B), spermidine (panels C and D) and spermine (panels E and F) were determined by HPLC after derivatisation with dansyl chloride. Results are means of five replicates ± SD and different letters indicate statistically significant differences between control and infected plants ($P \leq 0.05$), according to one-way ANOVA and Tukey's multiple comparison test.

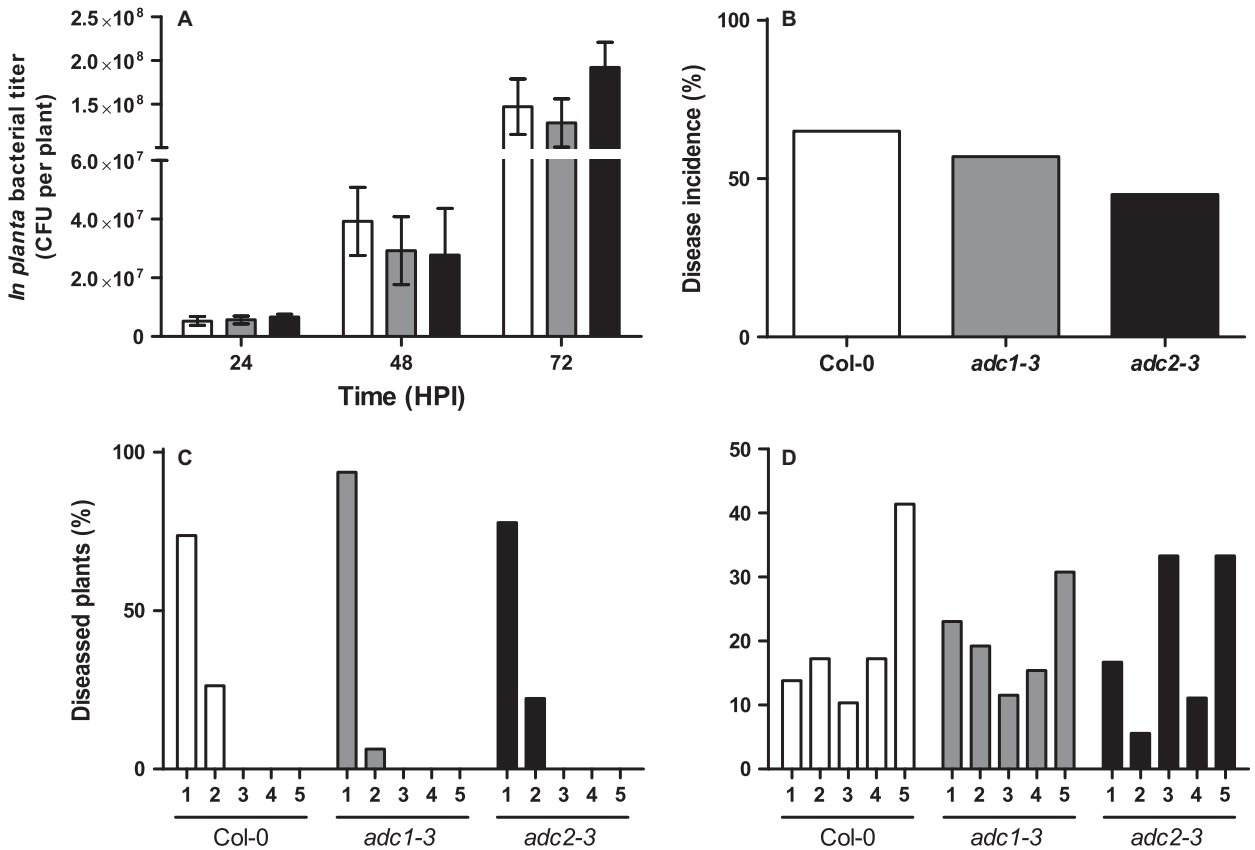

Fig. 5. Disease development in *P. viridiflava*-inoculated WT *A. thaliana* and *adc* mutants. (A) Propagation of *P. viridiflava*. WT (white bars), *adc1-3* (grey bars), and *adc2-3* (black bars) plants were inoculated with *P. viridiflava*, and the number of colony forming units (CFU) per plant was evaluated at 24, 48 and 72 HPI. (B) Disease incidence, evaluated at 48 HPI as a percentage of diseased plants over the total number of inoculated plants. (C–D) Disease severity, assessed by assigning a rank between 1 (lowest severity) and 5 (highest severity) to each diseased plant, and further analysing the number of plants comprised in each rank at 48 (C) and 144 (D) HPI. Results of propagation of *P. viridiflava* are means of four to five replicates ± SD. No differences in bacterial titers of *adc* mutants and WT Col-0 plants were detected according to one-way ANOVA and Dunnett's test. For disease incidence and severity, 20–25 plants were evaluated. No statistical differences between WT and *adc* mutants were detected according to the chi-square test in both measurements.

