Putrescine elicits ROS – dependent activation of the salicylic acid pathway in *Arabidopsis thaliana*

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Abstract

Polyamines are small amines that accumulate during stress and contribute to disease resistance through as yet unknown signaling pathways. Using a comprehensive RNA-sequencing analysis, we show that early transcriptional responses triggered by each of the most abundant polyamines (putrescine, spermidine, spermine, thermospermine and cadaverine) exhibit specific quantitative differences, suggesting that polyamines (rather than downstream metabolites) elicit defense responses. Signaling by putrescine, which accumulates in response to bacteria that trigger effector triggered immunity (ETI) and systemic acquired resistance (SAR), is largely dependent on the accumulation of hydrogen peroxide, and is partly dependent on salicylic acid (SA), the expression of *ENHANCED DISEASE*

SUSCEPTIBILITY (*EDS1*) and *NONEXPRESSOR of PR GENES1* (*NPR1*). Putrescine elicits local SA accumulation as well as local and systemic transcriptional reprogramming that overlaps with SAR. Loss-of-function mutations in arginine decarboxylase 2 (*ADC2*), which is required for putrescine synthesis and copper amine oxidase (*CuAO*), which is involved in putrescine oxidation, compromise basal defenses, as well as putrescine and pathogen – triggered systemic resistance. These findings confirm that putrescine elicits ROS-dependent SA pathways in the activation of plant defenses.

Keywords: Polyamines, defense, systemic acquired resistance, salicylic acid, putrescine

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Summary statement

Polyamines trigger stress signaling that involves defense sectors. Putrescine, which accumulates during ETI, contributes to defense through activation of a ROS, EDS1, SA and NPR1 - dependent pathway which leads to local SA accumulation and the establishment of systemic responses overlapping with SAR.

Authorship

R.A. designed the overall project. R.A., C.L., K.E.A., A.F.T and J.Z. designed the experiments. C.L., K.E.A, N.A., E.M., and R.A. performed research. C.L., R.A. and J.Z. analyzed and interpreted the data. R.A. wrote the manuscript with contributions from other authors.

INTRODUCTION

Plant pathogens are recognized by innate immune receptors resident at the cell surface or in the cytoplasm. Binding of conserved microbial molecules (pathogen-associated molecular patterns, PAMPs) to plasma membrane associated pattern recognition receptors leads to PAMP-triggered immunity (PTI), which provides resistance to non – adapted microbes (Dodds & Rathjen 2010). PTI can be suppressed by effectors, pathogenic virulence factors deployed into the cell that promote susceptibility (Macho & Zipfel 2014). Certain pathogen effectors are recognized by intracellular nucleotide-binding/leucine ‐ rich ‐ repeat (NLR) receptors, leading to effector triggered immunity (ETI). ETI boosts PTI, salicylic acid (SA) biosynthesis and reactive oxygen species (ROS) production, which ultimately leads to transcriptional reprogramming (Dodds & Rathjen 2010; Cui, Tsuda & Parker 2015). Local pathogen recognition also triggers systemic responses that provide broad-spectrum disease resistance against secondary infection in distal (systemic) tissues. This phenomenon, known as systemic acquired resistance (SAR), is associated with local and systemic SA biosynthesis, as well as extensive transcriptional reprogramming (Fu & Dong 2013). Even though PTI and ETI are activated upon recognition of different pathogen molecules, both share common signals including ROS and SA production (Herrera-Vásquez, Salinas & Holuigue 2015). Impaired SA biosynthesis strongly compromises SAR (Wildermuth *et al.* 2001), although SA itself is not the mobile signal responsible for SAR (Vlot, Dempsey & Klessig 2009). Other plant metabolites have been reported to orchestrate the establishment of SAR through SA-dependent and independent pathways (Shine *et al.* 2019). These metabolites include *N*-hydroxypipecolic acid (NHP) (Návarová *et al.* 2012; Hartmann *et al.* 2018), azelaic acid (AzA) (Jung *et al.* 2009), glycerol-3-phosphate (Chanda *et al.* 2011), dehydroabietinal (Chaturvedi *et al.* 2012), free radicals, nitric oxide (NO) and reactive oxygen species (ROS) (Wang *et al.* 2014).

EDS1 is a non-catalytic lipase-like protein required for basal resistance, ETI and SAR. EDS1 contributes to SA accumulation as part of a feedback loop that reinforces SA signaling (Falk *et al.* 1999; Feys *et al.* 2005). ETI triggered by recognition of the *Pseudomonas syringae* effector

protein AvrRpm1 is mediated by the coiled-coil (CC)-NLR RESISTANCE TO *PSEUDOMONAS SYRINGAE* pathovar MACULICOLA1 (RPM1) receptor. Although local resistance mediated by RPM1 is independent of EDS1 (Aarts *et al.* 1998), systemic immunity and local SAR signal generation are compromised in the *eds1* mutant (Truman *et al.* 2007; Breitenbach *et al.* 2014). NPR1 acts downstream of SA as transcription co-factor that triggers defense-related transcriptional reprogramming. Upon SA accumulation, NPR1 oligomers resident in the cytosol dissociate and NPR1 monomers are translocated to the nucleus, where they interact with TGA transcription factors leading to transcriptional reprogramming. As such, *npr1* mutations severely compromise SA responses (Zhang *et al.* 1999; Després *et al.* 2000; Fan & Dong 2002; Mou, Fan & Dong 2003; Tada *et al.* 2008; Fu & Dong 2013).

In addition to SA, polyamines also accumulate during defense responses (Jiménez-Bremont *et al.* 2014; Seifi & Shelp 2019). Polyamines are low molecular weight polycationic molecules bearing amino groups. Most abundant polyamines are the diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm). The diamine cadaverine (Cad) and tetraamine thermospermine (tSpm), a Spm isomer, are also present in higher plants. These compounds can be found in free or conjugated forms to hydroxycinnamic acids (Walters 2003; Tiburcio *et al.* 2014; Tiburcio & Alcázar 2018). Polyamine concentration is regulated by tight control of its biosynthesis, conjugation, transport and oxidation. Polyamines can be oxidatively deaminated by amine oxidases that produce H_2O_2 (Cona *et al.* 2006), which might lead to ROS-dependent stress signaling (Mittler *et al.* 2011; Wang *et al.* 2019). Amine oxidases are classified in copper-containing amine oxidases (CuAO, EC 1.4.3.6) or FAD-dependent polyamine oxidases (PAO, EC 1.5.3.11). In *Arabidopsis thaliana* (*Arabidopsis*), CuAO exhibit strong preference for Put and Spd as substrates, and catalyze the oxidation of primary amino groups producing the corresponding aldehydes along with H_2O_2 and NH_4^+ (Cona *et al.* 2006; Angelini *et al.* 2010; Planas-Portell *et al.* 2013). Plant PAO are involved in terminal catabolism or back-conversion reactions, depending on the species (Cona *et al.* 2006; Angelini *et al.* 2010; Moschou *et al.* 2012). In *Arabidopsis*, PAO mediate back-conversion reactions that reverse the biosynthesis pathway by

oxidation of the carbon at the *exo*-side of the N^4-nitrogen, producing 3-aminopropanal and H_2O_2 (Ono *et al.* 2012; Moschou *et al.* 2012; Ahou *et al.* 2014). Spm oxidation through PAO activity triggers the activation of mitogen-activated protein kinases (Zhang & Klessig 1997; Seo *et al.* 2007) and contributes to disease resistance against cucumber mosaic virus (CMV) (Mitsuya *et al.* 2009; Sagor *et al.* 2012), *Pseudomonas* (Marina *et al.* 2008; Moschou *et al.* 2009; González *et al.* 2011; Marco, Busó & Carrasco 2014; Lou *et al.* 2016), *Botrytis cinerea* (Seifi *et al.* 2019) and other microbial pathogens. Overall, most defense traits related to polyamines have been attributed to Spm oxidation. Even though Put accumulation is a conserved metabolic hallmark of plant stress, the contribution of Put to defense has remained elusive (Yoda, Yamaguchi & Sano 2003; Mitsuya *et al.* 2009; Sagor *et al.* 2012; Vilas *et al.* 2018; Seifi & Shelp 2019). We recently reported that Put accumulates during PTI and amplifies PTI responses in a ROS and *RESPIRATORY BURST OXIDASE HOMOLOG* (*RBOH*) *D* and *RBOHF* - dependent manner (Liu *et al.* 2019). Consistent with the contribution of Put to basal defenses, *adc2* mutants deficient in pathogen-triggered Put accumulation are more susceptible to *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection (Kim *et al.* 2013). Furthermore, accumulation of free Put in the *N-acetyltransferase activity1* (*nata1*) mutant, which is deficient in Put N-acetylation, correlated with enhanced disease resistance to this *Pseudomonas* strain (Lou *et al.* 2016). Despite the growing body of evidence demonstrating the participation of polyamines in stress protection, polyamine signaling pathways underlying such effects are largely unknown. In an attempt to get an insight into polyamine signaling and its contribution to defense in plants, we performed a comprehensive RNA-sequencing (RNA-seq) analysis to determine transcriptional changes elicited by each of the most abundant polyamines in *Arabidopsis*, in addition to cadaverine (Cad). Even though Cad is not present in *Arabidopsis*, this polyamine is found in many *Leguminosae* and microorganisms in the phyllosphere and rhizosphere (Jancewicz, Gibbs & Masson 2016). RNA-seq analyses indicated that the different polyamines elicit stress signaling in *Arabidopsis*. By focusing on Put, which accumulates in response to *AvrRpm1*-triggered ETI in a SA-independent manner, we find that transcriptional responses to this polyamine are H_2O_2, SA, EDS1 and NPR1 - dependent, thus highlighting the importance of ROS and SA pathways for Put signaling. Consistent with a role

for Put in defense, we find that Put treatment leads to local SA biosynthesis, as well as local and systemic transcriptional reprogramming that overlaps with SAR. The contribution of Put oxidation to defense is confirmed in *adc2* and *cuao* mutants, which are compromised in basal defenses as well as Put and pathogen -triggered systemic resistance. Overall, we provide new insights into polyamine signaling and the involvement of Put oxidation in defense.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

For plants grown on soil, seeds from the different genotypes were stratified for 3 days on a wet filter paper and directly sown on a mixture containing peat moss (40%), vermiculite (50%) and perlite (10 %). Plants were grown at 20–22°C under 8 h light (8:00 AM to 4:00 PM)/16 h dark cycles at 100–125 μmol photons m^{-2} s^{-1} of light intensity and 60 % - 70 % relative humidity. For *in vitro* culture, seeds were sterilized in 30 % sodium hypochlorite supplemented with 0.5 % Triton X-100 (Sigma-Aldrich) for 10 min, followed by three washes with sterile distilled H_2O. Seeds were sown on growth media [1/2 Murashige and Skoog salts (MS) supplemented with vitamins (Duchefa Biochemie), 1% sucrose, 0.6% plant agar (Duchefa Biochemie) and 0.05 % MES adjusted to pH 5.7 with 1 M KOH]. To synchronize germination, seeds were stratified in the dark at 4 °C for 2–3 days. Plates were incubated under 8 h light/16 h dark cycles at 20 – 22 °C at 100–125 μmol photons m^{-2} s^{-1} of light intensity. The *npr1-1* (Cao *et al.* 1997) mutant was kindly provided by Prof. Xinnian Dong (Duke University, USA). The *adc1-2* (SALK_085350), *adc2-4* (SALK_147171), *atao1-3* (SALK_082394C), *cuao1-3* (SALK_019030C), *cuao2-1* (SALK_012167C) and *cuao3-1* (SALK_095214C) mutants were obtained from the Nottingham *Arabidopsis* Stock Center (NASC, UK). The *adc1-3* and *adc2-3* mutants were previously reported (Cuevas *et al.* 2008).

Polyamine chemicals and stock solutions

Putrescine, spermidine, spermine and cadaverine pure chemicals were purchased from Sigma-Aldrich. Thermospermine was kindly provided by Prof. Masaru Niitsu (Josai University, Japan). Fresh polyamine stock solutions were prepared at 100 mM concentration in 5 mM MES pH 5.7 and sterilized by filtration.

