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DOCTORAT EN BIOTECNOLOGIA

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Extragenic Suppressors of Immune Hybrid Incompatibility

Memòria presentada per Changxin Liu per optar al títol de doctor per la

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Doctorand

To My Parents and Sisters

To Yuzhen

vi

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thanks for your heartfully support, it is one of the most meaningful things ever happen to me that we are family, I love you all.

Changxin Liu

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Layout of the thesis

The main topic of this thesis is to investigate the contribution of polyamines to defense in *Arabidopsis thaliana* and the requirement of callose deposition for full expression of effector-triggered immunity in autoimmune hybrids. Due to its accumulation during pathogen infection, I mainly focused on the polyamine putrescine. The interaction between polyamines, reactive oxygen species (ROS) production and salicylic acid pathway activation is also studied in the context of PAMP-triggered immunity (PTI) (**Chapter 1**) and systemic acquired resistance (SAR) (**Chapter 2**). The data support a role for putrescine as a priming agent contributing to resistance against pathogens, which can lead to practical applications in the development of PPP (plant protection products). In the last chapter, I report the involvement of *GLUCAN SYNTHASE-LIKE 2* and 10 (*GSL2* and *GSL10*), two of the twelve callose biosynthesis genes, in the temperature-dependent immune hybrid incompatibility between natural accessions of *Arabidopsis thaliana* from North Europe (L*er*) and Central Asia (Kas-2), which constitutes a model for the study of effector-triggered immunity (ETI). This work supports that PTI and ETI are not two separate branches of defense, but support each other through mutual potentiation.

In the **Introduction,** I summarize key aspects about the model plant *Arabidopsis thaliana*, and introduce reader to general concepts of plant immunity, including the different modes of pathogen recognition, initiation of PTI and ETI , as well as the establishment of SAR, the participation of ROS and current knowledge about the implication of polyamines in defense, which are furtherly discussed in **Chapters 1** and **2**. Afterwards, I introduce immune - related hybrid incompatibilities as a model for ETI studies, and what is known about glucan synthases involved in callose deposition in response to environmental inputs, which is further discussed in **Chapter 3**.

In **Chapter 1,** I show the contribution of the polyamine putrescine to a positive feedback loop which boosts PTI in a hydrogen peroxide, NADPH oxidase, RBOHD and RBOHF - dependent manner. I report the quantitation polyamine levels in response to a well-known PAMP (flagellin 22), non-virulent bacteria *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC* (*HrcC*), which lacks the TTSS (type three secretion system) and its wild-type strain *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Loss-of-function mutants in two isoforms of arginine decarboxylase gene (*ADC1* and *ADC2*) were used to reveal that the ADC2

isoform is the major contributor to basal defenses. I detected the typical PTI response in plants after treatment with exogenous putrescine, such as higher expression of PTI marker genes, callose deposition in leaves and enhanced disease resistance against bacterial pathogens. Moreover, RBOHD and RBOHF loss-of-function mutants were used to confirm that apoplastic ROS burst contributes to Put-induced signaling. Overall, the data are consistent with Put reinforcing PTI signaling during defense.

In **Chapter 2**, We used a comprehensive RNA-sequencing analysis to investigate early transcriptional responses triggered by each of the most abundant polyamines (putrescine, spermidine, spermine, thermospermine and cadaverine) in *Arabidopsis thaliana*. The data indicate that polyamines, rather than being mere metabolic markers of stress, elicit stress signaling involving defense sectors. Similar to *Pseudomonas syringae* pv. *tomato* DC3000 *AvrRpm1* (*Pst AvrRpm1*), Put induced SAR was largely dependent on the production of hydrogen peroxide and salicylic acid (SA), as well as the expression of *EDS1* (enhanced disease susceptibility) and *NPR1* (non-expressor of pathogenesis related genes 1). *Copper amine oxidase* (CuAO) mutants involved in putrescine oxidation were compromised in basal defenses and/or putrescine and pathogen – triggered systemic resistance. These results reveal the contribution of Put oxidation to SA-dependent systemic defenses in *Arabidopsis thaliana*.

In **Chapter 3,** I describe the contribution of *GLUCAN SYNTHASE-LIKE*, *GSL2*/*10* to the temperature conditioned immune-related hybrid incompatibility (HI) between *Arabidopsis thaliana* accession L*er* and Kas-2. Or in other words, the contribution of callose to the establishment of ETI. We performed a suppressor screen of L*er*/Kas-2 HI by EMS (ethyl methanesulfonate) mutagenesis and mapped causal genes by genome sequencing. Candidate genes were tested by the generation of artificial microRNA targeting *GSL2* or *GSL10* in the L*er*/Kas-2 HI (NIL) autoimmune background. Through measurement of SA level and expression of SA pathway reported genes, I concluded that ETI is suppressed by *GSL2* or *GSL10* mutation. Callose detection in response to mechanical wounding, flagellin 22 (flg22) and bacteria inoculation revealed that ETI but not callose deposition is impaired in these mutants.

In the **Discussion**, I summarize key findings from the three chapters and discuss about the outcomes of my research. I finalize with main conclusions derived from the different chapters.

List of abbreviations

ACL5: Acaulis 5

ADC: Arginine decarboxylase

AGD2: Aberrant growth and death 2

AIH: Agmatine iminohydrolase

ALD1: AGD2-like defense response protein 1

AO: Amine oxidase

AzA: Acid azelaic acid

Azi1: Azelaic acid induced 1

BAK1: *Brassinosteroid* insensitive associated receptor kinase 1

BDM: Bateson-Dobzhansky-Muller

BIK1: Botrytis-induced kinase 1

BTH: 2,1,3-benzothiadiazole

Cad: Cadaverine

CalS: Callose synthase

Cat: Catalase

CC: Coiled-coil domain

CDPKs: Calcium-dependent protein kinases

CERK1: Chitin elicitor receptor kinase 1

Clh: Chlorophyll

CMV: Cucumber mosaic virus

CNL: CC-NB-LRR

CPA: N-carbamoylputrescine amidohydrolase

CuAO: Copper amino oxidase

DA: Dehydroabietinal

DAMP: Damage-associated molecular pattern

DAO: Diamine oxidase

DFMA: DL-α-difluoromethylarginine

DGDG: Digalactosyl-diacylglycerol

DIR1: Defective in induced resistance 1

DM: Dangerous mix

DMTU: 1,3-dimethyl-2-thiourea

dpi: Days post-inoculation

EARLI1: Early *Arabidopsis* aluminium induced 1

EDS1: Enhanced disease susceptibility 1

EFR: EF-Tu receptor

EF-Tu: Elongation factor Tu

EMS: Ethyl methanesulfonate

ET: Ethylene

ETI: Effector-triggered immunity

ETS: Effector-triggered susceptibility

FA: Fatty acid

Flg22: Flagellin 22

FLS2: Flagellin sensing 2

FMO1: Flavin-dependent-Monooxygenase 1

G3P: Glycerol-3-phosphate (or a G3P-dependent factor)

GSL: Glucan synthase-like

Gw: Gorzów (Poland)

HI: hybrid incompatibility

Hpa: *Hyaloperonospora arabidopsidis*

HPLC: High performance liquid chromatography

HR: Hypersensitive response

HTD: 1.7-diaminoheptane

ICS1: Isochromatic synthase 1

ISR: Induced systemic resistance

JA: Jasmonic acid

JA-Ile: JA-isoleucine

LRR: Leucine-rich repeat domain

LTP: Lipid transfer protein

Lys: Lysine

MAMP: Microbe-associated molecular pattern

MAPK: Mitogen-activated protein kinase

MES: 2-(N-morpholino) ethanesulfonic acid

MeSA: Methyl SA

MGDG: Monogalactosyldiacyl - glycerol

MS: Murashige & Skoog media

Mya: Million years ago

NADP: Nicotinamide adenine dinucleotide phosphate

NB: Nucleotide binding domain

NDR1: Non-race-specific disease resistance 1

NGS: Next generation sequencing

NHP: *N*-hydroxypipecolic acid

NIL: Near isogenic line

NLR: Nucleotide binding leucin rich repeat

NO: Nitric oxide

NOA: NO associated protein 1

NPR1: Non expressor of pathogenesis-related 1

NR: Nitrate reductase

ODC: Ornithine decarboxylase

PA(s): Polyamine(s)

PAD4: Phytoalexin-deficient 4

PAL: Phenylalanine Ammonia-Lyase

PAMP: Pathogen-associated molecular pattern

PAO: Polyamine Oxidase

PBL1: PBS1-like 1

PBS1: AvrPphb susceptible 1 1

PCA: Principal component analysis

PCD: Programmed cell death

PD: Plasmodesmata

PDLP5: PD-localizing protein 5

Pip: Pipecolic acid

PMR: Powdery mildew resistant

POD: Peroxidase

PR1: Pathogenesis-related gene 1

PRR: Pattern recognition receptors

PRX: Peroxidases

Pst: *Pseudomonas syringae*

PTI: PAMP-triggered Immunity

Put: Putrescine

QTL: Quantitative trait locus

RBOHD/F: Respiratory burst oxidase homolog D and F

RIN4: RPM1 interacting protein 4

RLK: Receptor like kinase

RLP: Receptor like protein

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RPM1: Resistance to *Pseudomonas syringae maculicola* protein 1

RPP1: Recognition of *Peronospora parasitica* 1

RPS2: Resistance to *Pseudomonas syringae* 2

SA: Salicylic acid

SABP: SA binding protein

SAG101: Senescence associated gene101

SAM: *S*-adenosylmethionine

SAMDC: SAM decarboxylase

SAMT: SAM methyltransferases

SAR: Systemic acquired resistance

SARD4: SAR-deficient 4

SERK3: Somatic embryogenesis-related kinases 3

SID2: SA induction deficient 2

SIPK: Salicylic acid-induced protein kinase

SOD: Superoxide dismutase

SPDS: Spermidine synthase

TNL: TIR-NB-LRR

TSPMS: Thermo-spermine synthase

TTSS: Type three secretion system

WIPK: Wound-induced protein kinase

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General Introduction

1.1. The model plant *Arabidopsis thaliana*

Plants play a critical role in the world, not only providing food and sustainable agricultural resources but also mediating in the quality of the environment (Shameer et al., 2019). Land plants originated from streptophyte algae 870–962 million years ago (Mya) and diversified into living lineages during the mid-Palaeozoic era (471–480 Mya) (Kenrick and Crane, 1997; Wellman et al., 2003). The first seed-producing plant evolved between 310 to 350 Mya (Doyle, 2012; Magallón et al., 2013). The most recent common ancestor of angiosperms likely arose between 140 to 250 Mya (Sauquet et al., 2017; Foster et al., 2017). Plant species are various in size, configuration, structure and physiological behavior. They inhabit in most parts of lands, oceans, lakes and rivers (Willis and McElwain, 2014). Currently, the known number of plant species is ca 374,000, of which approximately 295,383 are flowering plants (Christenhusz and Byng, 2016).

The Brassicales, which originated around 70 Mya, is an important family in the plant kingdom. It contains five family groups: Brassicaceae (Cruciferae), Capparidaceae, and Cleomaceae; Akaniaceae and Tropaeolaceae (Edger et al., 2018). The Brassicaceae family contains ~3,628 species that mainly live in North temperate zones (Christenhusz and Byng, 2016). It contains approximately 328 genera within the Dicotyledons class, Dilleniidae subclass, Cleome gynandra order and Brassicales family. Many crops are included in this family, such as cabbage and mustard *(Brassica)*, radish (*Raphanus*) and woad (*Isatis tinctorial*, *Isatis*), which is widely used as dye and it has some medicinal properties (Hamburger, 2002). Notably, the most used model organism in plant sciences is the genus *Arabidopsis,* which also belongs to the Brassicaceae family (Warwick et al., 2006).

Arabidopsis evolved about 20 - 40 Mya when the Camelinae tribe diverged from the Brassicaceae family (Franzke et al., 2011). (**Figure 1**). *Arabidopsis* was first discovered by Johannes Thal in the Harz Mountains (Germany) in 1577 (Krämer, 2015). The present name *Arabidopsis thaliana* (L.) Heynh was first published in 1753 by Linnaeus. Currently, the number of Arabidopsis species is ten and contains more than 20 subspecies, such as *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Arabidopsis arenosa* and *Arabidopsis croatica* (Koch et al., 2006; Hohmann et al., 2015). *Arabidopsis thaliana* was first used in the research studies by Friedrich Laibach in the 1940s (Koornneef and Meinke, 2010), and systematically collected from the wild since then. *Arabidopsis thaliana* inhabits

regions in Europe, Asia and North Africa, but was introduced in North America and Australia (Mitchell-Olds, 2001; Koch et al., 2008).
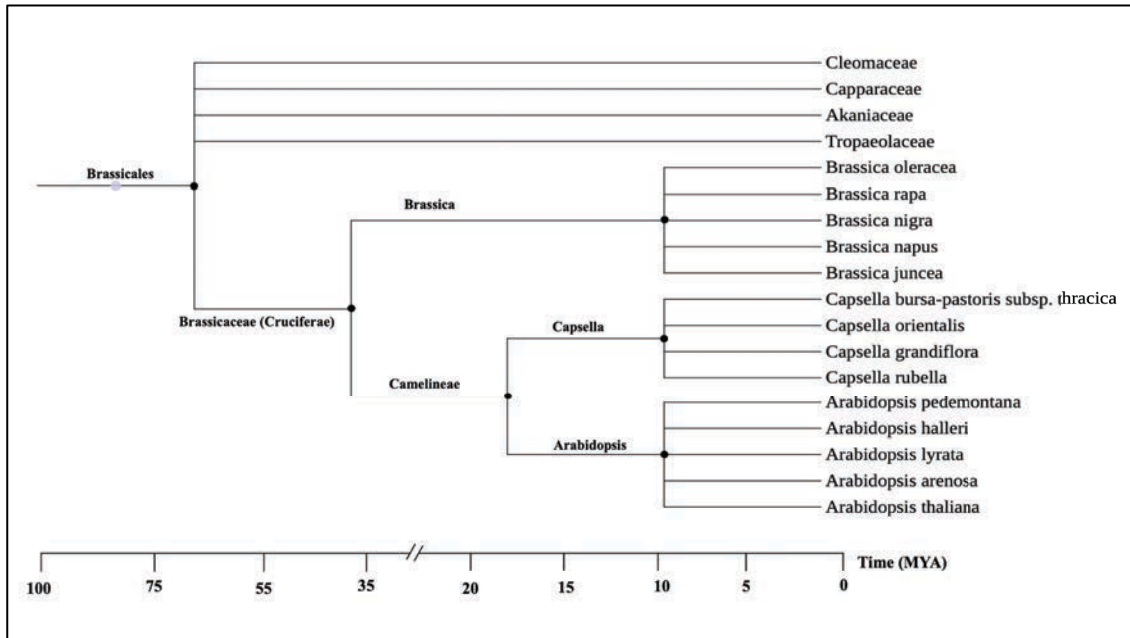
Figure 1. The phylogeny of Brassicaceae. Camelinae tribes originated from the Brassicaceae family (Cruciferae) around 37 Mya, and then diverged into the different species of *Arabidopsis, Capsella* and the *Brassica* genus ~10 Mya.

Arabidopsis thaliana (hereafter referred to as "Arabidopsis") development starts with a small basal rosette of 2-5 cm diameter, which produces 20 to 70- cm high flower stems. Flowers are typical crucifers, carrying four sepals, four petals and six stamens originated from intervals. Flowers are approximately 3 mm long and 1 mm wide as part of an inflorescence or a raceme. Many accessions or transgenic lines produce multiple flowering stems. Arabidopsis is self-fertile, yielding several hundred siliques upon fertilization (Krämer, 2015).

Arabidopsis is a diploid species (2n =10), and is believed to have the smallest nuclear genome among higher plants (100 Mb) with low repetitive DNA content (~25%) differing between accessions (Meyerowitz, 2001; Schmuths et al., 2004). The genome contains 25,498 genes encoding proteins from 11,000 families, which makes Arabidopsis an ideal subject for mutagenesis experiments or map-based cloning (Meinke et al., 1998). More importantly, Arabidopsis is easily transformed with *Agrobacterium tumefaciens*, which can be used not only to introduce specific transgenes, but also as a random

mutagenesis method for gene-tagging (Lloyd et al., 1986; Clough and Bent, 1998; Zhang et al., 2006). The capacity of self-fertilization maintains homozygosity of the different transgenic lines and wild accessions over generations, while it is also possible to perform crosses when necessary (Wright et al., 2003; Guo et al., 2009). On the other hand, compared with other model species such as maize or pea, the commonly used Arabidopsis accessions, such as Columbia (Col-0) and Landsberg *erecta* (L*er*), have a shorter life history, six to eight weeks, under optimal conditions, thus enabling rapid genetic analysis.

All of the features described above make Arabidopsis a remarkable model system to investigate the development, physiology, biochemistry and genotype-by-environment (G x E) interactions studies over forty years (Meinke et al., 1998; Alcázar et al., 2014; Dittrich et al., 2019).