disease incidence was similar to Col-0 at 48 HPI (Fig. 5B) and at all analysed times post-inoculation (data not shown). The analysis of disease severity at 48 HPI showed that diseased Col-0 plants fell within the low severity ranks 1 and 2 (Fig. 5C). Disease severity increased as a function of time after inoculation. In this way, more than 50% of plants that showed disease symptoms at 144 HPI were assigned to severity ranks 4 and 5 (Fig. 5D). Similarly to disease incidence, disease severity of *adc* mutants showed no significant difference to Col-0 plants at 48 and 144 HPI (Fig. 5C and D), nor at any analysed time post-inoculation (data not shown). In *adc1-3* mutants, basal levels of *PR-1* and *PDF1-2* expression were similar to WT plants. In contrast, *adc2-3* mutants exhibited basal *PR-1* and *PDF1-2* expression levels approximately 6.5-fold higher than WT plants (Fig. 6A). *P. viridiflava* infection of WT plants caused a 50- and 17-fold increase in *PR-1* and *PDF1-2* expression, respectively. *P. viridiflava* also increased *PR-1* and *PDF1-2* expression in both *adc* mutants (Fig. 6B). *P. viridiflava* induction of defence gene expression in the *adc1-3* mutant followed a similar trend to Col-0 plants, both *PR1* and *PDF1.2* reaching similar expression levels to the WT plants. In contrast, induction of defence gene expression in the *adc2-3* mutant (infected *versus* control plants) was less intense, as in Col-0 plants. However, after considering the basal (non-inoculated condition) expression levels of defence genes and their induction by *P. viridiflava* infection, *PR1* expression in *P. viridiflava*-infected *adc2-3* plants was lower than in Col-0. Conversely, *PDF1-2* expression in *P. viridiflava*-infected *adc2-3* plants was higher than in Col-0.

DISCUSSION

Results obtained in the present work suggest that the ADC2 isoform, but not ADC1, contributes a higher proportion to total ADC activity in *A. thaliana*. This is supported by the decrease in ADC activity exhibited by *adc2-3* mutants and the normal ADC activity exhibited by the *adc1-3* mutant under control conditions in the developmental stage analysed in this work (Fig. 3). Additional evidence for a major contribution of ADC2 to ADC activity is the decrease in Put levels detected in the *adc2-3*, but not in *adc1-3* mutant, under control conditions (Fig. 4A and B). Even though expression of *ADC* isoforms in WT plants and *adc* mutants had been evaluated previously (Urano *et al.* 2005; Cuevas *et al.* 2008), the contribution of each