RNA-seq analyses

All polyamine treatments were performed at the same time of the day (12:00 PM) and samples collected at different time points post-treatment, as described below. Early gene expression

changes triggered by the different polyamines were determined in 12-day-old Col-0 wild-type, Col-0 *eds1-2* (Bartsch *et al.* 2006), Col-0 *sid2-1* (Wildermuth *et al.* 2001) and Col-0 *npr1-1* (Cao *et al.* 1997) seedlings. The different genotypes were grown *in vitro* on a sterile nylon mesh placed on top of the growth media. For polyamine treatments, seedlings were transferred to growth media supplemented with or without the different polyamines at 100 µM. Samples were collected at 1 h post-treatment. Analyses were performed in three biological replicates, each containing three individual seedlings from three independent plates. Local and systemic transcriptional responses to Put were performed in 5-week-old *Arabidopsis* wild-type (Col-0) plants infiltrated with 500 µM Put or mock (5 mM MES pH=5.7). Leaves were collected at 24 h post-inoculation. Treatments were performed in three biological replicates, each containing three leaves from three independent plants. Total RNA was extracted using *TriZol* (ThermoFischer) and further purified using RNeasy kit (Qiagen) according to manufacturer's instructions. Total RNA was quantified in Qubit fluorometer (ThermoFisher) and checked for purity and integrity in a *Bioanalyzer-2100* device (Agilent Technologies). RNA samples were further processed by the *Centro Nacional de Análisis Genómico* CNAG (www.cnag.crg.eu, Spain) for library preparation and RNA sequencing. Libraries were prepared using the Illumina *TruSeq Sample Preparation Kit* according to manufacturer's instructions. Each library was paired-end sequenced (2 x 75 bp) on HiSeq2000 Illumina sequencers. Read mapping and expression analyses were performed using the *CLC Genomics Workbench 12 version 12.0.3* (Qiagen). Only significant expression differences (fold-change ≥ 2; *p*-value and FDR ≤ 0.05) were considered. Gene ontology analyses were performed using *CLC Genomics Workbench 12 version 12.0.3* (Qiagen) and Gene Ontology resource (GO; http://geneontology.org) using annotations from Araport11 (Cheng *et al.* 2017; Carbon *et al.* 2019). Array similarity searches were performed using the Genevestigator Signature tool (www.genevestigator.com) in 2,799 perturbation arrays performed in *Arabidopsis* (Col-0) wild-type genotype and containing a minimum of three biological replicates (Hruz *et al.* 2008).

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (ThermoFisher). Two micrograms of RNA were treated with DNAse I (ThermoFisher) and first-strand cDNA synthesized using Superscript IV reverse transcriptase (ThermoFisher) and oligo(dT) according to manufacturer's instructions. Quantitative real-time PCR using SYBR Green I dye method was performed on Roche LightCycler 480 II detector system following the PCR conditions: 95 °C 2 min, 40 cycles (95 °C, 15 s; 60 °C, 30 s; 68 °C, 20 s). Standard curves were performed for quantification. Primer sequences used for gene expression analyses are listed in **Table S1**. qRT-PCR analyses were always performed on at least three biological replicates, each with three technical replicates.

2,7'-dichlorofluorescein staining

Twelve-day-old wild-type (Col-0) seedlings grown *in vitro* were transferred to liquid growth media and incubated 24 h before treatment with the different polyamines at 100 µM, 100 µM Put + 5 mM DMTU or mock (5 mM MES pH 5.7) for one hour. Seedlings were then stained with 50 µM 2,7'-dichlorofluorescein diacetate (Sigma Aldrich) dissolved in 20 mM phosphate buffer pH 6.1 for 30 min, and then washed three times with distilled water. Leaves from 5-week-old plants were used for infiltration with the different polyamines (500 µM), 500 µM Put + 5 mM DMTU or mock (5 mM MES pH 5.7) using a 1-mL needless syringe. Leaf staining was performed at 24 h post-infiltration. ROS fluorescence was observed under a Leica Fluorescent microscope (DMi8) (leaves) or confocal microscope (Olympus FV1000) (roots) using 488 nm excitation and 510 nm emission wavelengths. A minimum of ten independent plants were visualized for every treatment. Figure captions represent a representative sample from each treatment.

DAB and NBT staining

Seedlings and leaves from the different treatments were immersed and vacuum infiltrated with 1 mg ml^{-1} DAB staining solution (pH 3.8). Samples were then bleached by boiling in acetic acid-glycerol-ethanol (1/1/3) (v/v/v) solution for 5 min, and then immersed in glycerol-ethanol (1/4) (v/v) solution for microscope visualization. For NBT staining, samples were immersed and infiltrated under vacuum with 3.5 mg ml^{-1} NBT staining solution in 10 mM potassium phosphate

buffer (pH 7.5) containing 10 mM NaN_3. Bleaching and microscope visualization was performed as described above.

Pipecolic acid and Salicylic acid quantitation

Leaves from five-week-old *Arabidopsis* plants were inoculated with 500 µM Put or mock (5 mM MES pH 5.7). Local inoculated (1º) and distal non-inoculated (2º) leaves were collected at 24 h and 48 h post-inoculation in three to four biological replicates, each containing three leaves from three independent plants. The leaf contents of SA, Pip and NHP were analyzed by gas chromatography/mass spectrometry (GC-MS) as described by Hartmann et al. (2018). Briefly, 50 mg of frozen, pulverized leaf tissue were extracted twice with 1 ml of MeOH/H_2O (80:20, v/v). 1 µg of D_4-SA, D_9-Pip, and D_9-NHP were added as internal standards. 600 µl of the extract was evaporated to dryness and the residue was supplemented with 20 µl of pyridine, 20 µl of N-methyl-N-trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane (v/v) and 60 µl of hexane. After heating the mixture to 70 °C for 30 min, samples were cooled and diluted with 300 µl of hexane. 2 µl of the solution was separated on a Phenomenex ZB-35 (30m x 0.25mm x 0.25µm) capillary column using a GC 7890A gas chromatograph (Agilent Technologies) and the following temperature program: 70 °C for 2 min, with 10 °C / min to 320 °C, 320 °C for 5 min. A 5975C Agilent mass spectrometer in the electron ionization (EI) mode was used for compound detection. Metabolite analysis and quantification was performed with the Agilent MSD ChemStation software. Analyte peaks of selected ion chromatograms were integrated, and peak areas were related to those of internal standards: SA (m/z 267) relative to D_4-SA (m/z 271), Pip (m/z 156) relative to D_9-Pip (m/z 165), and NHP (m/z 172) relative to D_9-NHP (m/z 181).

Determination of polyamine levels

The levels of free Put, Spd and Spm were determined by high-performance liquid chromatography (HPLC) separation of dansyl chloride-derived polyamines as described (Marcé *et al.* 1995; Alcázar *et al.* 2005). Analyses were performed in three to four biological replicates per treatment, each including three technical replicates.

Polyamine levels in β-estradiol inducible *AvrRpm1* lines

The β-estradiol inducible *AvrRpm1* line (a11) and *AvrRpm1 rpm1-1* (a11r) were obtained from NASC (CS68776 and CS68777) (Tornero *et al.* 2002). Five-week-old a11, a11r and wild-type plants were infiltrated with 10 μM β-estradiol or water (mock) using a 1-mL needless syringe. Local inoculated (1º) and distal non-inoculated (2º) leaves were harvested at 24 h post-infiltration for polyamine quantitation. Analyses were performed in three to four biological replicates per treatment, each including three technical replicates.

Pathogen inoculation assays

Three local leaves from at least eight independent *Arabidopsis* plants per genotype were inoculated with 100 μL of a *Pseudomonas syringae* pv *tomato* DC3000 *AvrRpm1* (*Pst AvrRpm1*) suspension at OD_{600} = 0.001 in 10 mM $MgCl_2$, 500 μM Put or mock (10 mM $MgCl_2$) using a 1-mL needless syringe. Two days post-inoculation, systemic non-inoculated leaves (2º) were infiltrated with *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) at OD_{600nm} = 0.0005 in 10 mM $MgCl_2$. *Pst* DC3000 colony forming units (cfu) per cm^2 were determined at 72 h post-inoculation as described (Alcázar *et al.* 2010b) using eight biological replicates per treatment and genotype.

Accession numbers

RNA-seq data have been deposited in ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-9267.

RESULTS

Transcriptional responses to different polyamines

In order to gain an insight into polyamine signaling, we determined early gene expression changes triggered by different polyamines in 12-day-old *Arabidopsis* wild-type seedlings treated with 100 μM putrescine (Put), 100 μM cadaverine (Cad), 100 μM spermidine (Spd), 100 μM spermine (Spm), 100 μM thermospermine (tSpm) or mock (5 mM MES pH 5.7) for 1 h. RNA-seq analyses identified 382 genes that exhibited significant expression differences in response to one or more polyamines (expression fold change ≥ 2, Bonferroni- corrected *p*-value < 0.05) (**Figure 1 and Figure S1; Tables S2.1 to S2.7)**. Put treatment led to the largest number of differentially expressed genes (227 genes; **Table S2.1**), followed by Spd (209 genes, **Table S2.2**), Cad (179 genes, **Table S2.5**), tSpm (156 genes, **Table S2.4**) and Spm (87 genes, **Table S2.3**) treatments. Many genes were responsive to various polyamines, although specific quantitative differences were evident (**Table S2.6**). Gene ontology (GO) analyses of polyamine responsive genes evidenced the enrichment in biological processes related with the stress response, defense, elicitation and hypoxia (**Figure S2** and **Table S3**). A survey for similar gene expression patterns in 2,799 publicly available arrays identified perturbations related to basal defenses, flg22 treatment, hypoxia and iron deficiency (**Figure S3**). Closer inspection of molecular functions and pathway analyses identified 86 genes coding for proteins with different catalytic activities, including an overrepresentation of pectin modifying and flavonoid biosynthesis enzymes, peroxidases and glutathione *S*-transferases **(Table S2.6)**. Other 30 genes encoded transcription factors, eleven of which belonged to the WRKY family. This was followed by 20 protein kinases, 16 defense-related genes, 16 transporters of different nature, 16 Cysteine/Histidine-rich C1 domain family proteins, and several genes related to ABA, auxin and ethylene signaling or metabolism, among other categories **(Table S2.6)**. Overall, these analyses indicated that polyamines elicit stress signaling, including biotic responses. The principal component analysis (PCA) (**Figure 2A**) and hierarchical clustering analysis (HCA) (**Figure 2B**) of RNA-seq data evidenced the absence of correlation between transcriptional responses and polyamine charge. Indeed, the two HCA sample clades grouped polyamines with different number of amino groups.

Clade I included the diamine Put and tetraamine Spm, whereas clade II contained the diamine Cad, triamine Spd and tetraamine tSpm (**Figure 2B**). We concluded that the different polyamines elicit stress signaling and exhibit quantitative rather than qualitative differences, which are not correlated with charge.

Put signaling is ROS dependent

The overlapping transcriptional responses triggered by the different polyamines (**Figures 1 and 2B; Table S2.6**) suggested their convergence into a common signal. Hydrogen peroxide is a common by-product of polyamine oxidation (Cona *et al.* 2006; Angelini *et al.* 2010; Wang *et al.* 2019). Staining with the 2',7'-dichlorofluorescein diacetate (DCFDA) dye identified sites of ROS production in leaves and roots treated with the different polyamines **(Figure S4A)**. Staining with 3-3'-diaminobenzidine (DAB) also exhibited dark brown precipitates in polyamine-treated roots and leaves, consistent with the production of H_2O_2 **(Figure S4B)**. Conversely, no evident differences were observed between mock and polyamine treatments in roots or leaves stained with nitroblue tetrazolium (NBT) **(Figure S4C)**. These histochemical analyses suggested that polyamines trigger the production of H_2O_2. To further investigate the importance of H_2O_2 in the transcriptional response elicited by polyamines, we focused on Put, which accumulation is a metabolic hallmark of stress (Alcázar *et al.* 2010a). We interrogated the dependence of transcriptional changes triggered by Put on H_2O_2 production, by using the H_2O_2 scavenger dimethylthiourea (DMTU). Importantly, DMTU treatment inhibited Put-triggered ROS staining in roots and leaves **(Figure S4A and S4B)**. Gene expression analyses indicated that, out of the 227 genes responsive to Put (**Table S2.1**), the deregulation of 205 genes (90.3 %) was significantly inhibited (≥ 2-fold) in the presence of 5 mM DMTU (**Table S4**). We concluded that most transcriptional responses to Put are H_2O_2 dependent, thus pointing to an important contribution of ROS to Put signaling.