1.2. The Plant immune system

Plants regularly confront various adverse stresses, generally categorized as biotic and abiotic. It is reported that 28 – 42% of annual worldwide crops losses (corn, wheat, soy, oats and barley) are due to disease caused by diverse pathogens (viruses, fungi, bacteria, oomycetes and nematodes) and herbivores. Another 6 – 20% loss is caused by abiotic stresses such as drought, flood, salinity, nutrient deficiencies, extremes in temperature and heavy metals (Mahajan and Tuteja 2005; Asensi-Fabado, Amtmann, and Perrella 2017; Shameer et al., 2019). Unlike animals, plants lack mobility or an adaptive immune system (Jones and Dangl, 2006). To make this through, plants acquired complex and effective defense systems during evolution.

Plants rarely grow without the threat of pathogen colonization in natural environments, and their confrontation relies on a multi-layered system of innate immunity (Wiermer et al., 2005). The multi-layered plant immune system is described as the "zig-zag" model (Jones and Dangl, 2006; Glazebrook and Roby, 2018).

Pathogens have adopted different strategies for plant colonization. For instance, pathogenic bacteria proliferate in the apoplast after entering through the stomata, hydathodes, or via wounding. Germinating spores from fungi can penetrate the epidermal cells directly (Jones and Dangl, 2006). After pathogen's break of the physical barriers, plant defense relies on two layers of pathogen recognition (Chisholm et al., 2006; Jones and Dangl, 2006; Yeats and Rose, 2013). Pathogen associated molecular patterns

(PAMPs) and microbial associated molecular patterns (MAMPs) are conserved microbial elicitors which are directly recognized by cell-surface-localized pattern recognition receptors (PRRs). These receptors are comprised of receptor kinases (RKs) and receptor-like proteins (RLP), which initiate PAMP-triggered immunity (PTI) (Boller and Felix, 2009; Macho and Zipfel, 2014). Similarly, the signals may be derived from the plant itself because of the damage caused by microbes, which involve damage-associated molecular patterns (DAMPs) released by damaged cells to warn their healthy neighbors (Lotze et al., 2007; Morimoto and van der Hoorn, 2019).

In the next stage, to enhance microbial fitness, pathogens deliver effectors or virulence proteins into the host cell through blocking or delaying PTI signaling and/or defense output. This effector-triggered susceptibility (ETS) makes the host susceptible to pathogens (the '*zag*'). However, the effectors are also directly or indirectly recognized by intracellular receptors proteins containing nucleotide-binding leucine-rich repeat (NB-LRR or NLR) domains, which are encoded by resistance (*R*) genes, and activate effector-triggered immunity (ETI) (Jones and Dangl, 2006). Most *R* genes code for NLR proteins that recognize the 'modified-self' by-products of ETS (another '*zig*'). The outcome from the interaction then depends on the total sum of [(PTI - ETS) + ETI] (Nishimura and Dangl, 2010) (**Figure 2**). This attack-and-response can occur in multiple rounds of ETS, accompanied by a series of defense events. For instance, global defense gene up-regulation, programmed cell death (hypersensitive response, HR), accumulation of reactive oxygen species (ROS) and phytohormones such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) (Jones and Dangl, 2006; Hein et al., 2009).

1.2.1. PAMP and DAMP - triggered immunity

PAMP or MAMP - triggered immunity (PTI) is a complex set of responses intended to resist against pathogen attack through a series physiological and biochemical reactions happening in due succession (Ausubel, 2005). A given PAMP/MAMP is recognized by a specific PRR to initiate PTI. For instance, elongation factor Tu (EF-Tu), an 18 amino acid long eliciting epitope (elf18) from *Escherichia coli*, and chitin (a component of fungal cell walls) are perceived by the EF-Tu receptor (EFR) (Zipfel et al., 2006; Katagiri and Tsuda, 2010). Flg22 is a conserved 22 - amino acid epitope from bacterial flagellin of *Pseudomonas aeruginosa* and it is recognized by the LRR receptor Flagellin sensing 2

(FLS2) (Felix et al., 1999; Gómez-Gómez et al., 2000). Similarly, the 23 amino acids peptide AtPep1, from the Pep family, was the first discovered DAMP which specifically binds two leucine-rich repeat receptor-like kinases, AtPEPR1 and AtPEPR2. Moreover, AtPep1 elicits a stronger immune response than flg22 (Yamaguchi and Huffaker, 2011; Poncini et al., 2017).
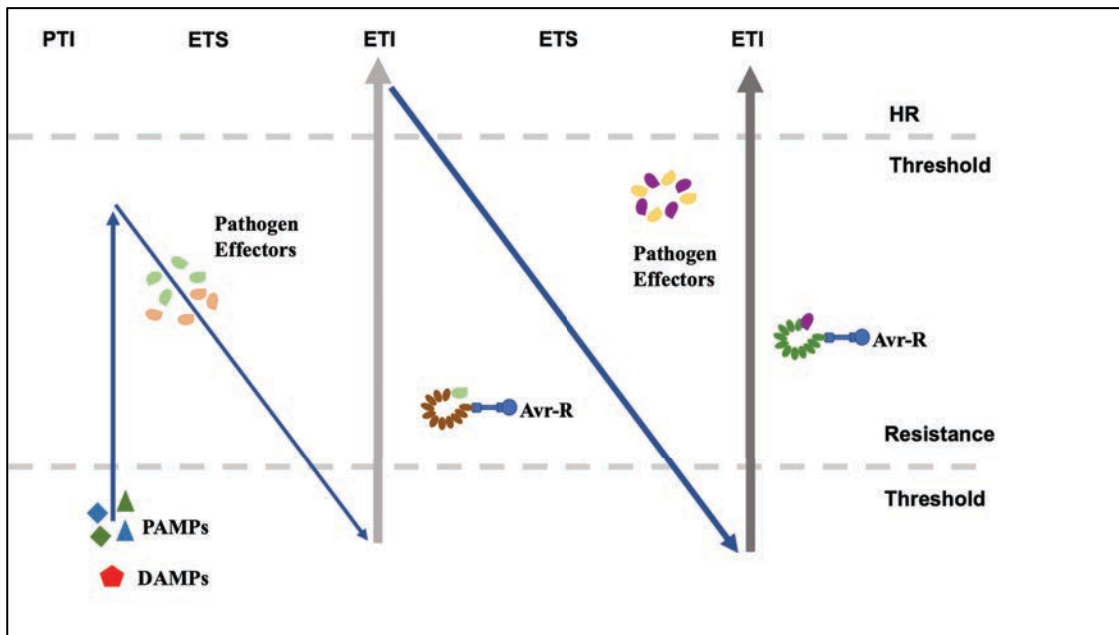
Figure 2. "zig-zag" model for the visual presentation of the output of the plant immune-system interaction with pathogen effectors according to Jones and Dangl (2006). DAMPs/PAMPs, Damage/Pathogen associated molecular patterns; PTI, PAMP triggered immunity; ETS, Effector triggered susceptibility; ETI, Effector-triggered immunity; HR, Hypersensitive response.

Flg22 recognition quickly triggers FLS2 association with the leucin-rich repeat receptor-like kinase (LRR -RK) BAK1 (brassinosteroid insensitive associated receptor kinase 1), which is also called SERK3 (somatic embryogenesis-related kinases) (Chinchilla et al., 2007; Heese et al., 2007). Likewise, elf18/elf26 are perceived by EF-Tu, and form an EFR/BAK1 complex immediately after stimulation (Schwessinger et al., 2011). In unstimulated cells, FLS2 and BAK1 are not associated but form a tight complex upon binding flg22, which then triggers phosphorylation of BIK1 (Botrytis induced kinase 1, a receptor-like cytoplasmic kinase), activating ion influx across the plasma membrane and mitogen-activated protein kinases (MAPKs) (Chinchilla et al., 2007).

After perception of flg22 and other PAMP treatments, four MAPKs: MAPK3, MAPK4, MAPK6, and MAPK11 are activated (Zipfel et al., 2006; Han et al., 2019). Flg22-induced

MAPK3, MAPK4, and MAPK6 activation is dependent on BIK1 and its homolog PBL1 (avrPphB suseptible 1-like 1) but not CDPKs (calcium-dependent protein kinases) (Boudsocq et al., 2010; Feng and Zhou, 2012). Several transcription factors are essential for the following signaling, like WRKY33 which interacts with MAPK3/MAPK6 leading to camalexin (phytoalexins) biosynthesis (Qiu et al., 2008; Mao et al., 2011).

The early responses to PAMP/DAMP, including ion influx (Ca^{2+} burst), generation of reactive oxygen species (ROS burst), and other small signaling molecules such as reactive nitrogen species (RNS), nitric oxide (NO) and lipids, like phosphatidic acid and ceramides (Di Meo et al., 2016). Ca^{2+} burst can depolarize the plasma membrane, and furtherly trigger the influx of H^+, efflux of K^+, Cl^- and NO_3^-, and activation of CDPKs (Okazaki and Saito, 2014; Bigeard et al., 2015).

ROS burst is one of the early hallmark events in the PTI response. In Arabidopsis, the plasma membrane-localized NADPH (nicotinamide adenine dinucleotide phosphate) oxidases RBOHD/F (Respiratory burst oxidase homolog D and F) are responsible for ROS production (Nühse et al., 2007; Ranf et al., 2011; Liu et al., 2019). To enter the cytosol, apoplastic superoxide ($O_2.^-$) is catalyzed to hydrogen peroxide (H_2O_2) by apoplastic superoxide dismutase (SOD) (Nühse et al., 2007). ROS can act as antimicrobial agents directly or may indirectly contribute to defense causing cell wall cross-linking. In addition, ROS may serve on secondary stress signals to induce various defense responses (Apel and Hirt, 2004). MAMP/DAMP-induced ROS burst has a positive feedback effect on cytosolic Ca^{2+} levels via inducing an additive cytosolic Ca^{2+} elevation (Ranf et al., 2011). The following events of PTI are the production of antimicrobial compounds including camalexin, defense-related proteins/peptides (PR1, pathogen related gene 1) (Cowan, 1999; Bednarek, 2012; Ahuja et al., 2012; Bigeard et al., 2015), activation of MAPKs (O'Brien et al., 2012), and a programmed cell death (PCD) to limit pathogen progression (Mur et al., 2008).

SA, JA and ET are the major phytohormones implicated in plant innate immunity. SA signaling is usually involved in the defense against biotrophic or hemibiotrophic pathogens, while JA and ET signaling are essential against necrotrophic pathogens (Glazebrook, 2005). The SA and JA/ET sectors are mutually inhibitory in many cases (Tsuda et al., 2009). These hormones make a positive contribution individually upon PAMP/DAMP perception, since production of SA, JA and ET are activated (Tsuda et al., 2009). In Arabidopsis, several thousand genes, including genes involved in SA, JA and

ET, are deregulated by flg22 treatment. ETI and PTI share SA signaling in Arabidopsis, as SA is required for the regulation of SA-responsive genes during PTI, while during ETI, most responsive genes (including *PR1*) are regulated by SA-independent mechanisms (Nomura et al., 2011; Tsuda et al., 2009; 2013).

Callose deposition is one of the later events in the PTI response. Callose and other secondary metabolites reinforce the cell wall at the infection sites. Polyamines also display strong accumulation in response to PAMP/DAMP, and this response requires H_2O_2 production (Brown et al. 1998; Iriti and Faoro 2009; Liu et al., 2019). Seedling growth is inhibited by PAMPs such as flg22 and elf26. This response may reflect a tradeoff between fitness growth and immune resistance of plants. This effect explains the inducible nature of defense responses in plants, and the stunted phenotype of autoimmune hybrids (Alcázar et al., 2009).

1.2.2. Effector-triggered immunity

To combat virulent pathogens, plants utilize a second layer of the innate immune system, known as effector-triggered immunity (ETI) (also known as *R* gene–mediated resistance). Effectors are molecules produced by pathogens, which can be proteins, nucleic acids, carbohydrates or metabolites that suppress the PTI response. These effectors are usually injected into the plant cell by the type three secretion system (TTSS), and can be detected by plasma membrane localized NLR receptor proteins (Bos et al., 2010; Prince et al., 2014; Li et al., 2016). Arabidopsis contains approximately 150 NLR proteins (Meyers et al., 2003), which are classified into two groups based on the structure of their N-terminus. The CNL (CC-NB-LRR) group, contains an N-terminal coiled-coil domain, whereas the TNL (TIR-NB-LRR) group contains an N-terminal Toll/interleukin-1 receptor (TIR) motif (van Wersch et al., 2020). Peng et al. (2018) also classified all known PRRs into receptor-like kinase (RLK) or receptor-like protein (RLP) families. On the other hand, plant pathogens have been shown to possess a considerable number of effectors. For example, there are between 30 to 50 effector proteins in the bacterial pathogen *Pseudomonas syringae* (*Pst*) (Buell et al., 2003). Recognition of effectors by NLR receptors are through three possible ways, i) receptor-mediated direct interaction (Dodds and Rathjen, 2010), ii) accessory non-canonical protein (or guardee/decoy) mediated indirect interaction (Dangl and Jones, 2001; Hogenhout et al., 2009), iii) via the

"integrated decoy" model in which the decoy is integrated in the NLR protein structure (Cesari et al., 2014).

Participation of RIN4 in ETI

One of the most well studied proteins mediating ETI is RIN4 (RPM1 interacting protein 4). Here, I summarize the main molecular characteristics of RIN4 and associated receptors as an example of the guard-guardee model. The host protein RIN4 (guardee), is targeted by multiple effectors from *Pseudomonas syringae* (*Pst*) including TTSS effectors AvrRpm1, AvrRpt2 and AvrB in Arabidopsis. AvrRpt2, a cysteine protease, cleaves and degrades RIN4, while AvrRpm1 and AvrB induce the phosphorylation and inactivation of RIN4 (Hou et al., 2011). Two plasma membrane-located NLR, RPM1 (resistance to *Pseudomonas syringae maculicola* protein 1) and RPS2 (resistance to *Pseudomonas syringae* 2) are associated with RIN4 and sense these changes in RIN4, thus triggering ETI (Mackey et al., 2002; Belkhadir et al., 2004; Jones et al., 2016). Therefore, RPM1 and RPS2 respond to perturbations of RIN4 'guarding' the plant against these bacteria effectors (Kim et al.,2009). RPS2- and RPM1- mediated disease resistance leads to phosphatidic acid production and influx of extracellular Ca^{2+} followed by ROS and NO signaling (Nomura et al., 2012). Moreover, a single NLR may activate distinct signaling pathways in the cytoplasm and nucleus (Heidrich et al., 2013).

Recognition and mode of action of other pathogen effectors

The molecular mechanisms by which pathogen effectors are recognized are very variable. For instance, the *Pst* effector AvrRps4 is recognized by the TNL receptor RPS4, which is localized at endomembrane and inside nucleus. The plant defense regulator EDS1 (enhanced disease susceptibility 1), a lipase-like protein, is an essential component of RPS4 (resistance to *Pseudomonas syringae* 4) - triggered ETI (Gassmann et al., 1999; Wirthmueller et al., 2007), forming the EDS1-RPS4 complex in the nuclei and cytoplasm with defense co-regulators PAD4 (phytoalexin deficient 4) and SAG101 (senescence associated gene 101) (Feys et al., 2001; 2005; Bhattacharjee et al., 2011; 2013; Heidrich et al., 2013).

The *Pst* effector HopAO1 is a protein effector with tyrosine phosphatase activity (Underwood et al., 2007), that targets a specific Tyr residue of the PRR EFR (also

probably FLS2) by phosphorylation, to inhibit ligand-induced activation of the PRR, and suppresses the subsequent immune response (Espinosa et al., 2003; Macho et al., 2014).

Instead of targeting NLR receptors, the HopAI1 can directly target the PRR downstream signaling cascades and inactivate MAPK3, MAPK4 and MAPK6 by dephosphorylating these kinases (Zhang et al., 2007). The HopF2 effector inactivates MKK5 (and probably other MKKs) to suppress downstream defense responses by inhibiting MAPK signaling (Wang et al., 2010; Mukhtar et al., 2011; Feng and Zhou, 2012). The effector AvrB was also reported to regulate hormone signaling by inducing MAPK4 phosphorylation, thus enhancing plant susceptibility (Cui et al., 2010).

An alternative way to suppress immunity is by targeting components upstream of MAPKs through pathogen effectors. For instance, *Pst* AvrPto and AvrPtoB could directly target the PAMP receptors FLS2, EFR and CERK1 (chitin elicitor receptor kinase 1) to block PTI. BAK1 kinase activity is inhibited by AvrPtoB, and likewise, BIK1 is cleaved and uridylylated by AvrPphB and AvrAC to inhibit PTI signaling (Meng and Zhang, 2013).