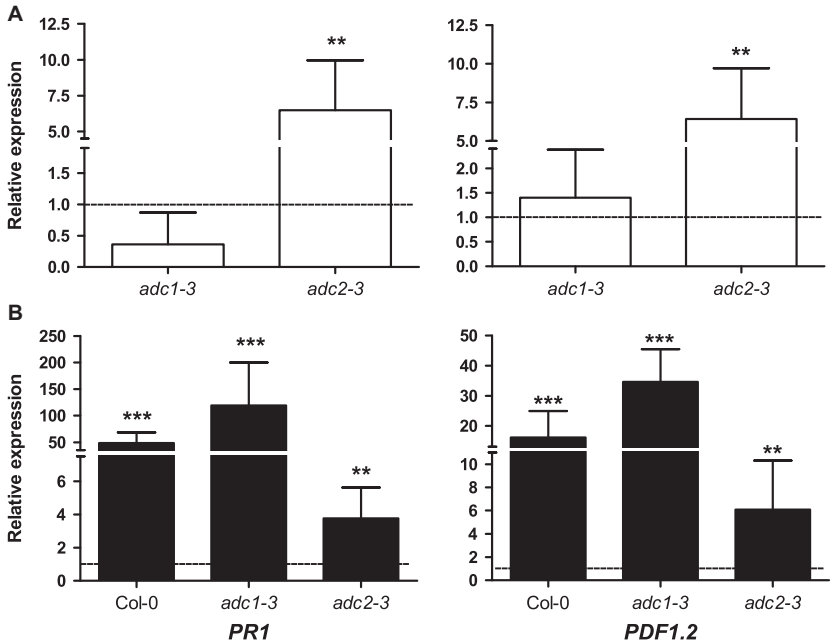

Fig. 6. Expression of PR genes in Col-0 *A. thaliana* plants and *adc* mutants under control conditions and in response to *P. viridiflava* infection. qRT-PCR was used to analyse the abundance of *PR-1* and *PDF1.2* transcripts in 15-day-old WT Col-0 and homozygous mutant lines for *ADC1* and *ADC2* genes (*adc1-3* and *adc2-3*, respectively). (A) Expression analysis of *PR* genes in non-inoculated plants. mRNA levels of both *PR* genes were analysed in *adc* mutants plants and WT plants. Results are expressed relative to *UBQ10* and normalised with respect to *PR* gene expression in WT plants, which was assigned to 1 and represented by a horizontal dotted line. (B) mRNA levels of *PR* genes in *adc* mutants and WT Col-0 plants infected with *P. viridiflava* at 48 HPI. Results are expressed relative to *UBQ10* and normalised with respect to mock-inoculated plants, which were assigned to 1 and are represented by a horizontal dotted line. Results presented in both panels are means ± SD of five replicates and statistically significant differences in gene expression, as analysed with the REST software, are shown as: ns, not significant, $**P \leq 0.01$.

ADC isoform to ADC activity remained unclear. An *A. thaliana adc2* knockout mutant obtained by Soyka & Heyer (1999) served to demonstrate that *ADC2* is responsible for induction of the PA biosynthesis pathway by osmotic stress. These authors also demonstrated that *adc2* mutants exhibited a 44% decrease in ADC activity under non-stress conditions, but the contribution of ADC1 to ADC activity was not evaluated (Soyka & Heyer 1999). Thus, the contribution of each of the two *A. thaliana* ADC isoforms to ADC activity was analysed for the first time in the present work. Results obtained are consistent with the high and generalised pattern of ADC2 expression, as opposed to the relatively low and organ-specific level of ADC1 expression detected using fusions of ADC1 and ADC2 promoters to the GUS reporter gene (El Amrani *et al.* 2002; Hummel *et al.* 2004).

Several studies reported the differential induction of *AtADC1* and *AtADC2* genes in response to diverse abiotic stimuli (Soyka & Heyer 1999; Perez-Amador *et al.* 2002; Urano *et al.* 2003; Alcázar *et al.* 2006, 2010; Cuevas *et al.* 2008), but the specific role of each ADC gene in defence responses to pathogen attack was only analysed in a single report (Kim *et al.* 2013). In the present work, infection of Col-0 *A. thaliana* by the phytopathogenic bacterium *P. viridiflava* induced *ADC1*, while *ADC2* expression remained unaffected (Fig. 2A). Taking into account the minor contribution of ADC1 to ADC activity, it is not surprising that induction of *ADC1* expression by bacterial infection did not cause an increase in ADC activity of Col-0 plants. However, when basal ADC activity was low (as is the case for *adc2-3*), the increase in *ADC1* expression induced by *P. viridiflava* infection was high enough to augment ADC activity (Fig. 3), and an increase in Put levels was also evident in *adc2-3* mutants in response to bacterial infection (Fig. 4A and B). Hence, it cannot be ruled out that under physiological or developmental conditions in which ADC activity is low, pathogen attack enhances ADC activity and Put levels as a consequence of an increase in *ADC1* expression. Thus, the increase in Put levels exhibited by *adc2-3* mutants, in addition to the previously discussed increase of ADC activity, demonstrate the specific contribution of ADC1 to Put accumulation in response to *P. viridiflava* infection. In this regard, Kim *et al.* (2013) found *Arabidopsis* infection by *Pseudomonas syringae* pv. *tomato* induced *ADC2* (but not *ADC1*) expression, which could seem inconsistent with the results here presented. However, as discussed in subsequent paragraphs, *ADC2* was found in the present work to be induced by *P. viridiflava* infection of *adc1* mutants. Therefore, it is clear that, as previously demonstrated by Kim *et al.* (2013), *ADC2* has the potential to be induced as part of the defence responses against bacteria. Differences in the experimental conditions, as well as the pathogenic organism employed in the two works could be responsible for the partial differences between their results. Fully developed WT plants such as those used by Kim *et al.* (2013) contain Put levels approximately three-fold lower than younger (four to six rosette leaves) plants such as those used in the present work (Fig. S1). Thus, differences in patterns of the induction of ADC isoforms between the two reports could be associated with the

differential Put content associated with each developmental stage. Moreover, although the bacterial species used in the two studies belong to the genus *Pseudomonas*, they are two different species. In this regard, *Arabidopsis* responses to *P. viridiflava* and *P. syringae* were previously shown to differ in their dependence on salicylate- and jasmonate-dependent signalling pathways (Jakob *et al.* 2007). Thus, *Arabidopsis* responses to these two closely related bacteria are not necessarily expected to be identical.