Involvement of SA pathway in putrescine signaling

GO and array similarity analyses of polyamine responsive genes highlighted many categories

related to defense (**Figure S2 and Figure S3, Table S3**). EDS1, SA and NPR1 are important regulators of defense also required to mount systemic responses to protect against secondary infection (Fu & Dong 2013). In order to study their contribution to Put signaling, we determined the expression changes induced by 100 μM Put in *eds1-2*, *sid2-1* and *npr1-1* mutants. Out of the 227 Put responsive genes (**Table S2.1**), the Put-triggered deregulation of 114 (50 %), 87 (38.3 %) and 59 (26 %) genes was compromised (≥ 2-fold) in *npr1-1, sid2-1* and *eds1-2* mutants, respectively. Only 37.8 % of Put responsive genes were independent of NPR1, SID2 or EDS1. The majority of EDS1 (66 %) and SA (87 %) gene expression sectors were also NPR1 dependent (**Figure 3 and Table S5**). Overall, we concluded that most transcriptional responses to Put require a functional EDS1/SA/NPR1 pathway.

Putrescine elicits local SA accumulation

The dependence of Put responsiveness on SA pathway led us to investigate a potential cross-modulation between these metabolites. For this, we infiltrated wild-type leaves with 500 μM Put or mock and determined free SA levels in local inoculated (1º) and systemic non-inoculated (2º) leaves at 24 h and 48 h post-treatment. Free SA levels were five-fold higher in 1º leaves infiltrated with Put than mock. Conversely, no differences in SA were detected between Put and mock treatments in 2º leaves **(Figure 4)**. The levels of *N*-hydroxipipecolic acid (NHP), another important SAR regulator, as well as its biosynthetic precursor pipecolic acid (Pip), were not affected by Put treatment in either tissue **(Figure 4)**. We concluded that Put elicits local SA accumulation, which supports the dependence of transcriptional responses to Put on SA-dependent pathways (**Figure 3**). The reciprocal modulation of polyamine metabolism by SA was also studied by inoculating wild-type leaves with 500 μM SA, 500 μM benzothiadiazole S-methyl ester (BTH) or mock (water), followed by the determination of polyamine levels. SA or BTH did not induce significant changes in polyamine content compared to mock (**Figure S5**). We concluded that Put elicits SA accumulation, but SA has no obvious influence on polyamine levels.

Polyamine levels in response to *Pst AvrRpm1*

To study whether ETI also associated with changes in polyamine levels, we inoculated wild-type leaves with the *Pseudomonas syringae* pathovar *tomato* (*Pst*) DC3000 carrying *AvrRpm1* (*Pst AvrRpm1*) or mock ($MgCl_2$) and determined Put, Spd and Spm concentration at 24 h post-inoculation (**Figure 5**). Because *Pst AvrRpm1* is a potent inducer of SAR, these analyses were performed in both local (1º) and systemic (2º) leaves. Primary leaves inoculated with *Pst AvrRpm1* accumulated three-fold more Put than leaves treated with mock, whereas Spd and Spm contents were unaffected. Local Put accumulation was also evident in *eds1-2, sid2-1* and *npr1-1* mutants inoculated with *Pst AvrRpm1*. Although AvrRpm1 recognition operates independently of EDS1, Put accumulation in *sid2-1* and *npr1-1* pointed to a SA and NPR1 independent response **(Figure 5A)**. In contrast to 1º leaves, polyamine levels in 2º leaves were not influenced by *Pst AvrRpm1* inoculation **(Figure 5B)**, indicating that Put accumulates in local but not systemic tissues. Local Put increases were also evident in 1º leaves of β-estradiol infiltrated *a11* transgenic plants expressing β-estradiol – inducible *AvrRpm1*, but not in 2º leaves from the same plants. Conversely, Put accumulation was attenuated in *a11 rpm1-1* (*a11r*) **(Figure S6)** (Tornero et al., 2002). These results indicated that AvrRpm1 recognition is sufficient to induce Put accumulation. These pathogen-free assays also support a major contribution of plant polyamine metabolism to local changes in Put levels during ETI.

Systemic transcriptional responses triggered by Put

Having found that Put accumulates in local leaves during ETI (**Figure 5 and S6**) and triggers local SA accumulation (**Figure 4**), we investigated whether Put responses were transmitted to systemic tissues. For this, we determined transcriptional changes triggered by 500 μM Put or mock in local (1º) and systemic (2º) wild-type leaves at 24 h post-treatment. A total of 185 and 216 genes were deregulated by Put in 1º and 2º leaves, respectively (**Figure 6**; **Tables S6.1 to S6.3**). GO analyses of these genes identified a strong overrepresentation of terms related with biotic stimulus, defense, SAR, response to SA and hypoxia in local and systemic tissues (**Tables S6.4 and S6.5**). Interestingly, 54.8 % of the 115 genes up-regulated by Put in 1º leaves and 72.3 % of the 177 genes up-regulated by Put in 2º leaves overlapped with SAR-responsive genes

(**Figure 6**) (Hartmann *et al.* 2018). However, these only represented a small fraction (4.5 %) of the full SAR transcriptional response (Hartmann *et al.* 2018). Quantitative RT-PCR analyses confirmed the transcriptional up-regulation of the SA marker gene *PATHOGENESIS RELATED1* (*PR1*) but also the SA biosynthesis gene *ISOCHORISMATE SYNTHASE 1* (*ICS1*) in 1º but not 2º leaves, as well as the up-regulation of SAR-related genes *FLAVIN-DEPENDENT MONOOXYGENASE 1* (*FMO1)* and *L-LYSINE ALPHA-AMINOTRANSFERASE ALD1* (*AGD2-LIKE DEFENSE RESPONSE PROTEIN 1*) in both local and systemic tissues (**Figure S7**). The data are consistent with a systemic response elicited by Put infiltration, which partly overlaps with SAR. However, transcriptional changes induced by Put in the absence of pathogen attack seem insufficient to trigger SA accumulation in 2º leaves **(Figure 4)**, or the full establishment of SAR responses **(Figure 6)**. This might prevent the cost of establishing systemic defenses due to transient fluctuations in Put levels not related to biotic stress.

Contribution of Put to the establishment of SAR in SA-pathway and Put biosynthesis mutants

To investigate the contribution of Put to the establishment of SAR, 1º leaves of the wild-type were pre-inoculated with 500 µM Put, *Pst AvrRpm1* or mock. After 48 h, 2º leaves were inoculated with *Pst* DC3000 and bacterial titers determined at 72 h post-inoculation (**Figure 7A**). Pre-treatment with Put led to lower *Pst* DC3000 growth in 2º leaves, similarly to *Pst AvrRpm1* pre-inoculation (**Figure 7A**). Put –elicited resistance was not evidenced in *eds1-2*, *sid2-1* or *npr1-1* mutants, which is consistent with the requirement of functional SA-dependent pathways for Put responses (**Figure 7A**). Furthermore, SAR elicited by *Pst AvrRpm1* was compromised in *adc2-3* and *adc2-4* mutants, but not in *adc1-2* or *adc1-3* (**Figure 7B**). The data suggested that Put contributes to the establishment of SAR.

Analysis of the SAR response in *copper amine oxidase* (*cuao*) mutants

Put can be converted into 4-aminobutanal, H_2O_2 and ammonia through an enzymatic reaction catalyzed by copper-containing amine oxidases (CuAOs). Among the characterized CuAO

enzymes in *Arabidopsis*, ARABIDOPSIS THALIANA AMINE OXIDASE1 (ATAO1), CuAO1, CuAO2 and CuAO3 exhibit high affinity for Put and Spd, and localize to the apoplast (Moller & McPherson 1998; Planas-Portell *et al.* 2013). By using *atao1-3*, *cuao1-3*, *cuao2-1* and *cuao3-1* loss-of-function mutants, we tested the contribution of CuAO to Put and *Pst AvrRpm1* – elicited systemic resistance. The results indicated that basal defenses and SAR were strongly compromised in *cuao1-3*. In addition, no significant differences in bacteria growth were detected between mock and Put or *Pst AvrRpm1* – elicited in *cuao2-1* and *cuao3-1* mutants (**Figure 8**). The data suggested that different CuAO family members contribute additively to the establishment of SAR, which highlights the importance of ROS generation for polyamine-triggered defense responses.

DISCUSSION

In order to investigate polyamine signaling, here we determined early transcriptional responses to most abundant polyamines (Put, Spd, Spm, tSpm and Cad) in *Arabidopsis*. Our data indicate that polyamines are active participants in stress signaling. The different polyamines elicited similar transcriptional responses but exhibited specific quantitative differences which were not correlated with charge (**Figure 2B and Table S2.6**). Remarkably, Cad also triggered a transcriptional response in *Arabidopsis*, although this polyamine is absent in this species (**Table S1.5**). This might be relevant in the context of plant-microbe interactions, as plants could take up Cad released from microorganisms in the rhizosphere or phyllosphere (Jancewicz, Gibbs & Masson. 2006). However, this possibility needs further investigation. By focusing on Put, which accumulates in response to a large variety of stresses (Alcázar *et al.* 2010a), we find that ROS production is necessary for Put-triggered transcriptional reprogramming **(Table S4)**. ROS play important signaling roles in plant growth, development and stress responses. Important sources of ROS are enzymatic activities from peroxisomal glycolate involved in photorespiration, acyl-CoA oxidases required for fatty acid β-oxidation, cell wall peroxidases, plasma membrane NADPH oxidases, copper-containing amine oxidases and FAD-dependent polyamine oxidases which produce H_2O_2 in the apoplast and peroxisomes (Cona *et al.* 2006; Del Río 2015). ROS generated through these and other enzymatic reactions in chloroplasts, mitochondria, peroxisomes and the apoplast are important chemical signals that contribute to PTI, ETI and SAR (Torres, Dangl & Jones 2002; Bindschedler *et al.* 2006; Rojas *et al.* 2012; Daudi *et al.* 2012; Macho *et al.* 2012; Wang *et al.* 2014; Mammarella *et al.* 2015).

In *Arabidopsis*, the biosynthesis of Put is stimulated during PTI (Liu *et al.* 2019) and ETI **(Figures 5 and S6)**. Activation of PTI or ETI is sufficient for the establishment of SAR, which involves extensive transcriptional reprogramming (Mishina & Zeier 2007; Zhang & Li 2019; Liu *et al.* 2019). The *catalase2* (*cat2*) mutant, which is impaired in peroxisomal H_2O_2 metabolism, exhibits high SA and Put contents in addition to cell death and activation of defenses (Chaouch *et al.* 2010). Put and SA have been suggested to operate in the same defense/cell death metabolic

response triggered by ROS (Chaouch *et al.* 2010). Here we find that Put elicits EDS1/SA/NPR1 dependent transcriptional reprogramming in *Arabidopsis* **(Figure 3)**, which leads to local SA increases **(Figure 4)** in the absence of programmed cell death (Liu *et al.* 2019). The *npr1-1* mutation suppressed the deregulation of a greater number of Put responsive genes (114 out of 227) than *sid2-1* (87 genes) or *eds1-2* (59 genes) mutations. Even though not all transcriptional responses to Put are SA dependent, 62.2 % of early Put-responsive genes require a functional EDS1/SA/NPR1 pathway. Interestingly, NPR1 localization is influenced by redox changes triggered by pathogen infection and SA accumulation, as well as S-nitrosylation (Tada et al., 2008). Remarkably, polyamines are sources of both ROS and NO (Cona *et al.* 2006; Wimalasekera, Tebartz & Scherer 2011). The involvement of EDS1 in Put signaling might be due to its contribution to SA accumulation as part of a feedback loop that boosts SA signaling (Falk *et al.* 1999; Feys *et al.* 2005). Consistent with this, EDS1, SA and NPR1-dependent sectors exhibit high overlap (**Figure 3**).