Plant pathogens produce not only protein effectors, but also small molecules such as the phytotoxin Phevamine A from *Pst*, which suppresses the polyamine biosynthesis pathway and plant immune response, therefore promoting bacteria colonization (O'Neill et al., 2018). In addition, coronatine, which is present in most bacteria, disrupts plant immune signaling through structurally mimicking the plant hormone JA-isoleucine (JA-Ile) (Petersen et al., 2000; Geng et al., 2014). NLR-mediated immune responses can induce overlapping of SA, JA and ET signaling pathways (Miché et al., 2018). In contrast to PTI, ETI induces more durable and long-lasting responses, thereby causing programmed cell death, leading to pathogen resistance (Zhang and Zhou, 2010; Peng et al., 2018; Ramirez-Prado et al., 2018).

1.2.3. Systemic acquired resistance (SAR)

Besides local defense mechanisms, immune responses can be induced at whole plant level against a broad-spectrum of pathogens. One of these systemic defense signaling responses is induced systemic resistance (ISR), which is triggered by beneficial rhizobacteria in roots (Van Wees et al., 2008). The other mechanism is systemic acquired resistance (SAR), which is induced by pathogen infection or certain chemicals applied to local tissues. SAR is a mechanism of long-lasting global resistance against a broad range

of pathogens and insect herbivores followed by primary treatment with chemicals, bacterial or fungal pathogens (Kohler et al., 2002; Fu and Dong, 2013; Conrath et al., 2015). SAR was first described by Frank Ross in 1961. Since then, SAR has been observed in a wide variety of plant species (Chester, 1933; Ross, 1961; Spoel et al., 2003; Pieterse et al., 2009; Fu and Dong, 2013). SAR is also induced by PTI and ETI, and involves the biosynthesis of SA, systemic expression of *PR* genes, ROS production and and/or cell death (Pieterse et al., 2014). SAR also downregulates photosynthesis and growth-related processes, and counteracts the fitness disadvantage resulted from disease invasion (Ryals et al., 1996; Fu and Dong, 2013).

Tobacco plants challenged with tobacco mosaic virus (TMV) induce SAR as early as 4 to 6 h after primary infection. Enhanced disease resistance in distant leaves (uninfected leaves) is detected about 48 h post primary inoculation, reaching a maximum of resistance at 7 to 10 days post-inoculation (dpi), which persists up to 20 dpi (Ross, 1961; Klessig et al., 2018). Moreover, epigenetic alterations by modulation of DNA methylation at the promoters of defense-related genes enable SAR transfer to the next generation (Luna et al., 2012; Slaughter et al., 2012).

To date, a number of SAR inducers have been reported. These include, SA and its derivative MeSA (Durrant and Dong, 2004; Park et al., 2007), the plastid lipid-derived oxylipin acid azelaic acid (AzA) (Jung et al., 2009; Cecchini et al., 2019), glycerol-3-phosphate (G3P) (Chanda et al., 2011), the abietane diterpenoid dehydroabietinal (DA) (Chaturvedi et al., 2012), the lysine (Lys) derivative N-hydroxypipecolic acid (NHP) (Návarová et al., 2013), free radicals, nitric oxide (NO) (Wendehenne et al., 2014) and reactive oxygen species (ROS) (Wang et al., 2014). Moreover, both AzA and G3P function downstream of NO and ROS (Lim et al., 2016a). Among these inducers, SA regulates one branch, whereas AzA, G3P, NO and ROS participation on SAR seem to be on another separated branch (Shine et al., 2019).

The lipid transfer protein (LTP) family homolog, DIR1 (defective in induced resistance 1), the LTP-like protein AZI1 (azelaic acid induced 1) and its closest paralog EARLI1 (early Arabidopsis aluminium induced 1) are required for the SAR signaling transport (Jung et al., 2009; Klessig et al., 2018). DIR1, AZI1 and EARLI1 are SAR-specific components proposed to form SAR complexes as part of a functional unit. Mutants of AZI1 and DIR1 not only lack SAR but are also not responsive to AzA.

The DIR1 - AZI1 - EARLI1 unit may play critical roles on mobilizing long distance SAR signals through the phloem to distant leaves (Cecchini et al., 2015b; Carella et al., 2017). DIR1 and AZI1 interact to modulate SAR signaling pathway induced by AzA which is dependent on MAPK3 and MAPK6 activities (Cecchini et al., 2015). The phloem is the major conduit for transporting SAR signal(s), the transport of AzA and G3P occur via the symplast, whereas SA is transported through the apoplast (**Figure 3**).
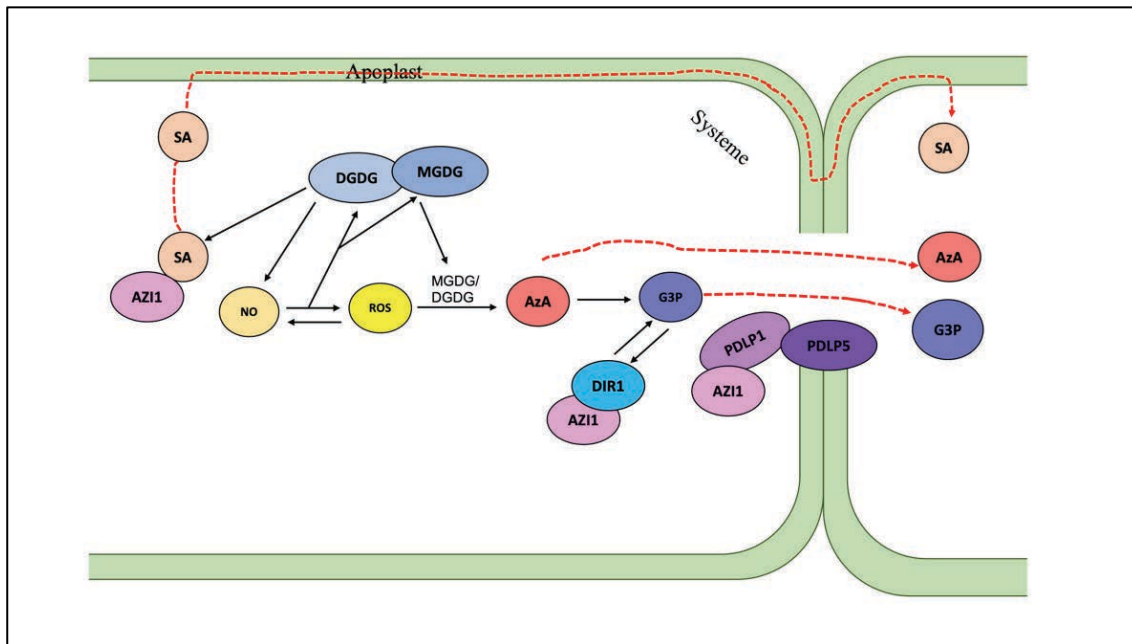
Figure 3. Model summarizing metabolite and protein-mediated signaling during systemic acquired resistance (Modified from Shine et al., 2019). SA, salicylic acid; NO, nitric oxide; ROS, reactive oxygen species; DGDG, galactolipid digalactosyldiacylglycerol; MGDG, monogalacto-syldiacylglycerol; AzA, azelaic acid; G3P, glycerol-3-phosphate; DIR1, Defective in induced resistance 1; AZI1, AZA induced 1 PDLP1, PDLP5; plasmodesmata localizing protein 1 and 5.

A successful induction of SAR not only depends on the inducers but also on the "perception" of the mobilization signals in distal tissues. SAR perception requires the membrane galactolipids digalactosyl-diacylglycerol (DGDG), monogalactosyldiacyl-glycerol (MGDG) and PD localized proteins (PDLPs). MGDG regulates the biosynthesis of AzA and G3P which are induced by ROS and NO. DGDG but not MGDG is required for AzA-induced SAR (Gao et al., 2014). The symplastic transport of AzA and G3P is regulated by PDLP5 and PDLP1. Notably, plants defective in these components are

unable to perceive though can generate the SAR signal (Singh et al., 2017; Shine et al., 2019).

1.2.4. Salicylic acid and SAR

SAR response is associated with the accumulation of SA. Both ICS1 (isochorismate synthase 1, also known as SA induction deficient 2, SID2) and PAL5 (phenylalanine ammonia-lyase 5) biosynthesis pathways contribute to SAR, while the former contributes the most to SA biosynthesis. Arabidopsis possesses two *ICS* genes, *ICS1* and *ICS2*, however, only *ICS1* is upregulated during pathogen-inoculation and in systemic leaves in the course of SAR (Hartmann and Zeier, 2019). Levels of SA and its derivative methyl SA (MeSA) increase in local and systemic tissues during SAR. However, SA levels in distal tissues are significantly lower than in infected ones (Gao et al., 2015). SA is not the mobile signal during SAR, since grafting experiments using transgenic tobacco plants expressing the *NahG* gene (salicylic hydroxylase) exhibit normal SAR (Yamamotoj et al., 1965; Vernooij et al., 1994; Kiefer and Slusarenko, 2003). However, SA accumulation is necessary for the induction of local resistance expression (Malamy et al., 1996). It is speculated that MeSA may function in delivering SA to distal tissues (Meuwly et al., 1995; Gao et al., 2015). MeSA synthesized from SA by benzoic acid/SA methyltransferases (SAMT/BSMT), is transported through the phloem, and then converted back to SA by the MeSA esterase SA binding protein (SABP) (Chen et al., 2003; Kumar and Klessig, 2003; Koo et al., 2007). SA signaling functions in parallel with the AzA/G3P-derived branch to regulate SAR (Hunt et al., 1996; Durrant and Dong, 2004; Conrath et al., 2006; Fu and Dong, 2013; Ding and Ding, 2020). Lim et al. (2016a) showed that the intracellular movement of SA occurs via the apoplastic compartment (**Figure 3**). On the other hand, pathogen induced SA accumulation in infected cells can hinder the intercellular movement of SAR signals solutes, such as AzA, G3P via the PD to the neighbor cells, since SA application reduces PD permeability (Lim et al., 2016a; Wang et al., 2013) by action of the PD-localizing protein 5 (PDLP5) (Lee et al., 2011) (**Figure 3**). SA-accumulation and immunity during SAR are positively regulated by lipase-like proteins EDS1, PAD4, the integrin-like protein NDR1 (non-race-specific disease resistance 1) and SID2 in distant leaves (Fu and Dong, 2013; Shah et al., 2014). In addition, SA signaling is positively regulated by MAPK3 and MAPK6 activity (Beckers et al., 2009), while MAPK4 cascade negatively regulates SA signaling (Petersen

et al., 2000). On the other hand, SA accumulation can drive NPR1 (non-expressor of pathogenesis-related 1), a SAR regulator, interact with TGA transcription factors, thus activate defense related transcription and redox changes, which in turn induce systemic PR protein expression (Després et al., 2003; Mou et al., 2003; Wang et al., 2005). Recently, Wang et al. (2020) reported that SA binds the core domain of NPR4 (non expressor of pathogenesis-related 4), whereas NPR1 displays minimal SA-binding activity compared to NPR4. Pathogen associated cell death is not required for SAR response, as the application of SA stimulated SAR downstream from cell death (Hartmann et al., 2018; Shine et al., 2019). On the other hand, during SAR, activation of SA suppresses pathogen-inducible JA responses in systemic tissues, while JA promotes SAR signaling by upregulating MeSA production in the inoculated leaves (Dempsey and Klessig, 2012; Bernsdorff et al., 2016).

1.2.5. ROS and SAR

Pathogen infection leads to the accumulation of SA and nitric oxide (NO), which trigger the accumulation of ROS via an amplification loop. ROS, in turn, generate AzA from unsaturated fatty acids (FAs). AzA then induces the synthesis and accumulation of G3P, which travels symplastically to the phloem and, together with SA, induces SAR (Yu et al., 2013; Lim et al., 2016c; Klessig et al., 2018).

Elevated levels of H_2O_2 function as a second messenger for SA during SAR signal transduction (Chen and Klessig, 1991; Ryan et al., 1995). However, it has been reported that the establishment of SAR does not necessarily correlate with increased H_2O_2 levels in tobacco leaves (Hunt et al., 1997). Nevertheless, very high phytotoxic levels of H_2O_2 (1 M) can induce *PR1* gene expression and this induction is dependent on the ability of the plant to accumulate SA, while potentiating the free radical burst associated with local defense responses (Marrè et al., 1998). RBOH generate ROS, and RBOHD/F are functionally non-redundant in SAR-related ROS generation. Additionally, NO also serves as a mobile reservoir for NO (Wang et al., 2013). Like ROS, NO is also highly concentration dependent. Too low or too high levels of NO can inhibit SAR (Wang et al., 2014). In plants, NO can be directly synthesized from nitrate by the nitrate reductases NIA1 and NIA2, which are functionally non-redundant in SAR. In addition, the GTPase AtNOA1 (NO associated protein 1) also contributes to NO levels via an unknown

mechanism (Shine et al., 2019). Pathogen-induced NO accumulation also depends on RBOHD and RBOHF (Wang et al., 2014) .

1.2.6. N-hydroxypipecolic acid and SAR

Pipecolic acid (Pip), is a non-peptide amino acid, which is the precursor of *N*-hydroxy-pipecolic acid (NHP), and plays an important role in inducing SAR signal transduction, since it accumulates in local and distal tissues upon pathogen inoculation in Arabidopsis (Návarová et al., 2013; Chen et al., 2018). Pip might be a mobile metabolite and it is indispensable for SAR since it is enriched in the petiole exudates of inoculated leaves (Návarová et al., 2013; Bernsdorff et al., 2016). The aminotransferase ALD1 (AGD2-like defense response protein 1) and SARD4 (SAR-deficient 4) are involved L-Pip biosynthesis from lysine in plastids (Song et al., 2004; Ding et al., 2016). Both *ALD1* and *FMO1* are induced in inoculated and systemic tissues during pathogen infection, and their induction is independent of SA accumulation (Mishina and Zeier, 2006). Notably, ALD1 is an essential SAR component which is upregulated in both local infected and systemic tissues (Song et al., 2004). It has been reported that one or more ALD1-generated, non-Pip metabolites or Pip precursor may regulate basal resistance (Cecchini et al., 2015a; Hartmann et al., 2017). FMO1 (flavin-dependent-monooxygenase 1) is also required for Pip accumulation in distal leaves, as Pip cannot trigger SAR in *fmo1* mutants (Návarová et al., 2013).

During SAR, SA synthesis appears to be regulated by a positive feedback loop involving Pip, ALD1, SARD4, FMO1 and PAD4 (Návarová et al., 2013; Ding et al., 2016; Hartmann et al., 2017). SA and Pip pathways contribute to basal resistance independently from each other and synergistically upon pathogen infection. On the other hand, petiole exudates from *ALD1* overexpressing Arabidopsis plants can enhance basal resistance though not elevating the levels of Pip or SA.

1.3. ROS generation in the apoplast

In plants, ROS include hydrogen peroxide (H_2O_2), superoxide ($O^{.-}$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($^{.}OH$). ROS is produced in various subcellular compartments, such as plasma membrane, cell wall, mitochondria, chloroplasts and peroxisomes /

glyoxysomes (Singh et al., 2016). ROS is produced in response to biotic and abiotic stresses to support cell wall remodeling, to act as signal molecules regulating plant growth, development, and to provide a toxic effect (Suzuki et al., 2011; Marino et al., 2012; Qi et al., 2017). There are three main sources of apoplastic ROS upon pathogen inoculation: plasma membrane-localized NADPH/NADH oxidases RBOHs (Kadota et al., 2015), cell wall peroxidases (PRX) and amine oxidases (AO) (Daudi et al., 2012; Qi et al., 2017).

The function of RBOH-dependent apoplastic ROS production has been studied in various plant species (Zhang and Zhou, 2010; Marino et al., 2012; Le Roux et al., 2015). Arabidopsis contains ten RBOH members encoded by *RBOHA* to *J* genes (Torres et al., 2005), among them, RBOHD and RBOHF play vital roles in the production of apoplastic ROS, regulating stomata closure and triggering hypersensitive response (HR) during plant-pathogen infections (Torres et al., 2002; Suzuki et al., 2011; Morales et al., 2016; Liu et al., 2019). RBOHs transfer electrons from cytosolic NADPH or NADH to apoplastic oxygen, leading to the production of superoxide ($O_2^{.-}$), which can be converted into H_2O_2 by superoxide dismutase (SOD) (Suzuki et al., 2011; Marino et al., 2012; Kadota et al., 2015). H_2O_2 often functions as an intercellular signal, and triggers long-distance signaling by entering neighboring cells (Miller et al., 2009; Suzuki et al., 2013; Mittler and Blumwald, 2015).

RBOHD-dependent ROS production in response to MAMPs/DAMPs regulates lignin biosynthesis, callose deposition (Luna et al., 2011; Poovaiah et al. 2014), proline accumulation and antioxidant defenses (Ben Rejeb et al., 2015). Moreover, phytohormones, such as ET and SA regulate ROS production through RBOHD activity (Boutrot et al., 2010; Yi et al., 2014). In addition to RBOHD, class III peroxidases (PRXs) are also widely responsible for apoplastic H_2O_2 accumulation during PTI. RBOHD-mediated rapid ROS production triggers a secondary PRX33/34-dependent ROS production (Nühse et al., 2007; Zhang et al., 2007). Both ROS and callose deposition are compromised in PRX34 defective lines upon flg22 treatment, thus leading to enhanced susceptibility to pathogens (Zhao et al., 2019).