Putrescine was the polyamine that was accumulated to a higher extent in response to bacterial infection in the present work, and it was of interest to analyse the contribution of each *ADC* gene to this response. A strong accumulation of this diamine was also found in maize plants infected by the smut fungus *Ustilago maydis* (Rodríguez-Kessler *et al.* 2008). Taking into account that ADC activity was not enhanced by bacterial infection in WT Col-0, the activity of this enzyme seems not to be limiting in this *Arabidopsis* line for enhancing Put levels in response to pathogen attack. Other potential sources of Put exist, such as Spd and Spm oxidation mediated by polyamine oxidases through the so-called back-conversion pathway (Kamada-Nobusada *et al.* 2008; Moschou *et al.* 2008; Takahashi *et al.* 2010; Fincato *et al.* 2011). In principle, this is not supported from the levels of Spd and Spm detected in infected plants in the present work, but further analysis of Spd and/or Spm conversion into Put in infected plants could contribute to test this hypothesis. Put accumulation in infected Col-0 plants could also result not from changes in PA metabolism of the host, but from PAs released from the bacterial cells. However, infected *adc1-3* mutants were colonised by bacteria to a similar degree as WT Col-0 (Fig. 5) without exhibiting an increase in Put levels, thus suggesting that Put accumulation is not derived from bacterial cells.

As discussed previously, the lack of a functional *ADC1* gene did not decrease basal resistance of *A. thaliana* to bacterial infection, as evidenced by the similar levels of bacterial colonisation, disease incidence and severity shown by the *adc1-3* mutant and WT Col-0 (Fig. 5). This could suggest that the observed induction of *ADC1* does not play a role in *A. thaliana* defence against *P. viridiflava*, but it should be kept in mind that this isoform is much less abundant than *ADC2*. Thus, the small increase in *ADC2* expression exhibited by *adc1-3* could compensate for the lack of a functional *ADC1*. Similarly to *adc1-3* mutants, basal resistance of *adc2-3* mutants was similar to WT Col-0 (Fig. 5). Taking into account the major contribution of *ADC2* to Put biosynthesis, this finding could suggest that Put plays no role in defence against bacterial infection. However, *adc2* mutants exhibited higher levels of PR gene expression than WT plants and *adc1-3* mutants in controls (non-inoculated plants), an issue not reported previously. Thus, the higher level of constitutive PR gene expression could counterbalance any potential decrease in resistance associated with the low Put levels of *adc2-3* mutants. Moreover, this finding suggests that Put can somehow regulate PR gene expression. It is noteworthy that *adc1-3* mutants, which have normal Put levels, showed similar levels of PR gene expression to WT plants, both in response to infection and under control conditions.

It is also important to point out that even though bacterial infection had no effect on *ADC2* expression in WT Col-0, it caused a slight increase in the expression of this *ADC* isoform in *adc1-3* mutants. Thus, it seems that in the absence of a functional *ADC1* gene, *ADC2* expression can be induced by *P. viridiflava* infection. Taking into account that *ADC1* is induced by bacterial infection of WT plants, it is tempting to speculate that a regulatory mechanism aimed to compensate for the lack of *ADC1* induction by an increase in *ADC2* expression could operate in *adc1-3* mutants. Moreover, the increase in *ADC2* expression induced by bacterial infection in *adc1-3* mutants was not associated with an increase in ADC activity and Put levels. In fact, *P. viridiflava* infection caused a decrease of ADC activity in *adc1-3* mutants, and no explanation for this effect ca be drawn from the data obtained in the present work. In this regard, results here presented were derived from analysis performed on whole plant extracts. Further exploration of the subcellular location of each ADC isoform in control and infected plants could improve our understanding of the contribution of each ADC isoform to plant defence.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Disease symptoms in plants assigned to different severity ranks.

Figure S2. Putrescine levels of WT *A. thaliana* Col-0 and *adc* mutants at different developmental stages.

Table S1. Primers used in quantitative real-time PCR.

Table S2. Raw data of gene expression analysis

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