Stimulation of local SA biosynthesis **(Figure 4)** might underlie the enhanced basal disease resistance of wild-type plants inoculated with Put (Liu *et al.* 2019) and/or the enhanced disease susceptibility of *adc2*, which is compromised in Put accumulation in response to *Pst* DC3000 bacteria (Kim *et al.* 2013). Even though Put elicited local but not distal SA accumulation (**Figure 4**), local Put treatment triggered systemic transcriptional reprogramming that overlapped with SAR (**Figure 6**). Remarkably, local or systemic NHP and Pip levels were unaffected by Put inoculation (**Figure 4**), which indicates that Put potentiates the SA-branch of SAR. In agreement with this, pre-treatment with Put increased the resistance to *Pst* DC3000 in systemic tissues in an EDS1, SA and NPR1 dependent manner (**Figure 7A**). In addition, SAR was compromised in *adc2* but not *adc1* mutants, thus highlighting the specific contribution of *ADC2* to systemic resistance (**Figure 7B**). *ADC1* has been shown to be required for Put accumulation in response to *Pseudomonas viridiflava* in *Arabidopsis*, although resistance was not conditioned by *ADC1* loss-of-function (Rossi, Marina & Pieckenstain, 2014). Therefore, *ADC1* may contribute to Put biosynthesis in response to certain pathogens in *Arabidopsis*, thus highlighting its specificity. The

involvement of ADC1 in the synthesis of N-acetylputrescine (Lou *et al*., 2019) may underlie such specificity.

The dependence of Put signaling on H_2O_2 production (**Table S4**) prompted us to investigate the potential participation of CuAO in Put- triggered defense responses. CuAO1 catalyzes the oxidative deamination of Put and Spd in the apoplast and its expression is strongly up-regulated in response to SA (Planas-Portell *et al.* 2013). Interestingly, the *cuao1-3* mutant was compromised in basal defenses to *Pst* DC3000 and the establishment of SAR triggered by Put and *Pst AvrRpm1* (**Figure 8**). Other CuAO family members (*CuAO2* and *CuAO3*) contributed additively to Put-elicited systemic responses (**Figure 8**). Based on these results, we argue that Put oxidation in the apoplast might be an important trigger of defense signaling. Polyamines are known to accumulate in the apoplast in response to pathogens (Yamakawa *et al.* 1998; Marina *et al.* 2008; Moschou *et al.* 2009; Liu *et al.* 2019). However, it is still a matter of debate the contribution of plant and pathogens to the total polyamine levels found in plant tissues. Here we show that inducible *AvrRpm1* expression in *Arabidopsis* is sufficient to induce Put accumulation, and this response is significantly mitigated in *rpm1-1* (**Figure S6**). We conclude that local Put accumulation triggered by *Pst AvrRpm1* inoculation is mainly of plant origin.

Earlier works already reported that high H_2O_2 stimulates the biosynthesis of SA (León, Lawton & Raskin 1995; Durner, Shah & Klessig 1997). Indeed, apoplastic ROS production is a hallmark of successful pathogen recognition and activation of defense responses (Torres 2010). Perception of PAMPs triggers the activation of NADPH oxidases and peroxidases leading to apoplastic H_2O_2 generation and defense signaling (Nühse *et al*. 2007; Zhang *et al*. 2007; O'Brien *et al.* 2012; Mammarella *et al.* 2015). In addition, apoplastic H_2O_2 bursts contribute to cell wall fortification and callose deposition at infection sites (Ellinger & Voigt 2014). SA also influences ROS levels through inhibition of the catalase activity (Chen, Silva & Klessig 1993) and the H_2O_2 scavenging activity of cytosolic ascorbate peroxidase (APX) (Vlot, Dempsey & Klessig 2009). Conversely, high SA stimulates reduced glutathione biosynthesis at long term, thus contributing to an antioxidative effect. Therefore, H_2O_2 and SA exhibit an intricate relationship reflected by a

514 biphasic (first oxidative and second reductive) redox dynamics (Herrera-Vásquez, Salinas &
515 Holuigue 2015).
516
517 In the context of plant-pathogen co-evolution, the polyamine pathway might be a good target for
518 pathogen effectors or small molecules which are delivered to manipulate host defenses. The
519 observed restriction in *Pseudomonas* growth triggered by Put is consistent with the activity of the
520 TALE-like Bgr11 effector from the plant pathogen *Ralstonia solanacearum* that boost Put
521 biosynthesis to inhibit the growth of microbial niche competitors (Wu *et al.* 2019). In addition,
522 the *P. syringae* virulence factor phevamine A, which is derived from Spd, suppresses the flg22-
523 induced ROS potentiation of defense responses triggered by Spd in *Arabidopsis* (O'Neill *et al.*
524 2018). Overall, we provide new insights into polyamine signaling and defense with a focus on
525 Put. Future research on this topic might help at the rational establishment of plant breeding and/or
526 engineering strategies for plant protection against disease.
527
528 **CONFLICT OF INTEREST STATEMENT**
529 The authors have no conflicts of interest to declare.

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765

766 **FIGURE LEGENDS**

767

768 **Figure 1. Venn diagram of polyamine responsive genes.** Venn diagram of unique and shared
769 genes responsive to 100 µM putrescine (Put), 100 µM cadaverine (Cad), 100 µM spermidine
770 (Spd), 100 µM spermine (Spm) and 100 µM thermospermine (tSpm) after 1 h of treatment **(**see
771 **Tables S2.1 to S2.6)**. RNA-seq analyses were performed in 12-day-old *Arabidopsis* wild-type
772 (Col-0) seedlings.

773

774 **Figure 2. (A) Principal component analysis (PCA) and (B) hierarchical clustering analysis**
775 **(HCA) of transcriptional responses to different polyamines.** PCA and HCA of RNA-seq data
776 from the treatments with 100 µM putrescine (Put), 100 µM cadaverine (Cad), 100 µM spermidine
777 (Spd), 100 µM spermine (Spm) and 100 µM thermospermine (tSpm). Ellipses in the PCA indicate
778 the 95% confidence interval. The chemical structure of the different polyamines is shown at the
779 bottom of the HCA clade.

780

781 **Figure 3. Dependence of Put-triggered transcriptional responses on EDS1, SA and NPR1.**
782 Distribution of Put responsive genes in EDS1, SA and NPR1- dependent gene expression sectors.
783 The data was obtained from RNA-seq analyses in *eds1-2*, *sid2-1* and *npr1-1* mutants treated with
784 100 µM Put or mock for 1 h. Expression differences are relative to mock in each genotype (see
785 **Table S5**).

786

787 **Figure 4. Quantitation of SAR-related metabolites salicylic acid (SA) and Pipecolic acid**
788 **(Pip) in Put-inoculated leaves.** Free SA and Pip levels in primary inoculated (1º) and systemic
789 non-inoculated (2º) leaves of 5-week-old *Arabidopsis* wild-type plants at 24 h and 48 h post-
790 treatment with 500 µM Put or mock (5 mM MES pH 5.7). Values are the mean of at least three
791 biological replicates ± standard deviation (SD). Letters indicate values that are significantly
792 different according to Tukey's HSD test at $P < 0.05$. The levels of the SAR-active Pip derivative

793 *N*-hydroxypipecolic acid (NHP) were below the limit of detection for all the samples under
794 investigation.
795
796 **Figure 5. Polyamine levels in response to *Pst AvrRpm1*.** Levels of putrescine (Put), spermidine
797 (Spd) and spermine (Spm) in local and systemic leaves of five-week-old *Arabidopsis* wild-type,
798 *npr1-1*, *sid2-1* and *eds1-2* plants at 24 h post-inoculation with ETI and SAR-inducing *Pst*
799 *AvrRpm1* bacteria (*AvrRpm1*) at $OD_{600\ nm}$= 0.001 or mock (10 mM $MgCl_2$). Values are the mean
800 from at least four biological replicates ± SD. Letters indicate values that are significantly different
801 according to Tukey's HSD test at $P < 0.05$.
802
803 **Figure 6. Local and systemic transcriptional responses to Put.** Five-week-old *Arabidopsis*
804 wild-type (Col-0) plants were infiltrated with 500 µM Put or mock (5 mM MES pH 5.7). Local
805 inoculated and systemic non-inoculated leaves were harvested at 24 h post-treatment for global
806 gene expression analyses by RNA-seq. Venn diagram represents the distribution of up- and
807 down-regulated genes in local (**Table S6.1**) and systemic (**Table S6.2**) leaves that exhibit
808 significant expression differences in response to Put inoculation compared to mock, and its
809 comparison with previously annotated SAR genes.
810
811 **Figure 7. Analysis of Put and *Pst AvrRpm1*-triggered systemic resistance in SA pathway and**
812 **Put deficient mutants. (A)** Five-week-old *Arabidopsis* wild-type, *eds1-2*, *sid2-1*, *npr1-1* and
813 **(B)** *adc1-2*, *adc1-3*, *adc2-3* and *adc2-4* plants were pre-treated with 500 µM Put (Put), *Pst*
814 *AvrRpm1* ($OD_{600\ nm}$= 0.001) or mock (10 mM $MgCl_2$). Two days later, systemic leaves were
815 inoculated with *Pst* DC3000 (OD_{600nm} = 0.0005). Bacterial numbers were assessed at 72 h post-
816 inoculation and expressed as colony forming units (cfu) per cm^2 leaf area. Values are the mean
817 from at least eight biological replicates ± SD. Letters indicate values that are significantly
818 different according to Tukey's HSD test at $P < 0.05$.
819

Figure 8. Analysis of Put and *Pst AvrRpm1*-triggered systemic resistance in *copper amine oxidase* mutants. Five-week-old *Arabidopsis* wild-type, *atao1-3*, *cuao1-3*, *cuao2-1* and *cuao3-1* mutants were pre-treated with 500 µM Put (Put), *Pst AvrRpm1* ($OD_{600\ nm}$= 0.001) or mock (10 mM $MgCl_2$). Two days later, systemic leaves were inoculated with *Pst* DC3000 (OD_{600nm} = 0.0005). Bacterial numbers were assessed at 72 h post-inoculation and expressed as colony forming units (cfu) per cm^2 leaf area. Values are the mean from at least eight biological replicates ± SD. Letters indicate values that are significantly different according to Tukey's HSD test at $P < 0.05$.

SUPPORTING INFORMATION

SUPPLEMENTAL FIGURES

Figure S1. Quantitative RT-PCR expression analyses of *ERF019, EXPA17, PER5* and *F6'H1* genes for RNA-seq validation. Analyses were performed in 12-day-old wild-type (Col-0) *Arabidopsis* seedlings treated with 100 µM Cad, 100 µM Put, 100 µM Spd, 100 µM Spm, 100 µM tSpm or mock for 1 h. Values are the mean from three independent biological replicates ± SD. Letters indicate values that are significantly different according to Tukey's HSD test at $P < 0.05$.

Figure S2. Gene ontology (GO) analyses of polyamine responsive genes. For each GO category, the number of differentially expressed genes (DE Genes) is shown. Green spots indicate significant GO associations (FDR<0.05).

Figure S3. Comparison analysis of the polyamine transcriptome with other perturbation conditions. Gene expression data from the different polyamine treatments was used to identify similar expression signatures in 2,799 publicly available perturbation assays (Genevestigator) obtained from wild-type genotypes in a minimum of three biological replicates. Log similarity values are relative to the average similarity.