Amine oxidases catalyze the oxidative de-amination of polyamines, ubiquitous polycationic compounds involved in crucial events in the life of cells (Seiler, 2004; Tiburcio et al., 2014). Amine oxidases include the copper-containing amine oxidases (CuAO) and the flavin-containing polyamine oxidases (PAO). In plants, apoplastic CuAO

and PAO directly produce H_2O_2 (Cona et al., 2006; Guo et al., 2014). (***See "biosynthesis of polyamines" section***)

Collectively, SAR is a critical event of the plant's overall immune response and it is involved in complex signaling networks and physiological processes. The parallel operation and transport of different chemical signals (NO-ROS-AZA-G3P and SA) during SAR is likely advantageous, as it enables plants to simultaneously engage multiple physiological processes in response to stress. The availability of multiple points at which the NO- and SA-derived pathways can be co-regulated facilitates a tighter control of SAR (Shine et al., 2019). The different transport routes of SA compared to AzA or G3P likely provide multiple avenues for controlling the transport of these defense chemicals and thereby minimizes the chances of non-specific activation of defense processes in systemic tissues.

1.4 Introduction to polyamines in plants

Polyamines (PAs) are water-soluble aliphatic polycationic amino groups, widely present in almost all eukaryotic organisms (Pegg and McCann, 1982; Wallace et al., 2003). PAs were first discovered by van Leeuwenhoek in 1678 from human semen (van Leeuwenhoek, 1678). The most common polyamines are the diamines Cadaverine (Cad) and putrescine (Put), triamine spermidine (Spd), and tetramine spermine (Spm) as well as its structural isomer thermospermine (tSpm). Notably, Arabidopsis does not contain Cad (**Figure 4A**). In plants, PAs generally occur as free or hydroxycinnamic acid conjugated forms. PAs play crucial roles in, i) cellular processes such as cell growth, differentiation, gene expression, DNA and protein synthesis; ii) physiological processes like organogenesis, embryogenesis, floral initiation and development, leaf senescence, pollen tube growth, fruit development and ripening; iii) in various stresses responses, as PAs can induce NO and ROS accumulation (Evans and Malmberg, 1989; Seiler, 2004; Alcázar et al., 2006a; 2010a; Kusano et al., 2007; Tiburcio et al., 2014; Agurla et al., 2018; Recalde et al., 2018; Takahashi, 2020).
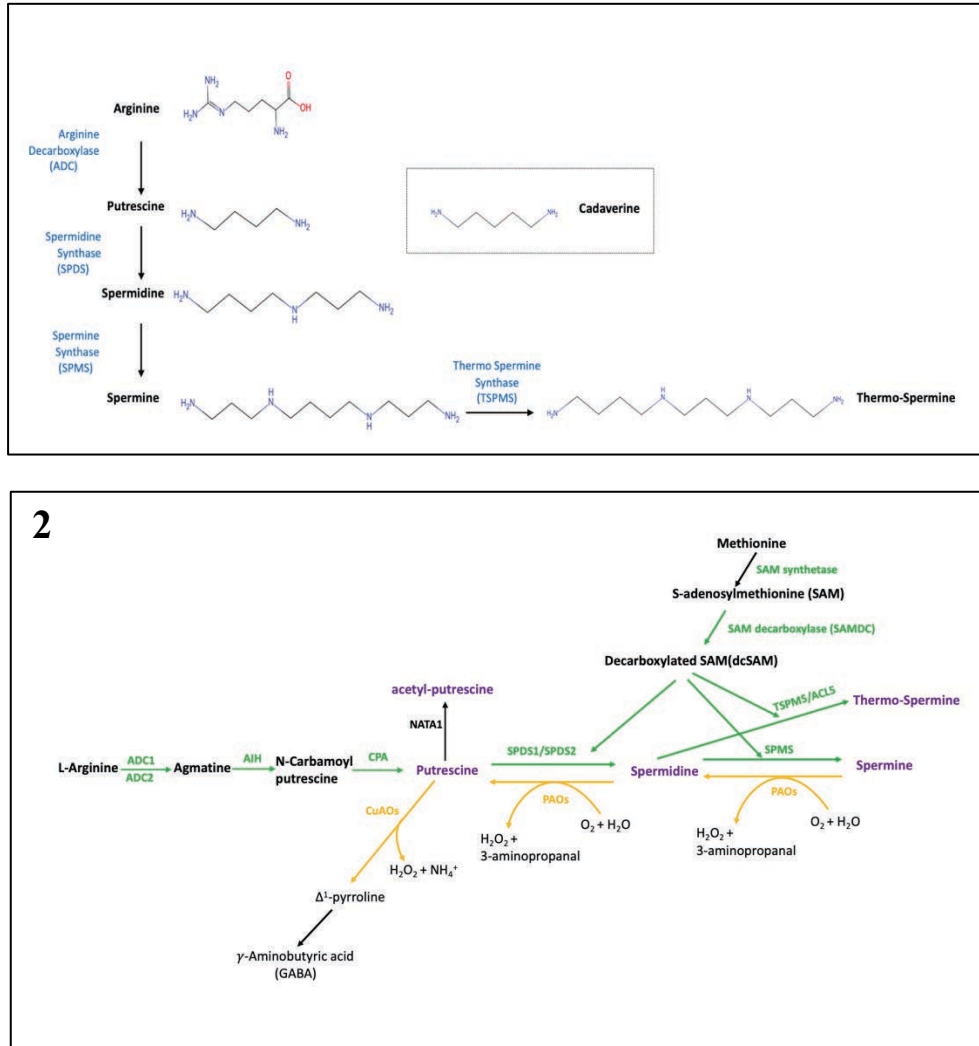
Figure 4. The plant polyamines and metabolic pathways in Arabidopsis (Modified form Alcázar et al., 2010a). The polyamines putrescine, spermidine, spermine and thermospermine in *Arabidopsis thaliana* (Cadaverine is not existed in Arabidopsis) (A). Biosynthetic and catabolic pathways for polyamines and related metabolites (B). ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, N-carbamoylputrescine amidohydrolase; NATA1: N-Acetyltransferase activity 1; SPDS, spermidine synthase SPMS, spermine synthase; ACL5, acaulis5; TSPMS, thermo-spermine synthase; SAMDC, *S*-adenosylmethionine decarboxylase; PAO, polyamine oxidases; CuAO, copper-containing amine oxidases.

1.4.1 Polyamine metabolism in plants

The first and limiting step in the biosynthesis of Put is the decarboxylation of arginine or ornithine mediated by arginine decarboxylase (ADC) or ornithine decarboxylase (ODC) enzymes, respectively. However, whether ODC route exists in Arabidopsis is still under debatable, since many attempts to clone the ODC gene have failed (Hanfrey et al., 2001), nonetheless a putative ODC activity is detected in Arabidopsis (Tassoni et al., 2003). Catalysis from Put to Spd requires Spd synthase (SPDS, EC 2.5.1.16), which

transfers aminopropyl residues to amine acceptors forming Spd, and then generates Spm by Spm synthase (SPMS, EC 2.1.22). The donor of the aminopropyl groups is decarboxylated S-adenosylmethionine (dcSAM), which is formed through the decarboxylation of S-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC; EC 4.1.1.50) (Slocum et al., 1984). In Arabidopsis, the key PAs biosynthesis genes are duplicated, for instance, two genes encoding ADC (*ADC1* and *ADC2*), SPDS (*SPDS1* and *SPDS2*), SPMS [*SPMS* and *acaulis 5* (*ACL5*)] and SAMDC (*SAMDC1* to *SAMDC4*) (**Figure 4B**) (Takahashi and Kakehi, 2010).

Polyamine degradation involves one or more diamine oxidases (DAO, EC 1.4.3.6) and FAD-containing polyamine oxidases (PAO; EC 1.5.3.11), which oxidize the carbon adjacent to the secondary or the primary amino groups (Moschou et al., 2012; Tavladoraki et al., 2012; 2016). Arabidopsis contains five *PAO* genes, termed *AtPAO1* to *AtPAO5*, which proteins are located in the cytoplasm or peroxisomes (Tavladoraki et al., 2006; Kamada-Nobusada et al., 2008; Takahashi and Kakehi, 2010; D. W. Kim et al., 2014). In some species including Arabidopsis, PAOs oxidize Spm to Spd and subsequently Spd to Put, producing H_2O_2 and 3-aminopropanal (Cona et al., 2006). PA catabolism by terminal oxidation or back-conversion are common pathway in plants (**Figure 4B**) (Moschou et al., 2008).

DAOs are copper-containing amine oxidases (CuAOs) localized in the apoplast or peroxisomes that typically catalyze the oxidation of diamines Put or Cad at the primary amino groups, yielding Δ^1-pyrroline, H_2O_2 and ammonia (NH^{4+}) (**Figure 4B**) (Cohen, 1998; Alcázar et al., 2010a). Arabidopsis possesses ten genes encoding CuAOs [*AtCuAOα1*, *AtCuAOα2*, *AtCuAOα3* (*AtCuAO2*), *AtCuAOβ* (*ATAO1* or *AtAO1*), *AtCuAOγ1* (*AtCuAO1*), *AtCuAOγ2*, *AtCuAOδ* (*AtCuAOδ2*), *AtCuAOζ* (*AtCuAO3*), *AtCuAOε1* (*AtCuAOε*) and *AtCuAOε2* (*AtCuAOδ1*)], which are located in extracellular or peroxisomal compartments (Planas-Portell et al., 2013; Ghuge et al., 2015; Tavladoraki et al., 2016).

1.4.2 Polyamines in response to stresses

PAs play many roles during the stress response, like modulation of the ion channels, active oxygen scavenging, elicitation of resistance gene expression, cell membrane stabilization and maintenance of cellular pH (Alcázar et al., 2010a; Liu et al., 2018; Chen

et al., 2019 and reference therein). The charges of polyamines are positive (Spm > Spd > Put), thus can bind to negatively charged nucleic acids, proteins, cell wall (Walden et al., 1997; Roussos and Pontikis, 2007), and modulate ion channels (Yamaguchi et al., 2006). PAs can regulate the size of ion channels, like K^+ and Ca^{2+}, thus strongly regulating pore opening and closure, which further controls water loss (Liu et al., 2000). Stress-induced PAs, especially Spm accumulation, are thought to be stabilizers rather than protecting the membrane system from denaturing (Hussain et al., 2011 and references therein; Romero et al., 2018). However, it has been reported that high level of Spd, and lower Put and Spm help to heat tolerance in alfalfa (Medicago sativa L.) (Zhuo et al., 2018). This suggests that the protective functions of PAs might be more evident in some species than others (Chen et al., 2019). In barley, the resistant cultivar accumulates higher Put and Spd levels than the susceptible one after infection of *Blumeria graminis* f. sp. *hordei* (Cowley and Walters, 2002).

Considering the advantages of polyamines in response to external stimuli, some works focused on the exogenous application of polyamines to enhance resistance. Spm protects against high salt stress, and double Arabidopsis knockout-mutant *acl5spms* exhibits higher sensitivity to high salt compared to wild type, whereas this deficiency is restored by exogenous Spm but not Put or Spd (Yamaguchi et al., 2006). In *Allium fistulosum*, exogenous application of Put reduces oxidative damage through enhancing the antioxidant capacity, thereby stabilizing cell membranes under flooding stress (Yiu et al., 2009). Moreover, it is also demonstrated that PAs regulate H_2O_2 and NO involved signal transduction during abiotic stress (Pál et al., 2015).

The rate-limiting enzyme at the beginning of the polyamine biosynthesis pathway is ADC2*,* which expression is strongly induced by several abiotic stresses like drought, high salinity, mechanical wounding and osmotic stress (Perez-Amador, 2002; Urano et al., 2005; Pál et al., 2018; Chen et al., 2019). On the other hand, during the response to pathogen infection, the expression and activity of both ADC1 and ADC2 are strongly upregulated (Fuell et al., 2010; Romero et al., 2018; Amrani et al., 2019). However, ADC2 makes more contribution to Put accumulation than ADC1 in the Arabidopsis response to certain bacterial pathogens (Rossi et al., 2015; Liu et al., 2019). Over-expression of *ADC2* leads to the accumulation of Put and higher tolerance to drought and cold stresses (Alcázar et al., 2006; 2010c). In contrast, Arabidopsis *adc2* knockout mutant shows enhanced susceptibility to *Pst* DC3000 (Kim et al., 2013). Similar to *ADC2*, the

expression of *SPDS1* and *SPMS* increases markedly under dehydration and high salinity. However, no changes in *ACL5* and *SPDS2* expression have been reported in response to any assayed stress (Urano et al., 2003; Alcázar et al., 2006a; 2006b). Expression of *SAMDC2* is induced mainly by cold and low salinity, whereas *SAMDC1* expression upregulation is caused by cold treatment in Arabidopsis cell suspensions (Vergnolle et al., 2005).

Under stress conditions, PA levels in plants are also regulated by catabolic pathways which produce ROS (H_2O_2) (Bagni and Tassoni, 2001; Cona et al., 2006). PAs like Spm and Spd are regarded as potent inducers of NO in plants (Tun et al., 2006). Accumulation of H_2O_2 and NO triggered by Spd or Spm plays a critical signaling role in plant-pathogen interactions (Walters, 2003; Romero-Puertas et al., 2004; Yamasaki and Cohen, 2006; Hussain et al., 2011). PAs synergistic interplay with ABA, H_2O_2 and NO, thus regulating stomatal aperture during abiotic stress (Alcázar et al., 2010a; 2010b). In Arabidopsis, silencing of cytoplasmic *PAOs* enhances salinity tolerance through dampening ROS production (Sagor et al., 2016). Likewise, loss-of-function of *PAO5* (*atpao5*-2 and *atpao5*-3) leads to constitutively higher tSpm levels which associated with increased salt tolerance (Zarza et al., 2017). Compared to untreated Arabidopsis plants, activities of CuAOs are higher in incompatible interactions between various plants and pathogens (Walters, 2003). *AtCuAOα2*, *AtCuAOα3*, *AtCuAOγ1* and *AtCuAOγ2* are also induced during dehydration recovery, wounding and Put treatment (Fraudentali et al., 2020), furtherly, *AtCuAOα2* regulates arginine-dependent nitric oxide production (Groß et al., 2017).

On the other hand, exogenous application of PAs increases the activity of peroxidase (POD) and catalase (Cat), thereby reducing H_2O_2 levels. For instance, exogenous Spd application elevates the accumulation of endogenous PAs (Put, Spd and Spm), and improves drought tolerance associated with antioxidant defense in creeping bentgrass (Li et al., 2015). Conjugated PAs are more efficient as antioxidants than free forms as scavengers of free radicals (Edreva et al., 2007). Collectively, homeostasis of PAs is maintained by biosynthesis and oxidation processes, thereby modulating plant resistance in response to stresses (Mayer and Michael, 2003).

Polyamines elicit defense signaling

Spm accumulation after lesion formation during TMV (tobacco mosaic virus) - tobacco infection, is reported as an endogenous inducer of defense related to transcriptional

expression and resistance, which is SA–independent (Yamakawa et al., 1998). Additionally, in Arabidopsis, Spm was found to function as a signaling molecule that induces defense reactions (cell death) during the cucumber mosaic virus (CMV) infection (Mitsuya et al., 2009). Two MAPKs are involved in Spm stimulation activity, WIPK (wound-induced protein kinase) (Seo et al., 1995) and SIPK (salicylic acid-induced protein kinase) (Zhang and Klessig, 1997), which trigger the expression of downstream defense genes (Takahashi and Kakehi, 2010).

1.4.3 The polyamine putrescine in response to stresses

In Arabidopsis, Put accumulation due to high ADC activity enhances tolerance to salt (Kasinathan and Wingler, 2004), mechanical wounding (Perez-Amador et al., 2002), dehydration and freezing stress (Alet et al., 2011). Similarly, in barley, the levels of Put and Spm are increased after infection with the powdery mildew fungus *Blumeria.graminis* f.sp. *hordei*, which is accompanied by increased ADC activity (Cowley and Walters, 2002). Rossi et al. (2015) reported that isoforms of ADC might play partially redundant functions after infection with *P. viridiflava.* In addition, Put synthesis is highly induced by *Pst* DC3000 inoculation, while *ADC1* transcript levels are only slightly upregulated (Kim et al., 2013). The *ADC2* knockout mutant (*adc2-1*) shows more sensitive to salt stress, which can be partially reversed by exogenous application of Put (Urano et al., 2004). Compared to the wild type, Put biosynthesis defective mutants (*adc1* and *adc2*) are more susceptible and exhibit reduced expression of *PR 1* after infection with *Pst* DC3000 (Kim et al., 2013). Moreover, overexpression of the homologous *ADC2* gene leads to the accumulation of Put but not Spd or Spm, and confers drought tolerance in Arabidopsis (Alcázar et al., 2010c). On the other hand, ADC1 also enhances Put accumulation during cold acclimation, leading to freezing tolerance in potato (Kou et al., 2018). The competitive inhibitor DL-α-difluoromethylarginine (DFMA) of ADC was used in rice seedling showed that inhibition of Put biosynthesis can enhance salt stress sensitivity and decrease Spd content (Yamamoto et al., 2017). Exogenous Put application increases not only the levels of endogenous PAs, but also the antioxidant enzyme activities, as well as proline content in *Anthurium andraeanum* (Chen et al., 2019).

Moreover, Put reduces H_2O_2 and lipid peroxidation under NaCl stress in Brassica seedlings, since Put increased the activity of antioxidant enzymes and carotenoids (Verma and Mishra, 2005). In wheat, Put is found to act as an inducer of trichothecene mycotoxin

production during the infection of fungal pathogen *Fusarium graminearum* (Gardiner et al., 2010). Interestingly, Walters et al. (2002) reported that the infection of the first leaves with methyl jasmonate in barley seedlings, leading to increased levels of Put and Spd conjugates, and increased activity of biosynthetic enzymes and DAO, further enhancing resistance in uninfected distal leaves. In addition, mechanical wounding of the first leaves in oilseed rape (*Brassica napus* ssp. *oleifera*), leads to significant, but transient, increases of ADC activity and free Put in both wounded first leaf and in unwounded secondary leaves. The activity of CuAOs is significantly reduced in both local and systemic leaves, thereby Put catabolism is reduced (Cowley and Walters, 2005). These data suggested that PAs (Put) might trigger systemic resistance. Collectively, Put accumulation associates with high ADC activity and enhanced resistance or tolerance against various stresses.

Despite the tremendous pioneering studies about polyamines during the plant stress response, the signaling pathways underlying polyamine functions have remained elusive. Here, we find that exogenous Put contributes to H_2O_2 and RBOHD/F dependent PTI response through a positive feedback loop. Furthermore, polyamines, and Put in particular trigger ROS-dependent salicylic acid pathway activation leading to systemic acquired resistance in Arabidopsis (***See Chapters 1*** and ***2***)

1.5. Callose biosynthesis during defense

The β-1,3 -D-glucan polysaccharide callose is synthesized by glucan synthase-like (GSL) enzymes, also known as callose synthases (CalS) (**ANNEX I; Table S1**) (Stone and Clarke, 1992). Callose deposits can be visualized by aniline blue staining under an epifluorescence microscope (Currier and Strugger, 1956). There are two types of callose: peripheral and interstitial callose (Nishikawa et al., 2005; Dong et al., 2005). Callose is an essential component during growth, development and defense response to stresses, which is synthesized by GSL proteins with UDP-glucose as substrate in plants (Xie and Hong, 2011). Callose accumulation is transient, with the polymer being removed once other polysaccharides such as hemicelluloses, pectins, and cellulose are deposited at the cell plate during cytokinesis (Samuels et al., 1995; Nishihama et al., 2002; Albersheim et al., 2010).

Arabidopsis has twelve glucan synthase family members (Hong et al., 2001), which are divided into two groups based on their biological functions. GSL1, GSL2, GSL6, GSL8 and GSL10 constitute the largest group, which is mainly responsible for callose biosynthesis during pollen development and cell division (Drábková and Honys, 2017). GSL4, GSL5, GSL7 and GSL12 are involved in callose deposition during plugging, barrier formation, and other types of structural reinforcements (Hong et al., 2001; Ellinger and Voigt, 2014). For the rest GSL members, GSL3, GSL9 and GSL11 functions are as yet unclear (Drábková and Honys, 2017). Also, the GSL family can be classified into four main subfamilies based on phylogenetic analyses (Drábková and Honys, 2017).

Callose contributes to the formation of sieve and cell plates, and it is involved in multiple developmental stages of male gametophytes (Chen and Kim, 2009). In addition, callose deposites at the plasma membrane and the cell wall as one of the many responses to wounding and pathogens, like fungi and oomycetes (Jacobs et al., 2003; Dong et al., 2005; Huang et al., 2009; Luna et al., 2011; Ellinger et al., 2013; Drábková and Honys, 2017). Moreover, callose formation in papilla of higher plants contributes to innate immunity and associates with global transcriptional changes (Jones and Dangl, 2006; Ellinger et al., 2013). Callose deposits are evidenced during DPI and PTI responses, and are considered as a marker of PTI activation (Clay et al., 2009; Luna et al., 2011). Callose is regarded as a potential target to achieve plant immunity in addition to its contribution to cell wall reinforcement, thus prevent further pathogen penetration (Chowdhury et al., 2016; Keppler et al., 2018). Indeed, callose inhibition studies in several Gramineae species including barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and oat (*Avena sativa*), reveal that penetration resistance to powdery mildew is especially suppressed in incompatible plant-fungus interactions (Zeyen et al., 2002). In addition, the effector Xanthan from *Xanthomonas campestris* pv. *campestris* induces plant susceptibility through suppressing callose deposition (Yun et al., 2006).

Nowadays, more attention is addressed to the role of callose in the modulation of plasmodesmal permeability during stress responses and the regulation of cell-to-cell communication. Plasmodesmata (PD) are the indispensable pathway for virus spread in plants (Benitez-Alfonso et al., 2010). Thereby, callose deposition in PD channels could slow down virus infection (Demchenko et al., 2014). Callose deposits in PD can also mediate cell trafficking in response to biotic and abiotic stresses regulated by ROS and SA (Cui and Lee, 2016).

Callose and phytohormones

Application of SA or BTH (2,1,3-benzothiadiazole) increases the production of callose deposits in response to wounding or microbes and significantly reduces virus movement (Kohler et al., 2002; Fernández-Crespo et al., 2017). Recently, Oblessuc et al. (2020) reported that callose deposition shares defense mechanisms with ICS1 and NPR1-dependent SA biosynthesis upon *Pst* DC3118 inoculation. Also, SA-dependent priming enhances flg22-triggered oxidative burst and callose deposition, while coronatine activation of JA signaling suppresses the flg22-induced callose deposition regulated by ROS. On the contrary, PAMP flg22-induced oxidative burst and callose response do not depend on SA signaling, but are suppressed by coronatine (Millet et al., 2010; Yi et al., 2014). On the other hand, under low Ca^{2+} conditions, callose accumulation alleviates cell wall damage, leading to SA/JA pathway suppression and cell death prevention (Shikanai et al., 2020).

1.5.1. GSL5 and plant defense

The plasma membrane located GSL5, also known as powdery mildew resistant (PMR) 4, is critical for callose synthesis in response to many external stimuli (Jacobs et al., 2003; Drábková and Honys, 2017). GSL5 has been shown to account for the nearly entire callose synthesis in response to wounding, pathogenic oomycetes and flg22 treatment (Jacobs et al., 2003; Nishimura et al., 2003; Clay et al., 2009; Luna et al., 2011). Overexpression of *GSL5* enhances the resistance against powdery mildew by elevating callose biosynthesis at the attempting penetration sites in Arabidopsis (Ellinger et al., 2013) and barley (Blümke et al., 2013). On the other hand, in Arabidopsis, no obvious callose deposition is observed in *gsl5* mutants, whereas SA and JA pathways are enhanced, which results in higher resistance to pathogens, thus suggesting that removal of callose can activate defense systems (Jacobs et al., 2003; Nishimura et al., 2003; Shikanai et al., 2020).

1.5.2. Co-working of GSLs

Among the twelve callose synthases members in Arabidopsis, GSL5 and GSL6 have been shown to be related to SA and/or biotic stresses (Dong et al., 2008). Apart from

GSL5, which plays major roles in callose formation in plants, other GSL family members make some extent of contribution. For instance, GSL2 is responsible for the establishment of the callose wall enclosing pollen mother cells and pollen tubes in Arabidopsis (Dong et al., 2005; Nishikawa et al., 2005), and the callose deposition in the wall and plugs of pollen tubes (Nishikawa et al., 2005). GSL8 mediates the deposition of callose at developing cell plates, root hairs, and plasmodesmata (De Storme et al., 2013). GSL7 and GSL8 are necessary for callose deposition in sieve plates, phloem transport and inflorescence growth (Paul Barratt et al., 2011; Xie and Hong, 2011; Shoala et al., 2018). GSL10 mostly localizes to the plasma membrane and is essential for microspore growth, alleviation of cell wall damage and defense responses in Arabidopsis (Töller et al., 2008; Huang et al., 2009; Chen and Kim, 2009; Shikanai et al., 2020). Increased callose deposition and reduced PD connectivity were found in gain-of-function mutants of *GSL12* (Vatén et al., 2011). In general, GSLs make contribution corporately in different cells. For instance, GSL1 works together with GSL5 in the synthesis of the callose wall that separates the microspores of the tetrad, and both are required for fertilization (Enns et al., 2005). GSL4 and GSL6 regulate callose accumulation at PD channels, thereby altering PD permeability under both pathogen infection and mechanical wounding (Cui and Lee, 2016). GSL8 and GSL10 play a role in the entry of microspores into mitosis (Huang et al., 2009), and silencing of *GSL8* or *GSL10* results in dwarfism (Töller et al., 2008). GSL4, GSL6, GSL8, and GSL12 are reported to contribute to callose formation at PD, but are not involved in SA- or ROS- dependent plasmodesmal regulation (Cui and Lee, 2016; Drábková and Honys, 2017). In addition, *gsl8* and *gsl10* mutants show a dwarf phenotype and abnormally shaped cotyledons under low calcium conditions, suggesting that GSL8 and GSL10 are required for the defense induction (Chen and Kim, 2009; Shikanai et al., 2020).

Overall, callose deposition, apart from supporting pollen formation and sieve development, also acts as one of the early responses during stimuli responses. Callose is deposited around parasite haustoria, reinforcing the cell wall as a physical barrier to slow the penetration of spores. On the other hand, it can modulate the cell transport of plasmodesmata, interacts with phytohormones and activates immunity. However, the contribution of GSL to hybrid incompatibilities and ETI have not been reported, which is the main of focus of *Chapter 3*.

1.6 Immune-related hybrid incompatibilities and the Bateson-Dobzhansky-Muller (BDM) model

Biological species are separated by pre-zygotic or post-zygotic reproductive barriers leading to reproductive isolation (RI) of individuals, which maintains gene flow barriers between species (Coyne and Orr, 2004; Vaid and Laitinen, 2019). How do these reproductive barriers contribute to plant adaptation and have evolved by natural selection has been a main focus of research by evolutionary biologists (Vaid and Laitinen, 2019). The hybrid between individuals showing reduced fitness or sterility compared to their parents is known as incompatible hybrid and this phenomenon referred to as hybrid incompatibility (HI) or hybrid necrosis (Maheshwari and Barbash, 2011). HI is a common phenomenon in nature. For instance, almost 2% of the hybrids between Arabidopsis accessions show HI (Bomblies and Weigel, 2007; Chae et al., 2014).
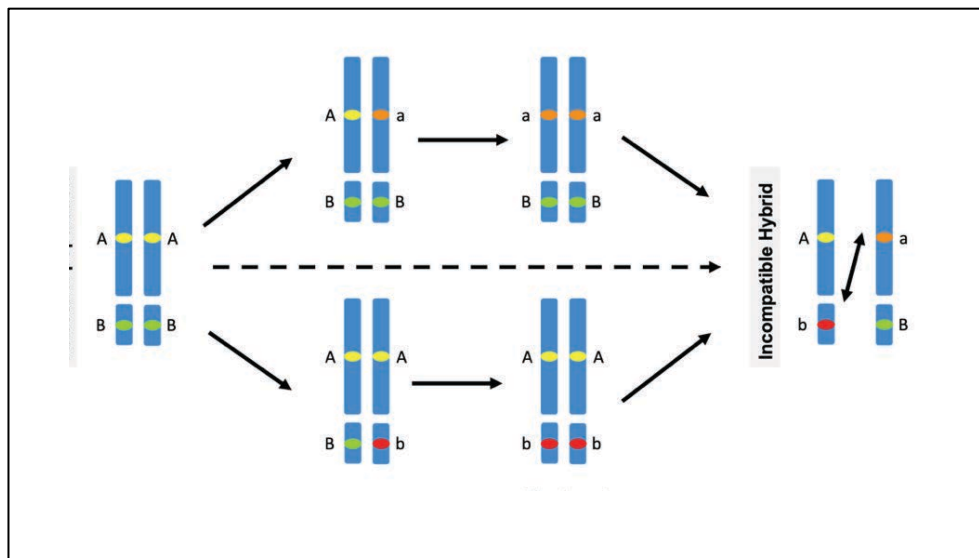
Figure 5. The Bateson-Dobzhansky-Muller (BDM) model for genetic incompatibilities. Two alleles (AABB) coexisted in ancestral populations, after split into two allopatric populations, the new populations contain independent alleles ("a" or "b"), the mutation could be fixed if they are under positive selection. Hybrid incompatibilities result from epistatic deleterious interactions when the individuals from these two divergent populations are crossed.

The HI interaction model is based on the Bateson–Dobzhansky–Muller (BDM) genetic model. According to the BDM model, independent mutations arise and fix at two or more interacting loci in each of two lineages from a common ancestor, the interaction between the newly formed alleles could cause, might be beneficially deleterious phenotypes in the F1 generation, or breakdown in the F2 generation (**Figure 5**) (Bateson, 2010; Dobzhansky, 1937; Muller, 1942). HI may arise as a by-product of natural selection, since

some of the known HI genes are likely under evolutionary selective pressure (Alcázar et al., 2010b).

Interaction Alleles	Interaction Accessions	References
DM2(RPP1) / DM1(SSI4)	UK-1 / UK-3	Bomblies et al., 2007
DM2 (RPP1) / DM9(ACD6) DM3 (At3g61540) / DM9(ACD6)	Bla-1 / Hh-0	de Felippes et al., 2012
DM2 (RPP1) / DM5	Dog-4 / ICE163	Chae et al., 2014
DM2 (RPP1) / DM4 (RPP8)	ICE163 / TueWa1-2	Chae et al., 2014
DM2 (RPP1-like) / SRF3	L*er* / Kas-2	Alcázar et al., 2009
DM6 (RPP7) / DM7 (RPW8)	KZ10 / Mrk-0	Chae et al., 2014
DM6 (RPP7) / DM7 (RPW8)	Lerik1-3 / Fei-0	Chae et al., 2014
DM8 (RPP4/5) / DM8 (RPP4/5)	Ey1.5-2 / ICE228	Chae et al., 2014
TAD3 / TAD3	Col-0 / Nok-1	Agorio et al., 2017
HISN6 / HISN6	Cvi-0 / Col-0	Bikard et al., 2009; Blevins et al., 2017

Table 1. Epistatic interactions leading to hybrid incompatibilities in Arabidopsis. (Adopted from Vaid and Laitinen, 2019). DM: Dangeous Mix; SSI4: Suppressor of salicylic acid insensitivity of npr1 4; RPP: Recognition of *Peronospora parasitica*; ACD6: Accelerated cell death 6; SRF3: Strubbelig receptor family 3; RPW8: Resistance to powdery mildew 8; TAD3: Trna adenosine deaminase 3

After the first causal gene pair underlying HI was reported in Arabidopsis by Bomblies et al. (2007), several additional genes underlying HI were mapped in various species. It has been revealed that almost all the HI-causing genes identified are likely to be associated with immune responses, since the pathogenesis-related (PR) genes are always activated in addition to the necrotic phenotype (Bomblies et al., 2007; Alcázar et al., 2009;

2010b; 2014; Chen et al., 2016; Atanasov et al., 2018). Many identified HI-related genes belong to NLR loci that, in interaction with other disease resistance genes (*R*), or genes with diverse functions, trigger the occurrence of immune-related HI (Bomblies and Weigel, 2007; Alcázar et al., 2009; 2010b; Yamamoto et al., 2010; Chae et al., 2014; Barragan et al., 2019). During the past decades, several BDM-type interaction gene pairs have been found causal for immune-related HI in Arabidopsis (**Table 1**).

Why is the immune system recruited for HI in various plant species? Microbe-driven selection accelerates the diversification of resistance genes (Jones and Dangl, 2006). The diversity of defense-related genes substantially increases the risk of a defense regulation mismatch between different populations, which may activate defense responses in the absence of infection and cause growth obstacle as a fitness cost (Chen et al., 2016).

Most of the immune-related HI cases that have been studied so far are temperature sensitive (Bomblies et al., 2007; Alcázar et al., 2009; Fu et al., 2013; Chen et al., 2014; 2016). Generally, low temperature promotes the expression of autoimmune symptoms, as high temperature can inhibit plant immunity (Traw et al., 2007; Alcázar and Parker, 2011; Hua, 2013). An exceptional contrasting case is found in rice, in which *Hwi1/Hwi2*-induced hybrid weakness is suppressed by low temperature (Chen et al., 2014). This two-locus/three-gene system was assumed to over-activate defense responses through PTI.