Figure S4. ROS detection in seedlings treated with exogenous polyamines. ROS was visualized by staining with **(A)** 2',7'-dichlorofluorescin diacetate (DCFDA), **(B)** 3,3'-diaminobenzidine (DAB) and **(C)** Nitroblue Tetrazolium (NBT) in 12-day-old *Arabidopsis* wild-type (Col-0) seedlings treated with 100 µM of the different polyamines, 100 µM Put + 5 mM DMTU and mock (10 mM MES pH 7.0) for 1 h, or 5-week-old *Arabidopsis* wild-type (Col-0) leaves infiltrated with the different polyamines at 500 µM, 500 µM Put + 5 mM DMTU and mock (10 mM MES pH 7.0) for 24 h.

Figure S5. Polyamine levels in response to SA and BTH. Levels of putrescine (Put), spermidine (Spd) and spermine (Spm) in wild-type plants treated with 500 µM benzothiadiazole S-methyl ester (BTH), 500 µM SA or mock (water). Values are the mean from at least four biological replicates ± SD. Letters indicate values that are significantly different according to Tukey's HSD test at $P < 0.05$.

Figure S6. Polyamine levels in local and systemic leaves at 24 h post-inoculation with 10 µM β -estradiol or mock in wild-type, β -estradiol inducible *AvrRpm1* (a11) and β -estradiol inducible *AvrRpm1 rpm1-1* (a11r). Values are the mean from at least four biological replicates ± SD. Letters indicate values that are significantly different according to Tukey's HSD test at $P < 0.05$.

Figure S7. Quantitative RT-PCR expression analyses of *ICS1*, *PR1*, *FMO1* and *ALD1* genes. Analyses were performed at 24 hours post-inoculation in local and systemic leaves of wild-type plants treated with 500 µM Put or mock (5 mM MES pH 5.7). Values are the mean from three independent biological replicates ± SD. For each gene, letters indicate values that are significantly different according to Tukey's HSD test at $P < 0.05$.

875

876 **SUPPLEMENTAL TABLES**

877 **Table S1.** List of oligonucleotides and their sequences used in this work.

878

879 **Table S2.1**. List of 227 genes that exhibit significant expression differences in response to 100
880 μM putrescine (Put) after 1 h of treatment. Expression differences are relative to mock treatment.

881

882 **Table S2.2.** List of 209 genes that exhibit significant expression differences in response to 100
883 μM spermidine (Spd) after 1 h of treatment. Expression differences are relative to mock treatment.

884

885 **Table S2.3.** List of 87 genes that exhibit significant expression differences in response to 100 μM
886 spermine (Spm) after 1 h of treatment. Expression differences are relative to mock treatment.

887

888 **Table S2.4.** List of 156 genes that exhibit significant expression differences in response to 100
889 μM thermospermine (tSpm) after 1 h of treatment. Expression differences are relative to mock
890 treatment.

891

892 **Table S2.5.** List of 179 genes that exhibit significant expression differences in response to 100
893 μM cadaverine (Cad) after 1 h of treatment. Expression differences are relative to mock treatment.

894

895 **Table S2.6.** List of 382 genes that exhibit significant expression differences in response to one or
896 more polyamines (100 μM) after 1 h of treatment. Genes are sorted according to their molecular
897 function or pathway. Log2 expression differences are relative to mock treatment.

898

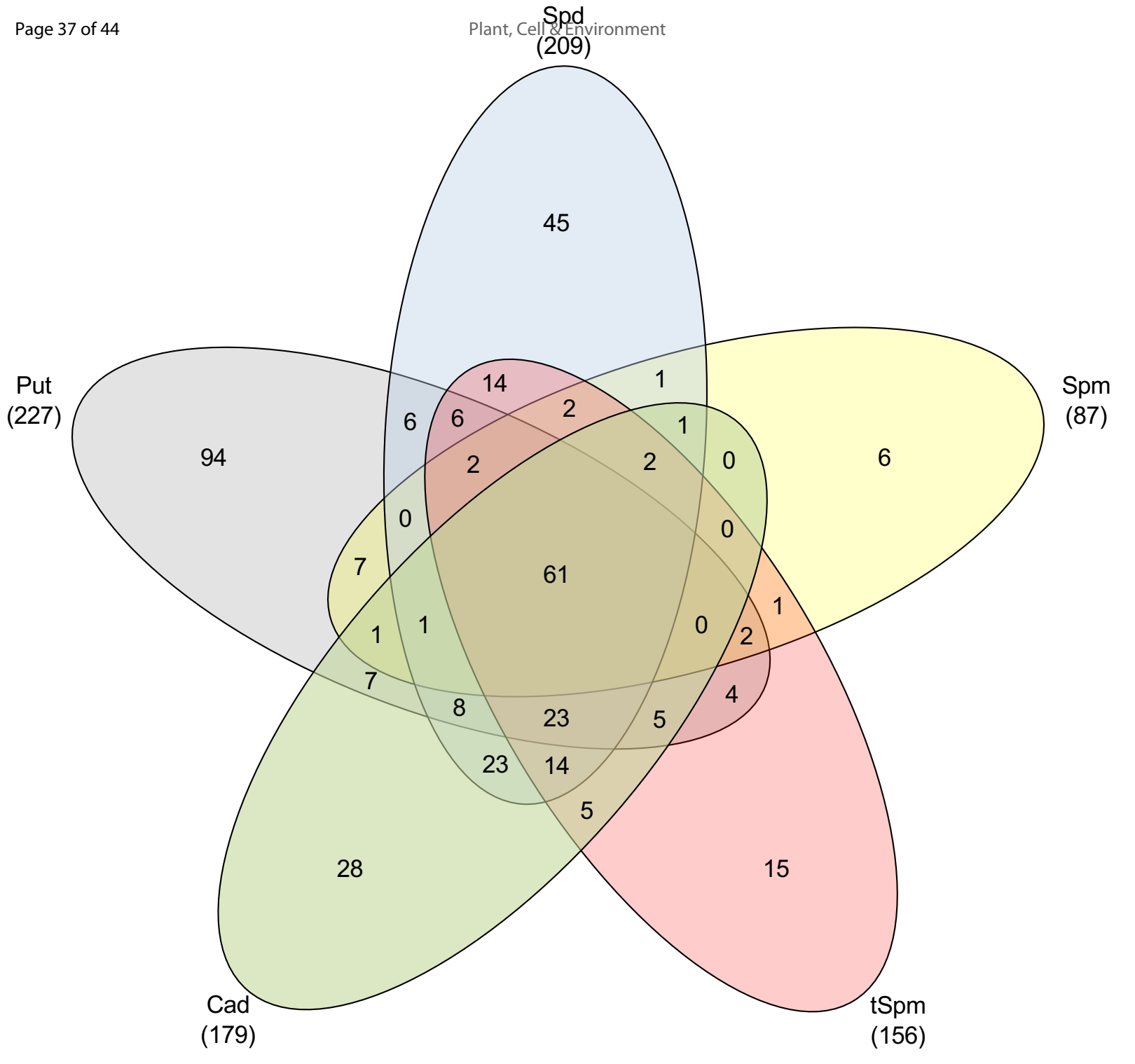
899 **Table S2.7.** Full list of genes and their expression changes triggered by 100 μM of putrescine,
900 spermidine, spermine, thermospermine, cadaverine and 100 μM Put + 5 mM DMTU after 1 h of
901 treatment. Expression differences are relative to mock or DMTU treatment, as indicated.

902

903 **Table S3.** Gene ontology (GO) analysis of polyamine responsive genes. Enrichment of GO terms
904 in the set of differentially expressed (DE) genes in the treatments with 100 μM Put, 100 μM Spd,
905 100 μM Spm, 100 μM tSpm or 100 μM Cad (related to **Tables S2.1 to S2.7**).
906
907 **Table S4.** Dependence of Put responsiveness on H_2O_2 production. Gene expression changes of
908 Put responsive genes in the presence of 5 mM DMTU + 100 μM Put after 1 h of treatment.
909 Expression changes are relative to treatment with 5 mM DMTU.
910
911 **Table S5.** Expression of Put responsive genes after 1 h of treatment with 100 μM Put in *eds1-2*,
912 *sid2-1* and *npr1-1* mutants. Expression differences are relative to mock treatment.
913
914 **Tables S6.1 to S6.5** List of genes significantly deregulated after 24 h of 500 μM Put infiltration
915 in local (**Table S6.1**) and systemic (**Table S6.2**) leaves of 5-week-old wild-type *Arabidopsis*. Full
916 list of genes is shown in **Table S6.3**. Expression differences are relative to mock treatment. Gene
917 ontology analyses of Put responsive genes in local (**Table S6.4**) and systemic (**Table S6.5**)
918 tissues.

Summary statement

Polyamines trigger stress signaling that involves defense sectors. Putrescine, which accumulates during ETI, contributes to defense through activation of a ROS, EDS1, SA and NPR1 - dependent pathway which leads to local SA accumulation and the establishment of systemic responses overlapping with SAR.