DM2 involved immune-related HIs is not only a hotspot in Arabidopsis, have also been reported in rice (*Oryza sativa*), lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), wheat (*Triticum aestivum*), the genus *Capsella*, and other species. In addition to *Hwi1/Hwi2*, Yamamoto et al. (2010) also reported that casein kinase I gene and an NLR cluster contribute to a two-way recessive interaction causing hybrid breakdown in rice. In lettuce interspecies (*Lactuca sativa* and *Lactuca saligna*), a specific combination of allelic RIN4 and a heterologous gene induces temperature-dependent hybrid necrosis (Jeuken et al., 2009). Cf-2 and Rcr3 interaction was observed in tomato interspecies, Rcr3 encodes a secreted papain-like cysteine endoprotease, which provides resistance to *Cladosporium fulvum* (Krüger et al., 2002). Closer to the Arabidopsis genus, the NPR1 allele shared by *Capsella grandiflora* and *Capsella orientalis* is incompatible with *RPP5* gene from *Capsella rubella*, leading to hybrid necrosis (Sicard et al., 2016).

1.6.1 L*er*/Kas2 hybrid incompatibility, near isogenic line (NIL)

Growth of plants is influenced by genotype-by-environment interactions (G X E). Alcázar et al. (2009; 2010b) identified a temperature-dependent autoimmune related hybrid incompatible interaction between *RPP1* (recognition of *Peronospora parasitica* 1) -*like* (RPP1-like) genes from Landsberg *erecta* (L*er*) accession and Kashmir-2 (Kas-2) or Kondara (Kond) alleles of Strubbelig receptor kinase family 3 (SRF3). The L*er*/Kas-2 near-isogenic line (NIL) carrying a single L*er RPP1* locus introgression on QTL 3 in a homogeneous Kas-2 background reconstituted dwarfism, cell death and constitutive activation of SA pathway at 14-16 ºC, which are all hallmarks of HI. However, the growth and reproductive loss of NIL could be suppressed at 20-22 ºC (Alcázar et al., 2009; 2010b). The *RPP1*-like locus contains eight TNL genes (*RPP1*-like, *R1-R8*). At least two *RPP1* genes (*R3* and *R8*) within the *RPP1*-like L*er* locus are responsible for incompatibility with *SRF3* Kas-2 (Alcázar et al., 2010b). Interestingly, *R3* overexpression in a neutral background (Col-0) triggers HI phenotypes of dwarfism and sterility, whereas expression of the rest *RPP1*-like genes L*er* has no effect on growth or pathogen resistance (Alcázar et al., 2014). These results indicate that some *RPP1*-like genes are more prone to induce HI than others by means of expression variation.

A previous screen for suppression of NIL phenotypes found that suppressing immune-related L*er*/Kas2 HI does not compromise basal resistance to local *Hyaloperonospora arabidopsidis* isolate (*Hpa* Gw), which depends on another locus (*RPP7*) not related to the incompatible ones. Furthermore, global metabolite profiling revealed that the growth inhibition of the incompatible hybrids is not due to limited C (carbon), N (nitrogen), or P (phosphate,) resources (Atanasov et al., 2018). To further study the genetic requirements of L*er*/Kas-2 HI, we mapped extragenic suppressors of L*er*/Kas2 incompatibility (*sulki*) mutants to *GSL2* and *GSL10* causal genes. (***see Chapter 3***)

Objectives

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Plants recognize conserved microbial molecules (pathogen-associated molecular patterns, PAMPs) via plasma membrane associated pattern recognition receptors leading to PAMP-triggered immunity (PTI), which initiates resistance to non–adapted microbes. PTI can be suppressed by effectors, pathogenic virulence factors deployed into the cell that promote susceptibility. Certain pathogen effectors are recognized by intracellular nucleotide-binding leucine-rich-repeat (NLR) receptors, initiating effector-triggered immunity (ETI). ETI boosts PTI, and shares salicylic acid (SA) pathway and reactive oxygen species (ROS) production with PTI, ultimately leading to transcriptional reprogramming. Local pathogen recognition also triggers systemic responses that provide broad-spectrum disease resistance against secondary infection in distal (systemic) tissues, known as systemic acquired resistance (SAR). SAR associates with local and systemic biosynthesis of SA, and extensive transcriptional reprogramming.

Polyamines are known to accumulate during different abiotic and biotic stresses, and contribute to disease resistance through as yet unknown signaling pathways. Polyamines are oxidatively deaminated by copper amino oxidases (CuAO) which produce H_2O_2. Some CuAOs are located to the apoplast and may function as a source for apoplastic ROS generation during the elicitation of plant defense. Previous studies revealed that Spm seems important for the establishment of HR and basal defenses to pathogens, but less information is reported about role of Put during plant – pathogens infections.

Immune related hybrid incompatibilities (HI) in plants are one of the main problems that plant breeders face when they attempt to obtain a new variety with improved traits. In basic research, such incompatibilities involving NLR genes, which can be used for the study of ETI in the absence of pathogen challenge. Constitutive activation of defense has a direct negative impact on plant growth and fitness, and in most cases is temperature dependent. Genetic screens in the identification of suppressors of HI might help at the discovery of novel components required for the establishment of ETI.

Based on the above considerations, the specific objectives followed for each chapter are described below:

1- Chapter1

The main objective of this chapter is to investigate the contribution of polyamines to PTI by using purified PAMPs and PTI-inducing bacteria.

1. Determine the potential involvement of polyamines in the PTI metabolic response. Determination of polyamines (putrescine, spermidine and spermine) levels in response to PTI-inducing pathogens and the PAMP flg22. Analysis of the contribution of ADC1 and ADC2 isoforms in such responses by using arginine decarboxylase mutants (*adc1-2*, *adc1-3*, *adc2-3* and *adc2-4*).

2. Determine the transcriptional responses triggered by Put inoculation. To this aim, I performed gene expression analyses of PTI marker genes and callose deposition in response to Put and flg22. I also analyzed the involvement of GSL5 in such responses.

3. Determine the contribution of RBOHD and RBOHF in the PTI response triggered by PAs. For this, I performed gene expression analyses of PTI marker genes in NADH oxidase loss-of-function mutants (*rbohd*, *rbohf* and *rbohd/f*) in response to Put.

4. Determine the potential use of Put as priming agent inducing local defenses.

2- Chapter2

The main objective of this chapter is to identify polyamine signaling pathways contributing to defense, with a focus on effector triggered immunity (ETI) and systemic acquired resistance.

1. Identify genes which are differentially expressed by each of the most abundant polyamines at an early time point of analysis (1 h) by RNA-seq. Gene Ontology and Pathway analyses of polyamine responsive genes.

2. Focusing on Put, determine the requirement of ROS production to Put transcriptional responses through the use of a hydrogen peroxide inhibitor.

3. Focusing on defense, determine the requirement of EDS1, SA and NPR1 pathway for the transcriptional responses to exogenously supplied Put.

4. Investigate the potential cross-modulation between Put and SA through PA and SA levels determination.

5. Focusing on ETI, determine the metabolic responses of polyamines to ETI-inducing bacteria.

6. Determine the systemic transcriptional responses triggered by locally applied Put in leaves. Comparison of transcriptional responses with SAR.

7. Investigate the involvement of ADC isoforms and CuAO to Put-triggered systemic resistance.

3- Chapter3

The main objective of this chapter is to identify new genetic components required for the establishment of immune-related HI (ETI), using the L*er*/Kas-2 HI model, and the impact of their loss-of function in defense and SA-dependent immune activation.

1. Mapping of causal genes underlying the suppression of L*er*/Kas-2 HI in two extragenic mutants isolated from an EMS-mutagenesis screen (*sulki3-1* and *sulki4-1*).

2. Confirm the causality of genes mapped to *GSL2* and *GSL10* through artificial microRNA (amiRNA) silencing in transgenic L*er*/Kas-2 HI (NIL) plants.

3. Determination of the immune status in *sulki3-1* and *sulki4-1* suppressed mutants and amiRNA lines by means of SA quantitation, expression of SA pathway marker genes and disease resistance against hemibiotrophic bacteria.

4. Determine callose deposition capacity in *sulki3-1*, *sulki4-1* and artificial microRNA (amiRNA) lines treated with PAMPs, wounding and bacteria inoculation.

Supervisor's report

Dr. Rubén Alcázar Hernández (Professor Agregat) and Dr. Antonio Fernández Tiburcio (Catedràtic d'Universitat), at the Department of Biologia, Sanitat i Medi Ambient of the Facultat de Farmàcia i Ciències de l'Alimentació (Universitat de Barcelona), Directors of the PhD Thesis entitled **"Involvement of Polyamines in PAMP-Triggered Immunity and Systemic Acquired Resistance (SAR). Extragenic Suppressors of Immune Hybrid Incompatibility"** declare that:

The Thesis hereby presented is the result of the work performed by **Changxin Liu** under our supervision and guidance.

The contribution of the PhD candidate to different articles and chapters included in this Thesis is detailed below.

Chapter 1: The Polyamine Putrescine Contributes to H_2O_2 and RbohD/F-Dependent Positive Feedback Loop in Arabidopsis PAMP-Triggered Immunity

Changxin Liu[*], Kostadin E. Atanasov, Antonio F. Tiburcio and Rubén Alcázar

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Quartile: 1

Contribution of listed authors (as stated in the published article): **CL** and KA performed the research. **CL** and RA planned the experiments. **CL**, KA, AT, and RA analyzed the data.

*This article is part of **Changxin Liu**'s PhD Thesis

Chapter 2: Putrescine Elicits ROS-Dependent Activation of The Salicylic Acid Pathway Contributing to Defense in *Arabidopsis thaliana*

Changxin Liu, Kostadin E. Atanasov, Nazanin Arafaty, Ester Murillo, Antonio F. Tiburcio, Jürgen Zeier and Rubén Alcázar[*]

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Impact Factor (2019): 6.362
Position: Plant Scis, 17 out of 483 (Scimago Journal & Country Rank)
Quartile: 1

Contribution of listed authors: **C.L.**, R.A., 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.

*This article is part of **Changxin Liu**'s PhD Thesis

Chapter 3: Mutations at *Glucan Synthase -like* (*GSL*) *2* and *GSL10* Suppress Immune-related Hybrid Incompatibility in *Arabidopsis thaliana*

Changxin Liu * and Rubén Alcázar

Publication status: Submission in preparation. This work is published as an attached chapter in this Thesis.

Contribution of listed authors (as stated in the published article): **C.L.** performed all the experimental research. **C.L.**, and R.A planned the experiments. **C.L.** and R.A analyzed the data.

*This article is part of **Changxin Liu**'s PhD Thesis

Barcelona, 28 d'Agost de 2020.

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Antonio Fernández Tiburcio

(Director)

CHAPTER 1

The Polyamine Putrescine Contributes to H_2O_2 and RbohD/F-Dependent Positive Feedback Loop in *Arabidopsis* PAMP-Triggered Immunity

The Polyamine Putrescine Contributes to H$_2$O$_2$ and *RbohD/F*-Dependent Positive Feedback Loop in *Arabidopsis* PAMP-Triggered Immunity

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Polyamines are involved in defense against pathogenic microorganisms in plants. However, the role of the polyamine putrescine (Put) during plant defense has remained elusive. In this work, we studied the implication of polyamines during pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in the model species *Arabidopsis thaliana*. Our data indicate that polyamines, particularly Put, accumulate in response to non-pathogenic *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC* and in response to the purified PAMP flagellin22. Exogenously supplied Put to *Arabidopsis* seedlings induces defense responses compatible with PTI activation, such as callose deposition and transcriptional up-regulation of several PTI marker genes. Consistent with this, we show that Put primes for resistance against pathogenic bacteria. Through chemical and genetic approaches, we find that PTI-related transcriptional responses induced by Put are hydrogen peroxide and NADPH oxidase (*RBOHD* and *RBOHF*) dependent, thus suggesting that apoplastic ROS mediates Put signaling. Overall, our data indicate that Put amplifies PTI responses through ROS production, leading to enhanced disease resistance against bacterial pathogens.

Keywords: polyamines, putrescine, defense, pathogen-associated molecular pattern, reactive oxygen species, PAMP-triggered immunity

INTRODUCTION

To face against biotic stress, plants have evolved complex and effective defense systems (Dodds and Rathjen, 2010). A first barrier of plant defense is the presence of the cuticle and the cell wall, which act as physical barriers (Yeats and Rose, 2013). However, when pathogens break these preformed barriers, sophisticated mechanisms of pathogen recognition are involved (Bigeard et al., 2015). Plasma membrane pathogen or pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) that lead to PAMP-triggered immunity (PTI) (Zipfel and Felix, 2005; Iakovidis et al., 2016). One of the most well-characterized PAMPs is flagellin, a structural component of the flagellum in Gram-negative bacteria. The peptide flagellin22 (flg22) is recognized by the leucine-rich repeat receptor kinase FLS2 (FLAGELLIN SENSING 2) (Felix et al., 1999; Gómez-Gómez and Boller, 2002). Known responses to PTI are the generation of

reactive oxygen species (ROS), cell wall reinforcement by callose deposition, and changes in the expression of defense-related genes (Boller and Felix, 2009; Nicaise et al., 2009; Ahuja et al., 2012). ROS production inhibits pathogen growth, stimulates cell wall cross-linking, and mediates the signal transduction for transcriptional changes (Apel and Hirt, 2004). NADPH oxidases are membrane-bound enzymes important for the generation of ROS during biotic and abiotic stresses, growth, and development. They transfer electrons from cytosolic NADPH or NADH to apoplastic oxygen, producing anion superoxide O_2^- in the apoplast, which can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (Kadota et al., 2015). *Arabidopsis thaliana* (*Arabidopsis*) carries 10 genes encoding NADPH oxidases, which belong to the *RBOH* (*RESPIRATORY BURST OXIDASE HOMOLOG*) family. Among them, RBOHD and, to a lesser extent, RBOHF are required for the generation of apoplastic ROS during incompatible plant–pathogen interactions (Torres et al., 2002). *RBOHD* is required for cell death control, cell wall damage-induced lignification, and systemic signaling in response to biotic and abiotic stresses (Torres et al., 2005; Miller et al., 2009; Denness et al., 2011). RBOHD and RBOHF fine-tune the spatial control of ROS production and hypersensitive response (HR) in and around infection sites (Torres et al., 2002, 2005, 2006; Chaouch et al., 2012). In addition to NADPH oxidases, apoplastic ROS can also be originated from polyamine oxidation. Polyamines are small polycationic molecules bearing amino groups. Most abundant plant polyamines are putrescine (Put), spermidine (Spd), and spermine (Spm), and they can be found in free forms or conjugated to hydroxycinnamic acids. Polyamines accumulate in response to different abiotic and biotic stresses and can be oxidatively deaminated by amine oxidases generating H_2O_2 (Tiburcio et al., 2014). Based on the cofactor involved, amine oxidases are classified in copper-containing amine oxidases (CuAOs) and FAD-dependent polyamine oxidases (PAOs). CuAOs catalyze the oxidation of Put at its primary amino group, producing 4-aminobutanal along with H_2O_2 and NH_4^+ (Cona et al., 2006; Angelini et al., 2010). In *Arabidopsis*, PAOs are involved in back-conversion reactions that convert Spm, thermospermine (tSpm), and Spd in their immediate precursors, producing 3-aminopropanal and H_2O_2 (Moschou et al., 2012; Ono et al., 2012; Ahou et al., 2014; Kim D.W et al., 2014). Some amine oxidases are located in the apoplast and may function as a source for apoplastic H_2O_2 during the elicitation of plant defense. For instance, inoculation of tobacco plants carrying the *N* resistance gene with tobacco mosaic virus (TMV) triggers HR and the accumulation of Spm in the apoplast (Yamakawa et al., 1998). In this species, Spm activates mitogen-activated protein kinases (MAPKs) SIPK (SA-induced protein kinase) and WIPK (wound-induced protein kinase) (Zhang and Klessig, 1997; Seo et al., 2007) and induces changes in the expression of Spm-responsive genes, some coding for acidic pathogenesis-related proteins (Yamakawa et al., 1998). Also in tobacco, inoculation with the hemibiotrophic bacteria *Pseudomonas viridiflava* and *Pseudomonas syringae* pv. *tabaci* leads to increases in Spm levels in the apoplast, which associate with disease resistance compromised by PAO inhibitors (Marina et al., 2008; Moschou et al., 2009). In *Arabidopsis*, Spm

and its isomer tSpm trigger transcriptional changes that restrict the multiplication of cucumber mosaic virus (CMV) (Mitsuya et al., 2009; Sagor et al., 2012). Also in this species, transgenic plants that accumulate Spm by overexpression of *SAMDC1* (*S-ADENOSYLMETHIONINE DECARBOXYLASE 1*) or *SPMS* (*SPERMINE SYNTHASE*) exhibit enhanced disease resistance against *P. syringae* pv. *maculicola* ES4326, *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), and *P. viridiflava* (Gonzalez et al., 2011; Marco et al., 2014). Overall, the polyamine Spm seems important for the establishment of HR and basal defense responses to hemibiotrophic pathogens in tobacco and *Arabidopsis*. Conversely, Put has not been observed to have such defense-promoting activities, although its content is remarkably increased in response to pathogens (Yoda et al., 2003; Mitsuya et al., 2009; Sagor et al., 2012; Vilas et al., 2018; Seifi and Shelp, 2019).

In this work, we studied the involvement of polyamines during PTI in *Arabidopsis*. We report that Put accumulates in response to inoculation with the type three secretor system (TTSS) defective *P. syringae* DC3000 *hrcC* mutant strain (*hrcC*), which induces a strong PTI response (Yuan and He, 1996; Tsuda et al., 2008), and this accumulation is not suppressed by *Pst* DC3000 type III effectors (Cunnac et al., 2009). Consistent with a potential role for Put during PTI, we show that this polyamine also accumulates in response to flg22, one of the most well-characterized PAMPs. Through the analysis of *arginine decarboxylase 1* (*adc1*) and *arginine decarboxylase 2* (*adc2*) loss-of-function mutants, deficient in Put biosynthesis, we find that the *ADC2* isoform is the major contributor to Put biosynthesis triggered by flg22. We show that Put induces the formation of callose deposits, a typical response of PTI, when applied to *Arabidopsis* seedlings. In addition, we demonstrate that Put quickly induces the expression of several PTI marker genes (Huffaker and Ryan, 2007; Xiao et al., 2007; Denoux et al., 2008; Wang et al., 2009; Boudsocq et al., 2010; Cheng et al., 2013; Po-Wen et al., 2013; Shi et al., 2015), and these transcriptional changes are compromised in the presence of the H_2O_2 scavenger dimethylthiourea (DMTU), and in *atrbohD*, *atrbohF*, and double *atrbohD/F* NADPH oxidase loss-of-function mutants. We finally report that Put can be regarded as a priming agent that contributes to basal disease resistance against bacterial pathogens. Overall, we provide evidence that Put contributes to H_2O_2 and *RBOHD/F*-dependent positive feedback loop amplification of PTI.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plants were grown on soil (peat moss:vermiculite:perlite, 40:50:10) at 20–22°C under 12-h dark/12-h light cycles at 100–125 μmol photons m^{-1} s^{-2} of light intensity and 70% relative humidity. For *in vitro* culture, seeds were sterilized with a solution containing 30% sodium hypochlorite supplemented with 0.5% Triton X-100 for 10 min, followed by three washes

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[1]www.arabidopsis.info

[2]www.anaspec.com

with sterile distilled H_2O. Sterilized seeds were sown on growth media [GM, 1/2 Murashige and Skoog supplemented with vitamins, 1% sucrose, 0.6% plant agar (Duchefa Biochemie), and pH 5.7 adjusted with 1 M KOH]. Plates were kept in the dark at 4°C for stratification for 2–3 days. Seedlings were grown under 12-h dark/12-h light cycles at 20–22°C, 100–125 µmol photons m^{-2} s^{-1} of light intensity. flg22 peptide was purchased from Anaspec[1]. The *fls2* mutant was kindly provided by Jane Parker (Zipfel et al., 2004). The *adc1-2* (SALK_085350), *adc2-4* (SALK_147171), *atrbohD* (SALK_109396 and SALK_005253), *atrbohF* (SALK_044584 and SALK_077748), and double *atrbohD/F* (N9558) (Torres et al., 2002) mutants were obtained from the Nottingham *Arabidopsis* Stock Center[2]. The *adc1-3* and *adc2-3* mutants were previously reported (Cuevas et al., 2008). The *gsl5* mutant was kindly provided by Christian Voigt.

Polyamine Levels Determination

Polyamines were derivatized with dansyl chloride and analyzed by high-performance liquid chromatography (HPLC) as previously described (Marcé et al., 1995; Zarza et al., 2017). All harvested tissues were washed three times with sterile distilled H_2O before processing or freezing in liquid nitrogen. Apoplastic polyamines were determined according to Yoda et al. (2009). All polyamine analyses were performed in at least three biological replicates.

Histochemical Analyses

For aniline blue staining, seedlings were fixed and cleared in a solution of acetic acid/ethanol (1:3) overnight, followed by two washes of 30 min in 150 mM K_2HPO_4 and staining with 0.01% aniline blue (Sigma) for 2 h in the same buffer. Observations were performed under an epifluorescence microscope and images were captured with a NIKON microscopy camera coupled to the NIS software 4.45 (NIKON). Callose intensity quantification was performed according to Daudi et al. (2012). Callose intensity was calculated with ImageJ by counting the number of callose spots and assigning a value from 1 to 10 (10, saturated signal; 9, over 250 spots; 8, between 200 and 249 spots; 7, between 150 and 199 spots; 5, between 100 and 149 spots; 3, between 50 and 99 spots; 2, between 5 and 49 spots; 1, between 0 and 5 spots). Average callose measurements were based on at least 20 leaf pictures taken from 12 different seedlings. Trypan blue staining for cell death visualization was performed as previously described (Alcázar et al., 2009).

Real-Time qPCR Expression Analyses

Total RNA isolated from 10-day-old seedlings was extracted using *TRIzol* reagent (Thermo Fisher). Two micrograms of RNA was treated with DNAse I (Invitrogen) and first-strand cDNA was synthesized using Superscript IV (Invitrogen) and oligo dT. 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 for 2 min, 40 cycles (95°C for 15 s; 60°C for 30 s). qRT-PCR analyses were always performed on at least three biological replicates with three technical replicates each using *ACTIN2* (At3g18780) as the internal control gene.

FIGURE 1 | Polyamine levels in response to *Pst* DC3000 and *hrcC* inoculation. Levels of free putrescine (Put), spermidine (Spd), and spermine (Spm) in 3-week old *Arabidopsis* wild-type (Col-0) plants after 0 h to 3 days of spray inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), *Pst* DC3000 *hrcC* mutant (*hrcC*), or mock. Values are the mean of three biological replicates ± SD (standard deviation). Letters indicate values that are significantly different according to Student–Newman–Keuls test at P value <0.05.

Relative expression was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primer sequences used for expression analyses are shown in **Supplementary Table 1**.

Pseudomonas syringae pv. *tomato* DC3000 and *hrcC* Inoculation Assays

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) and *P. syringae* pv. *tomato* DC3000 *hrcC* (*hrcC*) bacteria were streaked
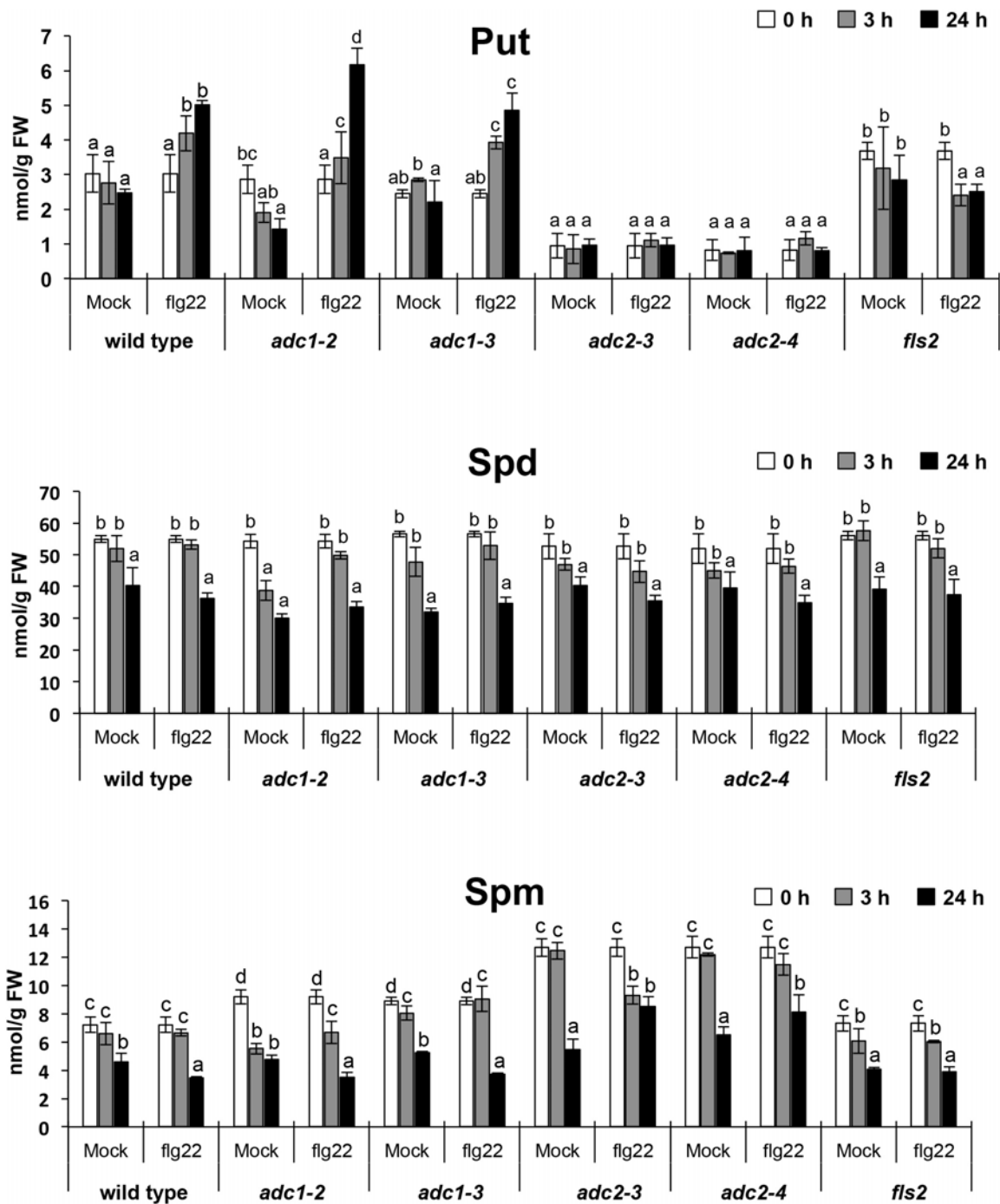
FIGURE 2 | Polyamine levels in response to flg22 treatment. Levels of free putrescine (Put), spermidine (Spd), and spermine (Spm) in 10-day-old wild-type, *adc1-2*, *adc1-3*, *adc2-3*, *adc2-4* (Cuevas et al., 2008), and *fls2* (Zipfel et al., 2004) seedlings treated with 1 μM flg22. Seedlings were grown *in vitro* on a nylon mesh in ½ Murashige and Skoog media and transferred to the same media supplemented with 1 μM flg22 or mock for 24 h. Samples were harvested after 0, 3, and 24 h of treatment for polyamine analyses. Results are mean of three biological replicates ± SD (standard deviation). Letters indicate values that are significantly different according to Student–Newman–Keuls test at *P* value <0.05.

on solid NYGA medium (5 g/L bacto peptone, 3 g/L yeast extract, and 20 mL/L glycerol, with 15 g/L agar for solid medium) containing 25 μg/ml rifampicin. Single colonies were transferred to liquid NYGA supplemented with 25 μg/ml rifampicin and

grown overnight at 28°C. Bacterial suspensions were washed two times with water and suspended on 10 mM $MgCl_2$ to $OD_{600} = 0.2$. Silwet L-77 was added to a final concentration of 0.04% (v/v) before spray inoculation of 3-week-old *Arabidopsis* plants. Leaves

FIGURE 3 | Callose deposition in response to Put treatment. Aniline blue staining of 10-day-old wild-type and *gsl5* loss-of-function mutants treated with Put or flg22 for 24 h. Leaves were drop inoculated with 5 µl of 100 µM Put or 1 µM flg22. Callose deposition was quantified based on image captions from at least 20 leaf pictures from 12 different seedlings per genotype and treatment. Letters indicate values that are significantly different according to Student–Newman–Keuls test at *P* value <0.05.

were harvested after 3 h and 72 h of pathogen inoculation for the determination of bacterial growth as described in Alcázar et al. (2010). At least three biological replicates were determined for each time point of analysis.

RESULTS

Polyamine Levels in Response to *Pst* DC3000 and *Pst* DC3000 *hrcC* Bacteria

The *P. syringae* pv. *tomato* DC3000 *hrcC* mutant (*hrcC*) is defective in the TTSS and mainly induces a PAMP-triggered response by failing to secrete defense-suppressing type III effectors into the plant cell (Yuan and He, 1996). In order to analyze the involvement of polyamines during PTI, we inoculated *Arabidopsis* wild type (Col-0) with *hrcC* and monitored the levels of free Put, Spd, and Spm for 3 days (**Figure 1**). One-day post-inoculation, the levels of Put, Spd, and Spm were 2.1-, 1.4-, and 1.7-fold higher in plants inoculated with *hrcC* than in mock inoculated plants (**Figure 1**). These results indicated that polyamines, and particularly Put, accumulated transiently in response to non-pathogenic *hrcC* bacteria, thus suggesting the participation of polyamines in the metabolic reprogramming induced during PTI.

In order to determine whether type III effector proteins suppress the changes in polyamine levels observed after *hrcC* inoculation, we determined Put, Spd, and Spm contents in plants inoculated with *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), which carries a functional TTSS (**Figure 1**). Compared to mocks, the Put levels increased up to 1.6- and 2.7-fold 1 and 2 days after

inoculation with *Pst* DC3000, respectively. Spd and Spm levels also increased up to 1.4- and 1.6-fold 1 day post-inoculation. These results indicated that type III effectors delivered by *Pst* DC3000 do not suppress increases in polyamine levels triggered by *hrcC*. Rather, Put accumulation was higher in the strain provided with a functional TTSS.

Determination of Apoplastic Polyamines

Some polyamines have been reported to accumulate in the apoplast of *Arabidopsis*, tobacco, tomato, and rice during defense (Yoda et al., 2009; Vilas et al., 2018). Under basal conditions (0 h), the levels of free polyamines in the apoplastic enriched fractions were undetectable. However, apoplastic Put and Spd contents remarkably increased after 24 h of *Pst* DC3000 and *hrcC* inoculation. The levels of Put remained high in *Pst* DC3000 but not in *hrcC* inoculated plants. Apoplastic Spm was not detectable in any treatment (**Supplementary Figure S1**). We concluded that Put and Spd accumulate in the apoplast in response to *Pst* DC3000 and *hrcC* inoculation. These data suggested that polyamines could trigger some defense response from the cell surface against bacterial infection.

Polyamine Levels in Response to flg22

To further investigate the involvement of polyamines during PTI, we analyzed polyamine levels in response to the PAMP flg22. Free Put, Spd, and Spm levels were determined in wild type and *fls2* seedlings treated with 1 µM flg22 or mock (**Figure 2**). In the wild type, Put accumulated up to twofold in response to 1 µM flg22 treatment after 24 h. This increase was not evidenced in the *fls2* mutant (**Figure 2**), which indicated that Put accumulation

FIGURE 4 | Expression analyses of PTI marker genes in response to 100 μM Put. Ten-day-old wild-type seedlings were treated with 100 μM Put or mock, and samples collected at 0 h, 10 min, 30 min, 1 h, 3 h, 8 h, 24 h, and 72 h post-treatment to analyze the expression of *PROPEP2*, *PROPEP3*, *CBP60g*, *WRKY22*, *WRKY29*, *NHL10*, *CYP81F2*, *FRK1*, and *WRKY53* as PTI marker genes by qRT-PCR. Seedlings were grown *in vitro* on a nylon mesh in ½ Murashige and Skoog media and transferred to the same media supplemented with the polyamine or mock up to 72 h. Expression values are relative to 0 h. Results are means of three biological replicates ± SD (standard deviation). Letters indicate values that are significantly different according to Student–Newman–Keuls test at *P* value <0.05.

triggered by flg22 was due to *FLS2*-dependent activation of PTI. The levels of Spd and Spm in seedlings treated with 1 μM flg22 did not exhibit significant changes compared with the mock control (**Figure 2**). Therefore, flg22 did not favor the synthesis or accumulation of Spd and Spm. However, increases in these polyamines were detected after 24 h of inoculation with *Pst DC3000* and *hrcC* bacteria (**Figure 1**). We suggest that other molecules produced by *P. syringae* (Xin and He, 2013) and perceived by the plant might trigger the synthesis of Spd and Spm in *Arabidopsis*.