Spd
(209)
Put
(227)
Spm
(87)
Cad
(179)
tSpm
(156)
45
94
6
28
15
14
1
6
6
2
1
2
2
0
0
0
7
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1
1
1
0
2
7
4
8
23
5
23
14
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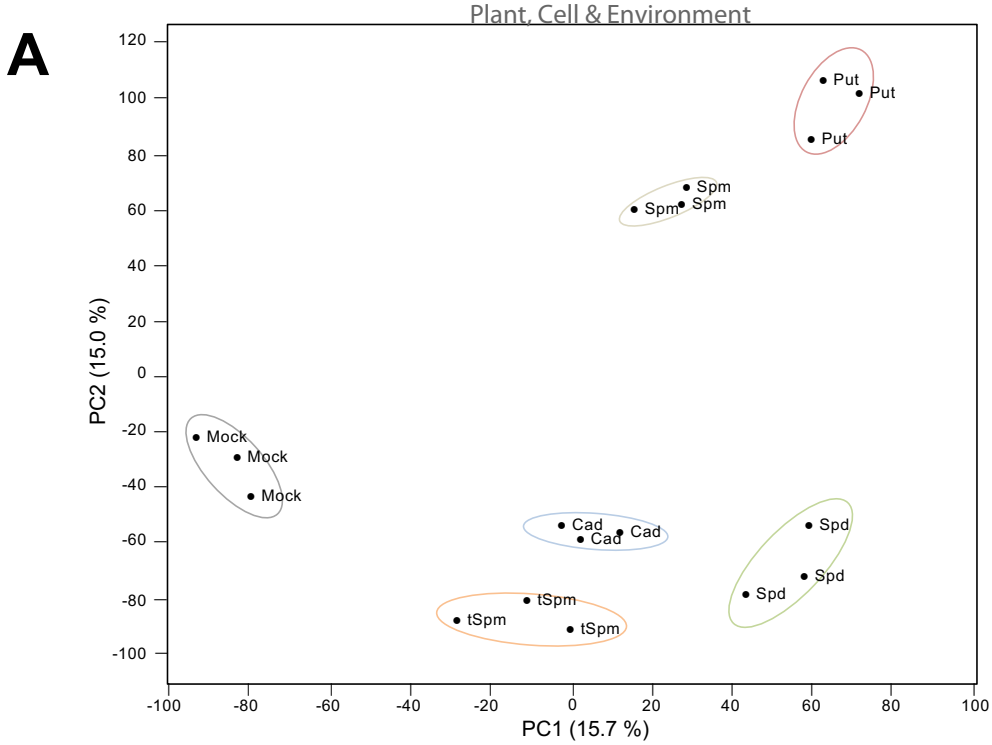
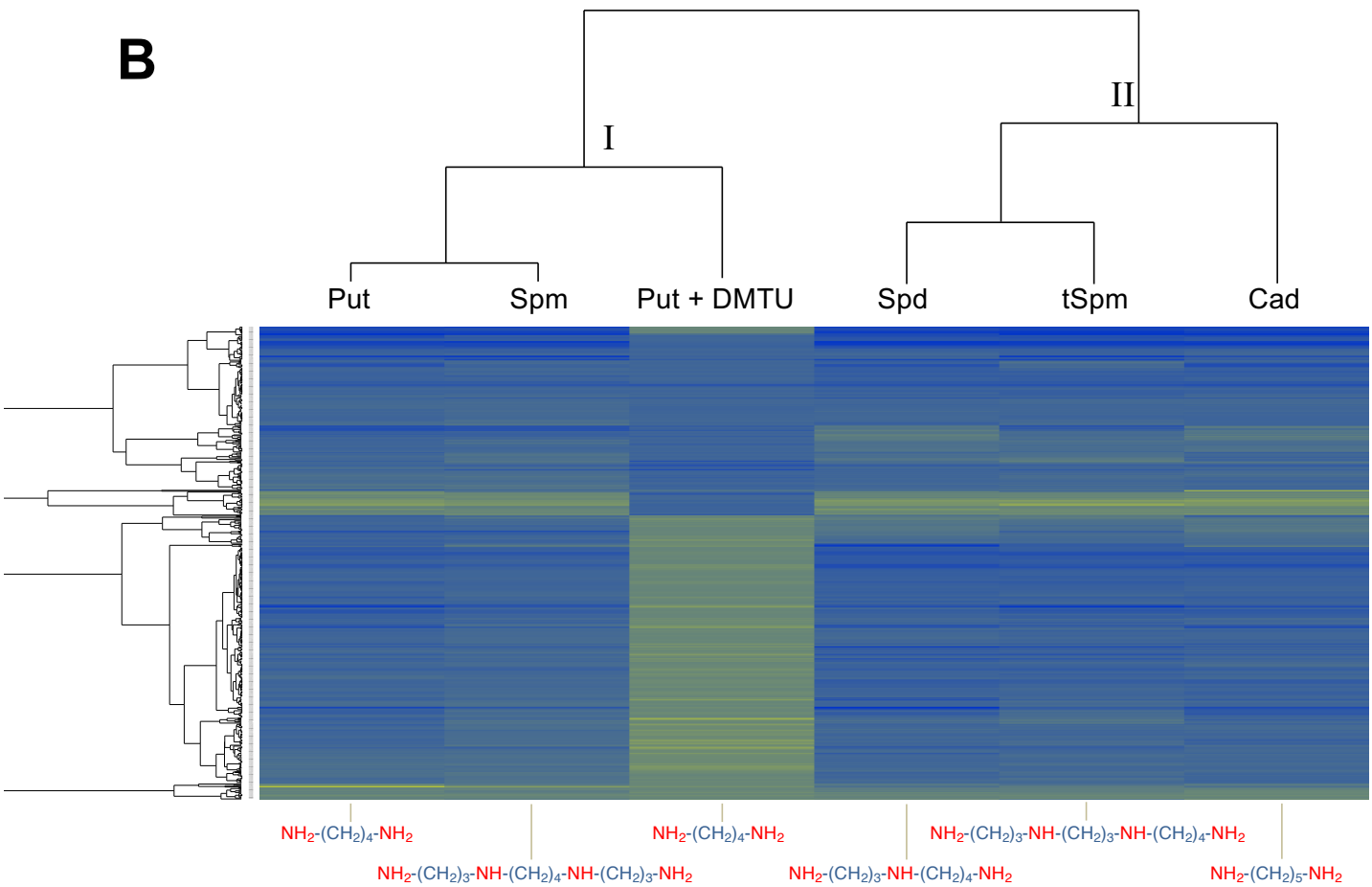
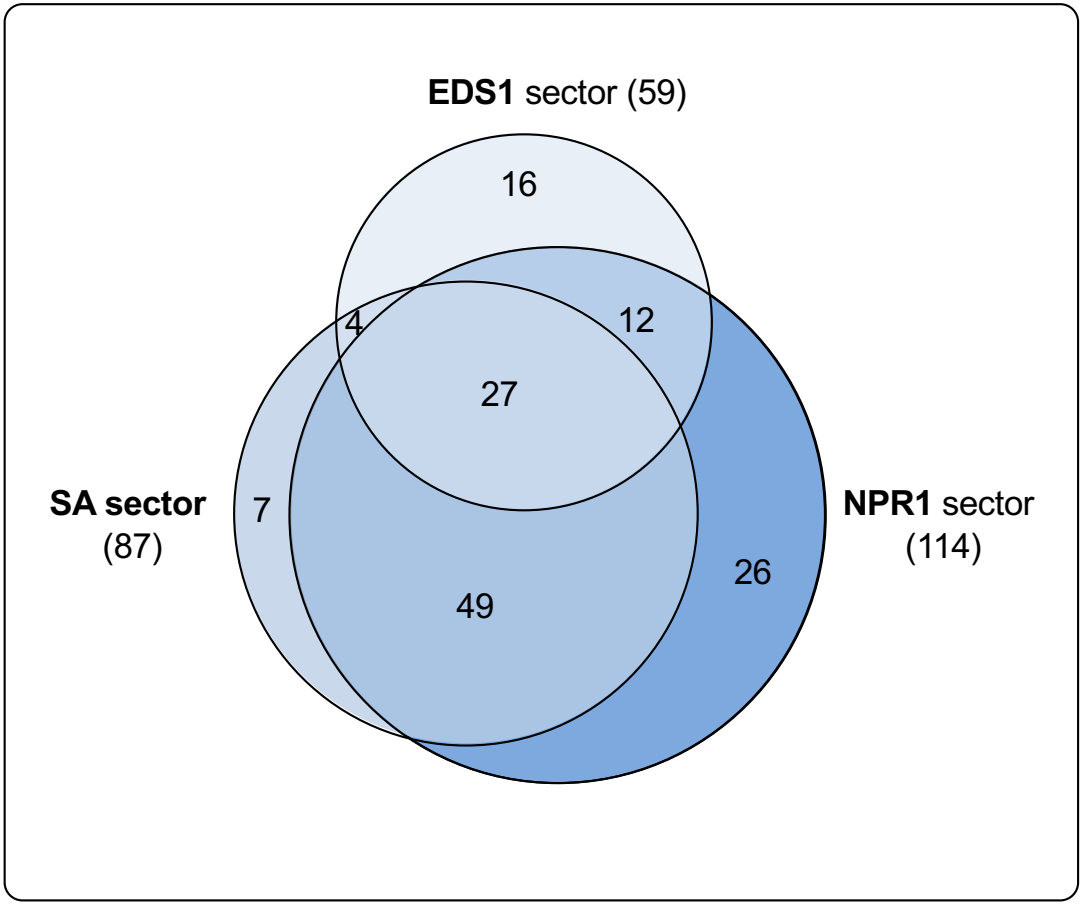
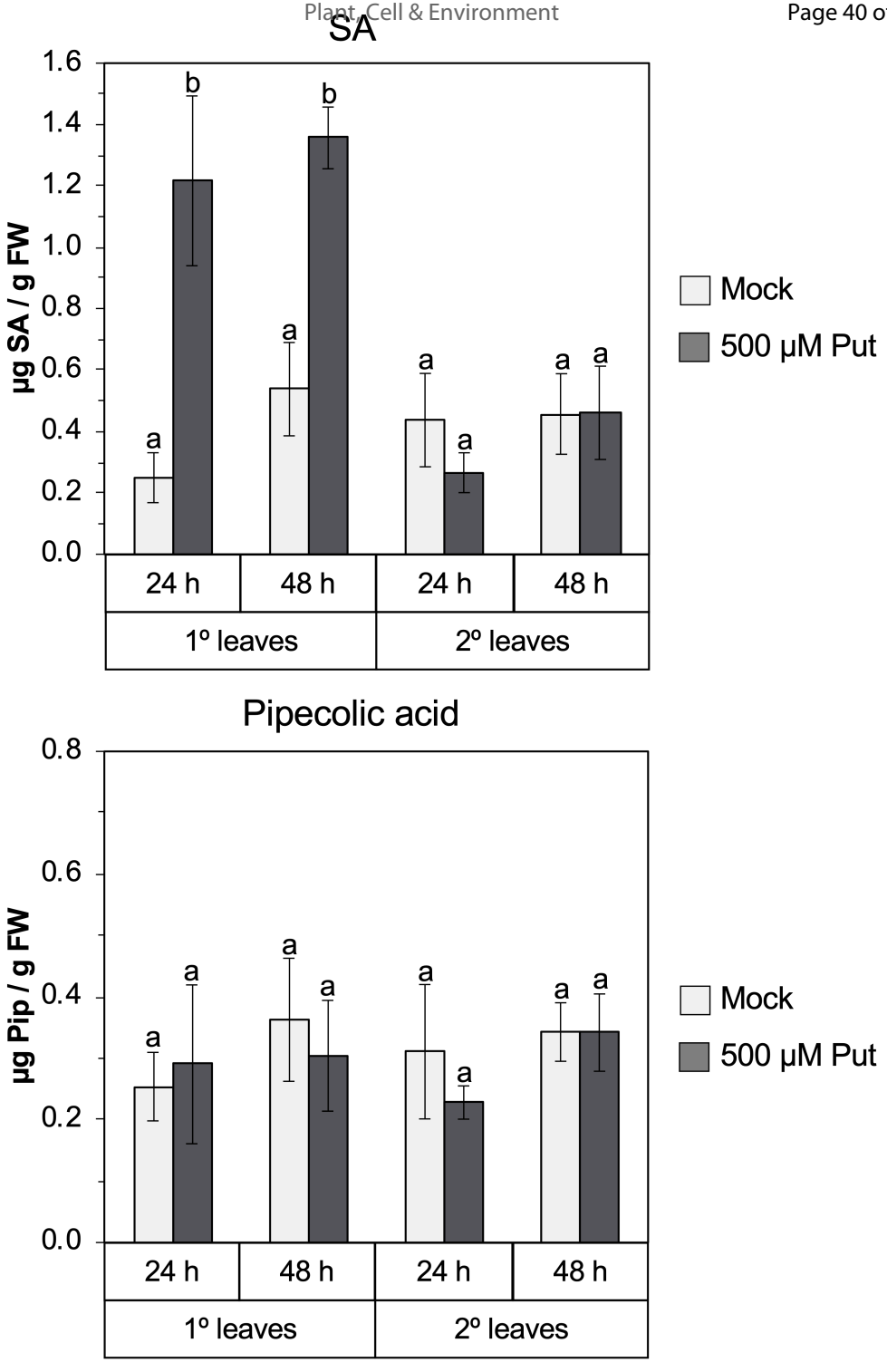
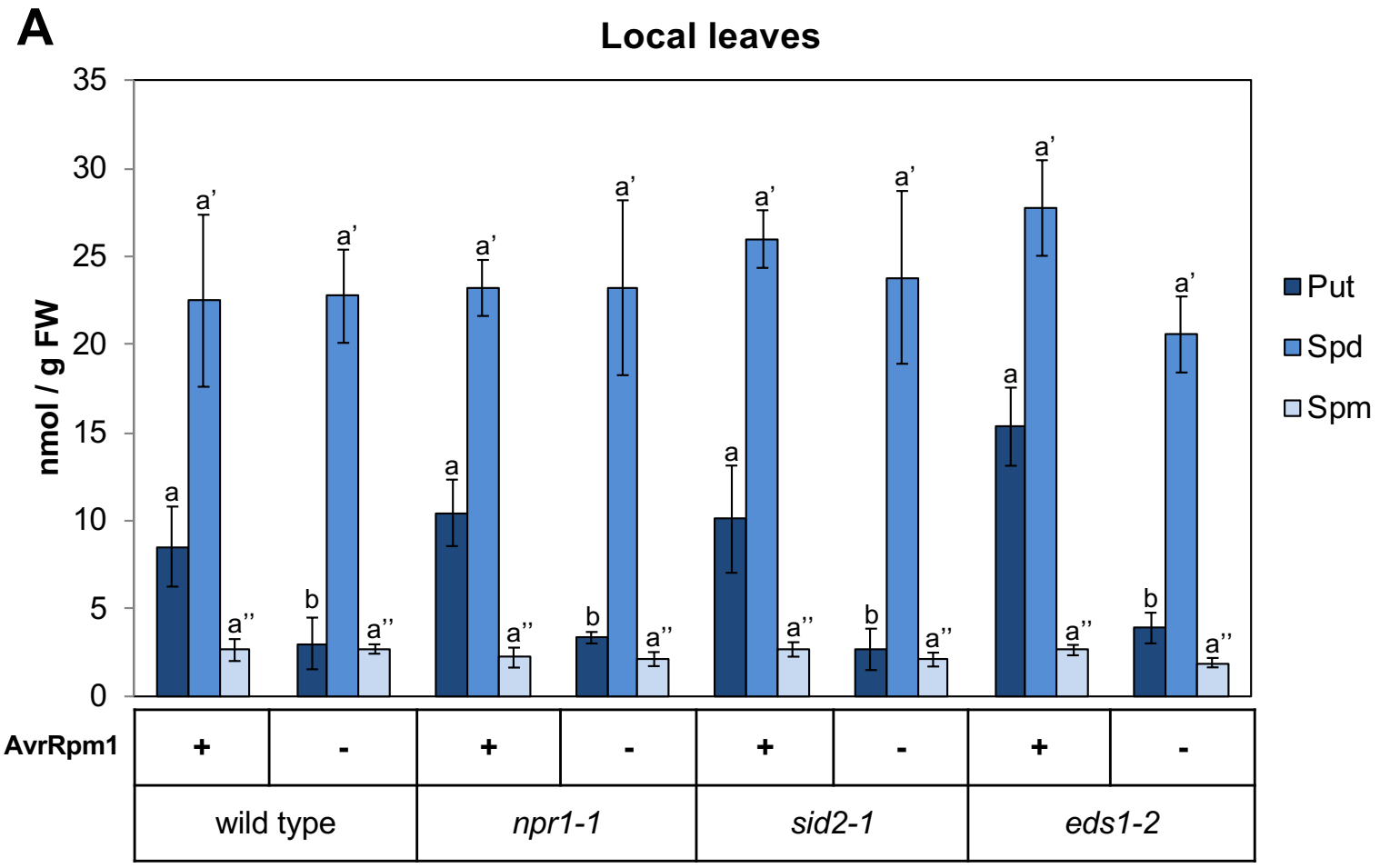
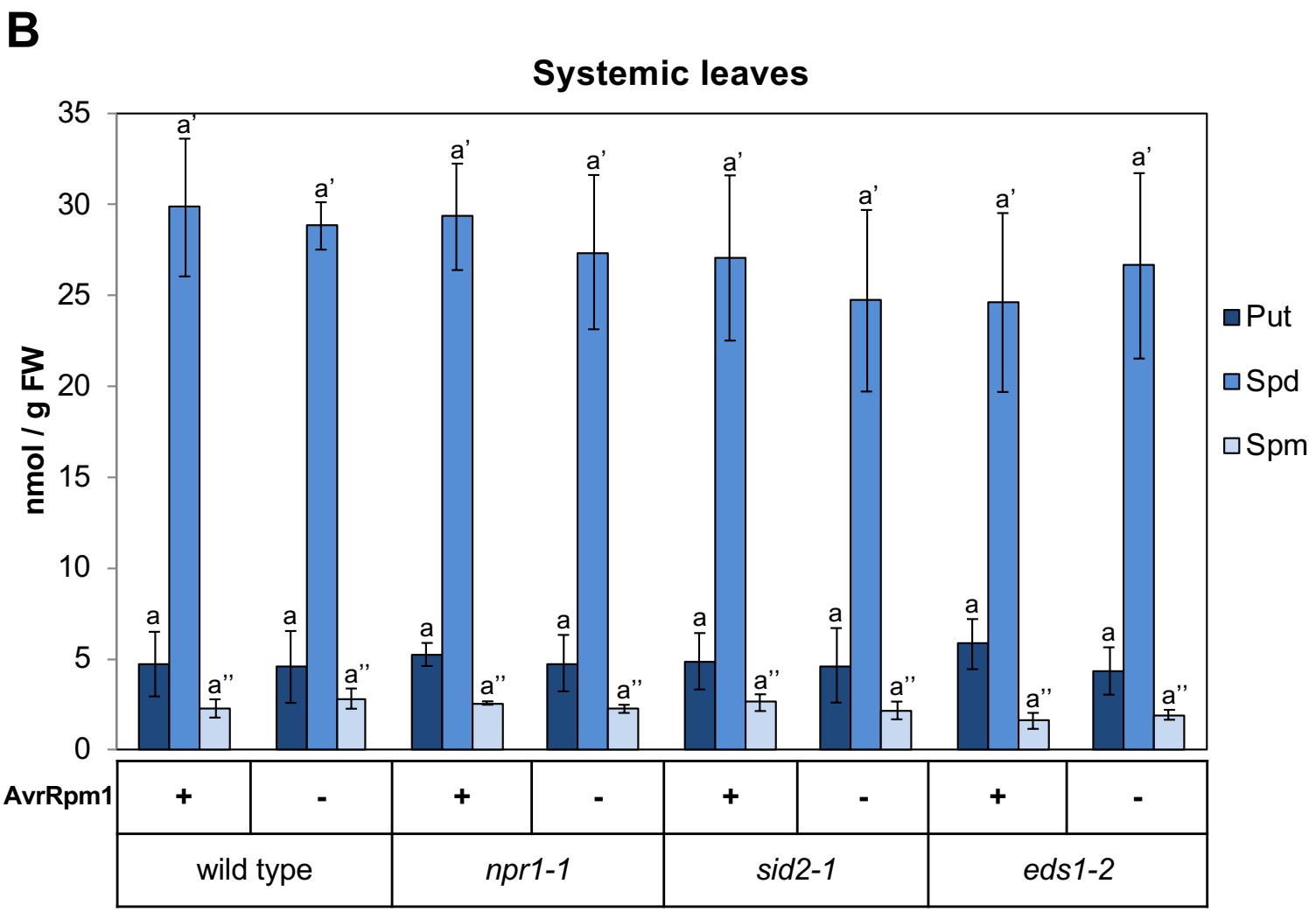

A
PC2 (15.0 %)
PC1 (15.7 %)
Put
Put
Put
Spm
Spm
Spm
Mock
Mock
Mock
Cad
Cad
Cad
Spd
Spd
Spd
tSpm
tSpm
tSpm


B
I
II
Put
Spm
Put + DMTU
Spd
tSpm
Cad
NH2-(CH2)4-NH2
NH2-(CH2)3-NH-(CH2)4-NH-(CH2)3-NH2
NH2-(CH2)4-NH2
NH2-(CH2)3-NH-(CH2)4-NH2
NH2-(CH2)3-NH-(CH2)3-NH-(CH2)4-NH2
NH2-(CH2)5-NH2

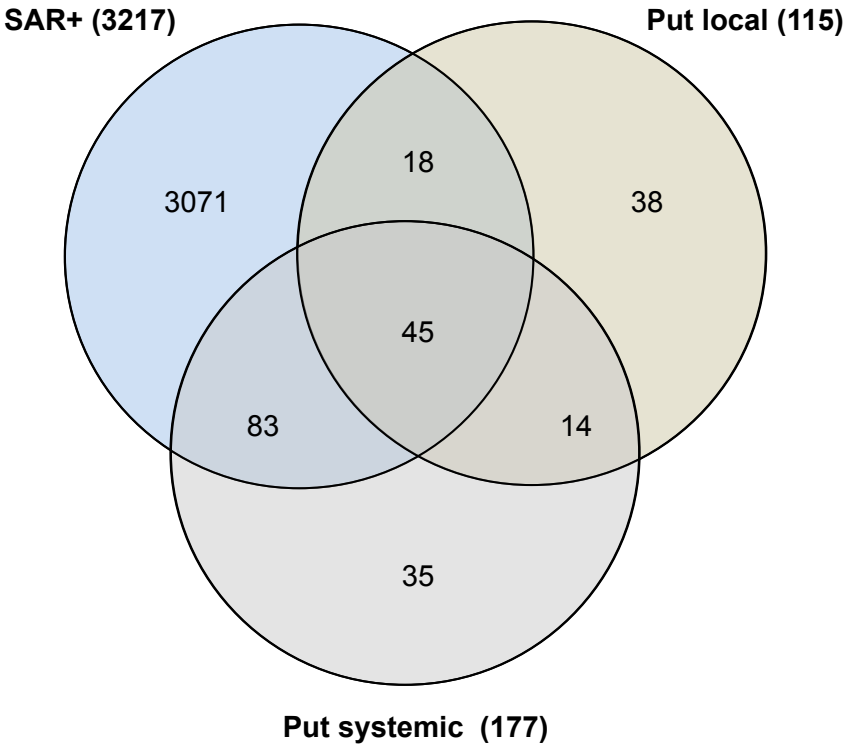

Put responsive (227)
EDS1 sector (59)
16
4
12
27
SA sector
(87)
7
49
26
NPR1 sector
(114)


SA
µg SA / g FW
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
b
a
a
a
a
a
a
24 h
48 h
24 h
48 h
1º leaves
2º leaves
Mock
500 µM Put
Pipecolic acid
µg Pip / g FW
0.8
0.6
0.4
0.2
0.0
a
a
a
a
a
a
a
a
24 h
48 h
24 h
48 h
1º leaves
2º leaves
Mock
500 µM Put

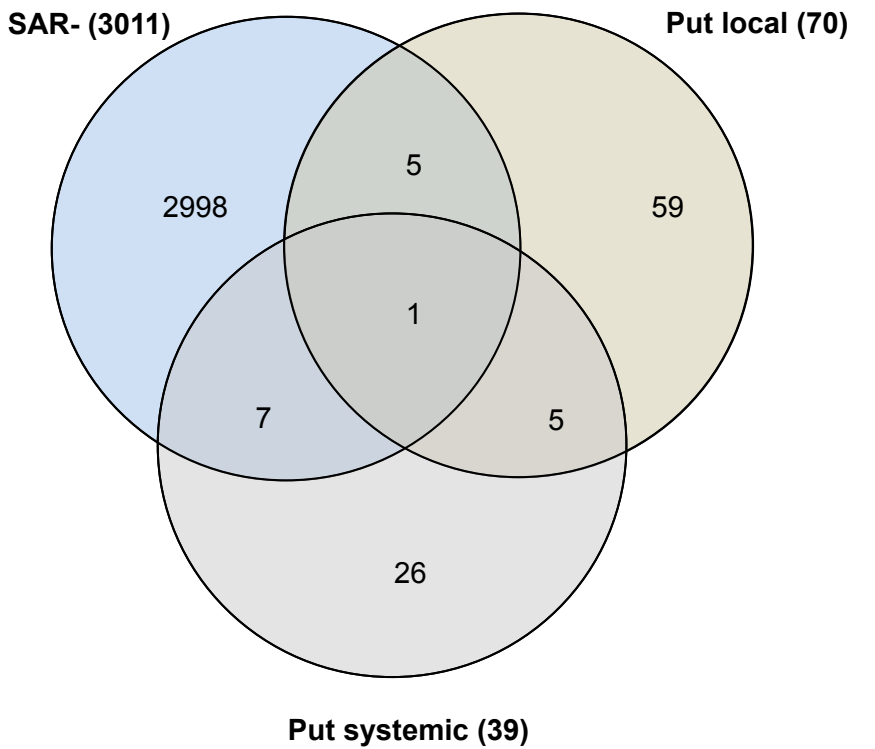

A
Local leaves
nmol / g FW
35
30
25
20
15
10
5
0
Put
Spd
Spm
AvrRpm1
+
-
wild type
npr1-1
sid2-1
eds1-2

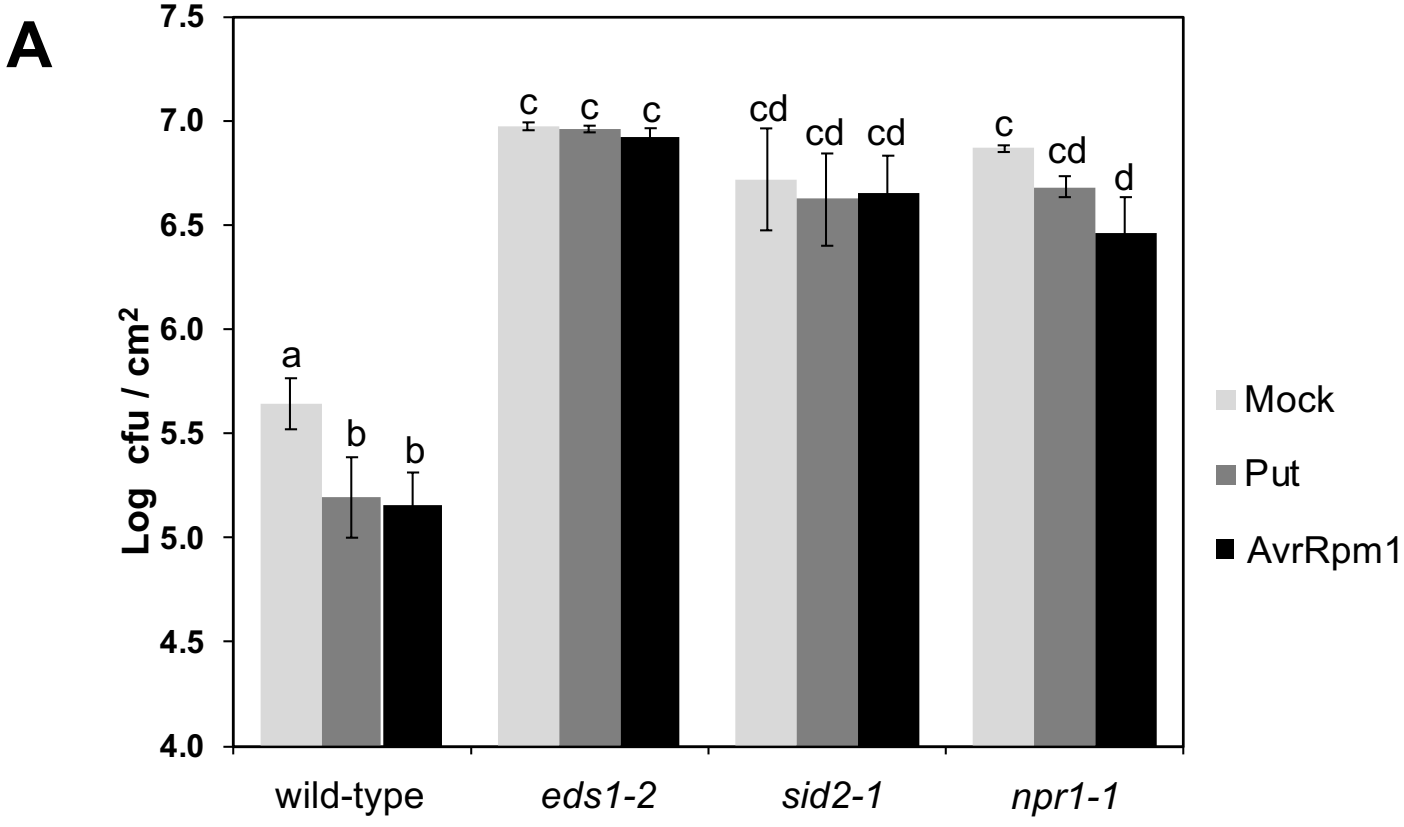

B
Systemic leaves
nmol / g FW
35
30
25
20
15
10
5
0
Put
Spd
Spm
AvrRpm1
+
-
wild type
npr1-1
sid2-1
eds1-2

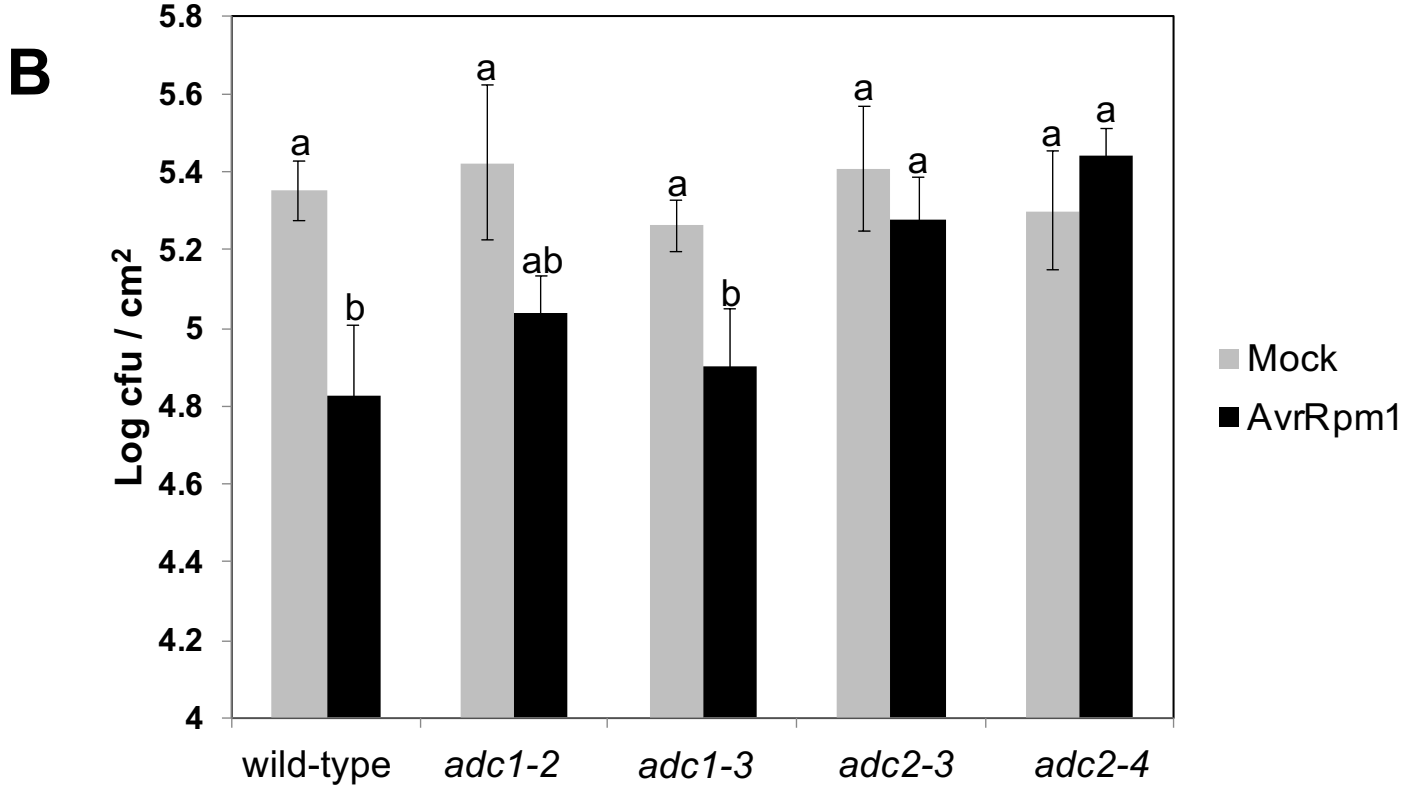
up-regulated

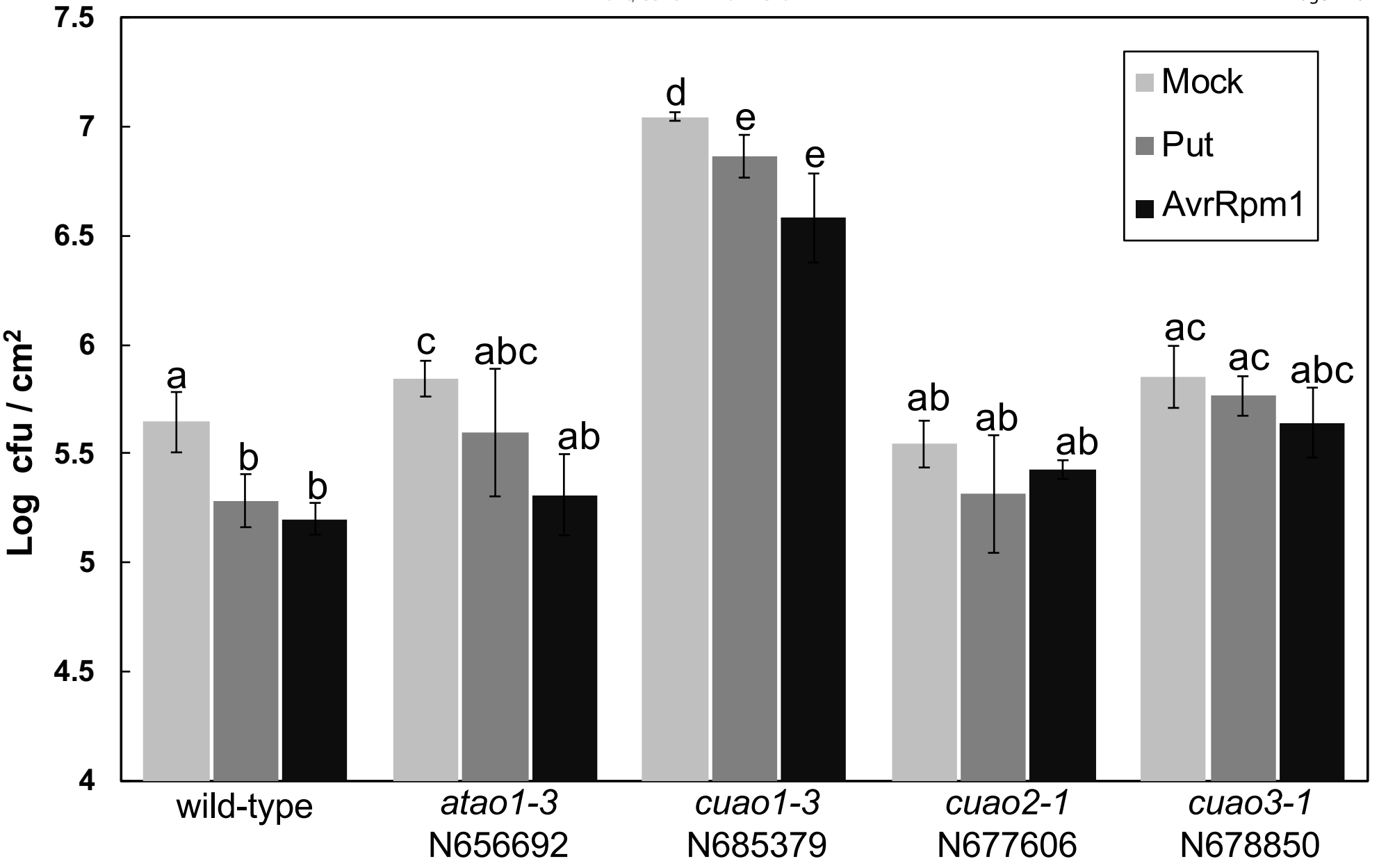


down-regulated




A
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
Log cfu / cm²
a
b
b
c
c
c
cd
cd
cd
c
cd
d
wild-type
eds1-2
sid2-1
npr1-1
Mock
Put
AvrRpm1


B
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
Log cfu / cm²
a
b
a
ab
a
b
a
a
a
a
wild-type
adc1-2
adc1-3
adc2-3
adc2-4
Mock
AvrRpm1


7.5
7
6.5
6
5.5
5
4.5
4
Log cfu / cm²
Mock
Put
AvrRpm1
a
b
b
c
abc
ab
d
e
e
ab
ab
ab
ac
ac
abc
wild-type
atao1-3
N656692
cuao1-3
N685379
cuao2-1
N677606
cuao3-1
N678850