Involvement of *ADC* Isoforms in Put Biosynthesis Triggered by flg22

Arginine decarboxylase (ADC) catalyzes the conversion of arginine into agmatine, which is a limiting step in the biosynthesis of Put. In *Arabidopsis*, two *ADC* isoforms are found (*ADC1* and *ADC2*) that catalyze the same enzymatic reaction (Alcázar et al., 2006). To analyze the contribution of each isoform to Put

synthesis in response to flg22, we treated *arginine decarboxylase 1 (adc1-2, adc1-3)* and *arginine decarboxylase 2 (adc2-3, adc2-4)* loss-of-function mutants (Cuevas et al., 2008) with 1 μM flg22 and quantified the polyamine levels between 0 and 24 h (**Figure 2**). In *adc2-3* and *adc2-4*, the basal level of Put was much lower than in the wild type (Cuevas et al., 2008) and Put content did not increase in response to 1 μM flg22. Conversely, in *adc1-2* and *adc1-3*, Put content increased to a similar extent as the wild type in response to 1 μM flg22 (**Figure 2**). These results indicated that Put accumulation in response to flg22 is mainly contributed by ADC2 activity. Therefore, ADC1 and ADC2 forms do not act redundantly during PTI.

Callose Deposition but Not Cell Death Is Induced by Put

The increases in Put triggered by flg22 perception prompted us to investigate its potential role during PTI. Deposition of the (1,3)-β-glucan callose is induced in response to flg22, and

FIGURE 5 | Expression analyses of PTI marker genes in response to 100 μM Put in the presence of 10 mM DMTU. Ten-day-old wild-type seedlings grown *in vitro* on a nylon mesh in ½ Murashige and Skoog media were transferred to the same media containing 10 mM DMTU for 3 h before treatment with 100 μM Put + 10 mM DMTU or 10 mM DMTU. Samples were collected after 0 h, 10 min, and 0.5 h of Put treatment for expression analyses of *WRKY29*, *PROPEP2*, *PROPEP3*, and *CYP81F2* by qRT-PCR. Expression values are relative to DMTU treatment at each time point of analysis. Results are the mean of three biological replicates ± SD (standard deviation). Letters indicate values that are significantly different according to Student–Newman–Keuls test at *P* value <0.05.

it can be visualized by histochemical analysis based on aniline blue staining. We observed higher callose deposition in wild-type seedlings treated for 24 h with 100 μM Put or 1 μM flg22 than in seedlings treated with mock (**Figure 3**). Callose deposition induced by Put was compromised in the *glucan synthase like 5* (*gsl5*) mutant, which is defective in inducible callose accumulation upon wounding and biotic stress (Jacobs et al., 2003) (**Figure 3**). Conversely, callose deposition in response to flg22 was not obviously compromised in *adc1* or *adc2* mutants (**Supplementary Figure S2**). This indicated that flg22 responses are not impaired in *adc* mutants. To determine whether callose deposition triggered by Put was accompanied with cell death, we performed trypan blue staining in wild-type seedlings after 24 h of infiltration with 100 μM Put or mock (**Supplementary Figure S3**). Trypan blue staining did not reveal evident symptoms of cell death related with ETI in *Arabidopsis* leaves treated with 100 μM Put. These data indicated that Put infiltration does not induce HR in *Arabidopsis*.

Expression of PTI Marker Genes in Response to Put

Accumulation of callose by Put suggested that PTI responses were activated by this polyamine. To further investigate this hypothesis, we analyzed the expression of several PTI marker genes (*PROPEP2*, *PROPEP3*, *CBP60g*, *WRKY22*, *WRKY29*, *WRKY53*, *CYP81F2*, *FRK1*, and *NHL10*) (Huffaker and Ryan, 2007; Xiao et al., 2007; Denoux et al., 2008; Wang et al., 2009; Boudsocq et al., 2010; Cheng et al., 2013; Po-Wen et al., 2013; Shi et al., 2015) in wild-type seedlings treated with 100 μM Put or mock between 0 and 72 h (**Figure 4**). For most of the genes analyzed, their transcripts increased rapidly in response to 100 μM Put, with the highest expression peaks observed upon

10 min to 1 h of treatment (**Figure 4**). These results indicated that Put induces transcriptional changes consistent with activation of PTI. Because Put can be oxidized by amine oxidases, we then studied whether transcriptional responses were compromised in the presence of the H_2O_2 scavenger dimethylthiourea (DMTU). For this, we determined the expression of *WRKY29*, *PROPEP2*, *PROPEP3*, and *CYP81F2* (Huffaker and Ryan, 2007; Denoux et al., 2008; Cheng et al., 2013) in wild-type seedlings treated or not with 100 μM Put in the presence of 10 mM DMTU (**Figure 5**). The increase in the transcript levels of these genes triggered by Put was compromised in the presence of DMTU (**Figure 5**). We concluded that H_2O_2 production is required for Put-triggered transcriptional up-regulation of PTI marker genes.

Expression of PTI Marker Genes in Response to Put in *atrboh D*, *atrboh F*, and *atrboh D/F* Mutants

Plasma membrane RBOHD and RBOHF are important sources of ROS during plant–pathogen interactions (Kadota et al., 2015). To determine the contribution of these NADPH oxidases to changes in the expression of PTI marker genes induced by Put, we analyzed the expression of *WRKY22* and *CYP81F2* in *atrbohD* (SALK_109396C and SALK_005253C), *atrbohF* (SALK_034674 and SALK_077748), and double *atrbohD/F* loss-of-function mutants (Torres et al., 2002) treated with 100 μM Put or mock (**Figure 6**). In contrast with the wild type, up-regulation of *WRKY22* and *CYP81F2* expression by Put treatment was strongly compromised in *atrbohD*, *atrbohF*, and double *atrbohD/F* mutants (**Figure 6**). These results indicated that Put requires functional RBOHD and RBOHF NADPH oxidases for signaling.

Disease Resistance to *P. syringae* pv. *tomato* DC3000 and *hrcC* in Put Treated Plants

So far, our data pointed to a role for Put contributing to amplify PTI responses. To analyze how this was translated into disease resistance, we performed pathoassays using *Pst DC3000* and *hrcC* bacteria in wild-type plants treated with 500 μM Put, 1 μM flg22 or mock. As shown in **Figure 7**, Put treatment limited the growth of *Pst* DC3000 to a similar extent as 1 μM flg22, whereas no differences were detected by inoculation with the non-pathogenic *hrcC* strain. We concluded that Put could be regarded as a priming agent contributing to basal defense responses against some pathogenic bacteria.

DISCUSSION

Plants are provided with an innate immune system that recognizes pathogens and activates defense responses. A first layer of the innate immunity involves the recognition of PAMPs, which are conserved signatures within a taxonomic group of pathogens. PAMPs include the flagellin peptide flg22, the elongation factor Tu (EF-Tu) peptides elf18 and elf26, lipopolysaccharides, fungal chitin, and peptidoglycan, among others (Boller and Felix, 2009). PAMPs induce the

FIGURE 6 | Expression analyses of PTI marker genes in response to 100 μM Put in *rbohD*, *rbohF*, and double *rbohD/F* loss-of-function mutants. Ten-day-old wild-type, *atrbohD* (SALK_109396 and SALK_005253), *atrbohF* (SALK_044584 and SALK_077748), and double *atrbohD/F* seedlings were treated with 100 μM Put or mock as described in **Figure 4** and samples were collected at 0 and 1 h post-treatment to analyze the expression of *WRKY22 and CYP81F2* by qRT-PCR. Expression values are relative to the wild type at 0 h. Results are means of three biological replicates ± SD (standard deviation). Letters indicate values that are significantly different according to Student–Newman–Keuls test at *P* value <0.05.

production of ROS, which participate in defense signaling and transcriptional reprogramming (Bigeard et al., 2015). During defense, ROS are predominantly generated by NADPH oxidases RBOHD and RBOHF (Torres et al., 2002; Kadota et al., 2015). However, other sources of apoplastic ROS include the activity of apoplastic peroxidases (Daudi et al., 2012) and amine oxidases (Cona et al., 2006). The different sources of ROS might be related to the necessity of specific ROS synthesis at different stages of the defense response (Cona et al., 2006). In *Arabidopsis*, the copper-containing amine oxidases (CuAO) ATAO1/AtCuAOβ (*At4g14940*) and *CuAO1/AtCuAOγ1* (*At1g62810*) have been localized in the apoplast (Moller and McPherson, 1998; Planas-Portell et al., 2013), whereas PAO enzymes have been found in the cytosol and peroxisomes (Tavladoraki et al., 2006; Kamada-Nobusada et al., 2008;

Moschou et al., 2008; Takahashi et al., 2010; Ahou et al., 2014; Tiburcio et al., 2014). The apoplastic CuAOs preferentially catalyze the oxidation of Put (*ATAO1*) or Put and Spd (*CuAO1*) (Moller and McPherson, 1998; Planas-Portell et al., 2013), consistent with the occurrence of these polyamines in extracellular fluids (Yoda et al., 2009). Interestingly, *CuAO1* expression is induced by flg22 treatment (Planas-Portell et al., 2013), which suggests its participation in PAMP-triggered ROS signaling. The involvement of CuAO activities in the defense response of incompatible plant–pathogen interactions has previously been documented. In the incompatible interaction between barley and the powdery mildew fungus *B. graminis* f. sp. *hordei*, the levels of Put, Spd, as well as diamine oxidase and PAO activities were shown to increase and to contribute to defense through H_2O_2 production, leading to

FIGURE 7 | Growth of *Pseudomonas syringae* pv. *tomato* DC3000 and *hrcC* in wild-type pre-treated with Put or flg22. Three-week-old *Arabidopsis* plants were spray inoculated with 500 μM putrescine, 1 μM flg22, or mock 24 h before spray inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 or *hrcC*. Bacterial counting was performed at 3 h and 3 days post-inoculation. Results are the mean of four replicates ± SD (standard deviation). Letters indicate values that are significantly different according to Student–Newman–Keuls test at *P* value <0.05.

cell wall cross-linking of polysaccharides and proteins (Cowley and Walters, 2002). In chickpea, inhibition of CuAO activity was associated with decreased defense capacity against the necrotrophic fungus *Ascochyta rabiei* (Rea et al., 2002). The amount of free polyamines in the apoplast seems to be a limiting factor for CuAO activity (Rea, 2004). Indeed, it has been proposed that under stress conditions, polyamine excretion is activated in plant cells (Yoda et al., 2003). Consistent with this, the levels of apoplastic Put and Spd increase in response to avirulent *Pst* DC3000 *AvrRPM1* inoculation in *Arabidopsis* (Yoda et al., 2009).

Despite the growing body of evidence that shows the involvement of polyamines in defense, few studies have focused on the involvement of polyamines during PTI. In this work, we show that Put synthesis is stimulated by *Pst* DC3000 *hrcC* inoculation (**Figure 1**), a TTSS defective bacteria strain that

mainly triggers a PTI response by failing to secrete effectors (Yuan and He, 1996). Consistent with this, Put level also increased by treatment with the purified PAMP flg22 (**Figure 2**). These data suggested that polyamines are part of the metabolic reprogramming response during PTI. Interestingly, inoculation with *Pst* DC3000, which carries a functional TTSS and can deploy effectors into the plant cell (Xin and He, 2013), did not suppress the increase in polyamine levels observed with *hrcC*. Rather, polyamine levels became higher (**Figure 1**). These results indicate that *Pst* DC3000 effectors are unlikely to suppress polyamine pathway activation. Rather, effectors might be promoting agents in polyamine biosynthesis. For example, the ADC1 isoform from *Capsicum annuum* is targeted by the *AvrBsT* effector from *Xanthomonas campestris* pv. *vesicatoria*. Their co-expression in *Nicotiana benthamiana* leaves promotes polyamine biosynthesis, thus leading to enhanced cell death and H_2O_2 production (Kim N.H. et al., 2013). However, it is not known whether Arabidopsis ADC isoforms might be targets of bacterial effectors. In Arabidopsis, the *ADC2* isoform is the major contributor to Put synthesis in response to flg22 (**Figure 2**). Consistent with this, Kim S.H. et al. (2013) showed that the *adc2* mutant (SALK_073977) in *Arabidopsis* compromises resistance to *Pst* DC3000, which can be rescued by infiltration with 2 μM Put.

The Put accumulation triggered by flg22 and *hrcC* prompted us to investigate the role of this polyamine during PTI. Interestingly, we found that exogenously supplied Put induces callose deposition in *Arabidopsis* seedlings (**Figure 3**). The formation of callose deposits is a typical physiological response of PTI. Callose is synthesized at the cell wall by callose synthases. The *Arabidopsis* genome contains 12 callose synthase (*CalS*) genes, also referred to as Glucan synthase-like (*GSL*) (Ellinger and Voigt, 2014). Among them, *GSL5* (*PATHOGEN MILDEW RESISTANCE 4, PMR4*) is required for wound and papillary callose deposition (Jacobs et al., 2003). We found that callose deposition induced by Put supply was compromised in the *gsl5* (*pmr4*) loss-of-function mutant (Jacobs et al., 2003) (**Figure 3**). To further investigate the involvement of Put during PTI, we selected a number of PTI marker genes based on previous reports (Huffaker and Ryan, 2007; Xiao et al., 2007; Wang et al., 2009; Boudsocq et al., 2010; Cheng et al., 2013; Po-Wen et al., 2013; Shi et al., 2015). Exogenously supplied Put rapidly led to the up-regulation of PTI marker genes tested (**Figure 4**). Interestingly, such responses were suppressed in the presence of the H_2O_2 scavenger, DMTU (Tate et al., 1982) (**Figure 5**). Hydrogen peroxide is likely derived from amine oxidase activity, thus pointing to an important role for polyamine oxidation during the transcriptional response triggered by Put. Interestingly, up-regulation of PTI marker genes was also compromised in *atrbohD*, *atrbohF*, and double *atrbohD/F* loss-of-function mutants (**Figure 6**). These data indicate that plasma membrane NADPH oxidases are required for at least some transcriptional responses induced by Put. In tobacco, the NADPH oxidases RBOHD/F have been suggested to act upstream of apoplastic PAO during salt stress, contributing to cell death (Gémes et al., 2016). Our data indicate that *Arabidopsis* RBOHD/F are

downstream of Put or act in a concerted manner with apoplastic CuAOs during PTI. Collectively, we observed that PAMPs (flg22) induce Put biosynthesis and that Put triggers responses compatible with PTI activation, which are ROS and RBOHD/F dependent. Hence, a positive feedback loop is proposed in which Put amplifies PAMP-triggered signaling through ROS production, leading to enhanced basal disease resistance against bacterial pathogens (**Figure 7**). In this regard, apoplastic Put could act similarly to damage-associated molecular patterns (DAMPs) triggering a ROS-dependent defense response (Choi and Klessig, 2016; Versluys et al., 2017).

Collectively, our results gain insight into mechanistic processes by which polyamines contribute to disease resistance in plants. Such type of analyses should contribute to pave the road for the uses of polyamines as potential priming agents in agriculture.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

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AUTHOR CONTRIBUTIONS

CL and KA performed the research. CL and RA planned the experiments. CL, KA, AT, and RA analyzed the data. RA wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00894/full#supplementary-material

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CHAPTER 2

Putrescine Elicits ROS – Dependent Activation of The Salicylic Acid Pathway Contributing to Defense in *Arabidopsis thaliana*

ORIGINAL ARTICLE

Plant, Cell & Environment   PC/E   WILEY

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

defense, polyamines, systemic acquired resistance

1 | 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 (Cui, Tsuda, & Parker, 2015; Dodds & Rathjen, 2010). 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, Dewdney, Wu, & Ausubel, 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, Xiao, Kachroo, & Kachroo, 2019). These metabolites include N-hydroxypipecolic acid (NHP) (Hartmann et al., 2018; Návarová, Bernsdorff, Döring, & Zeier, 2012), azelaic acid (AzA) (Jung, Tschaplinski, Wang, Glazebrook, & Greenberg, 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 (Breitenbach et al., 2014; Truman, Bennet, Kubigsteltig, Turnbull, & Grant, 2007). 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 (Després, DeLong, Glaze, Liu, & Fobert, 2000; Fan & Dong, 2002; Fu & Dong, 2013; Mou, Fan, & Dong, 2003; Tada et al., 2008; Zhang, Fan, Kinkema, Li, & Dong, 1999).

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 (Tiburcio & Alcázar, 2018; Tiburcio, Altabella, Bitrián, & Alcázar, 2014; Walters, 2003). 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, Rea, Angelini, Federico, & Tavladoraki, 2006), which might lead to ROS-dependent stress signaling (Mittler et al., 2011; Wang, Paschalidis, Feng, Song, & Liu, 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^+ (Angelini et al., 2010; Cona et al., 2006; Planas-

Portell, Gallart, Tiburcio, & Altabella, 2013). Plant PAO are involved in terminal catabolism or back-conversion reactions, depending on the species (Angelini et al., 2010; Cona et al., 2006; 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 (Ahou et al., 2014; Moschou et al., 2012; Ono et al., 2012). Spm oxidation through PAO activity triggers the activation of mitogen-activated protein kinases (Seo, Katou, Seto, Gomi, & Ohashi, 2007; Zhang & Klessig, 1997) and contributes to disease resistance against cucumber mosaic virus (CMV) (Mitsuya et al., 2009; Sagor et al., 2012), *Pseudomonas* (González et al., 2011; Lou, Bor, Yan, Preuss, & Jander, 2016; Marco, Busó, & Carrasco, 2014; Marina et al., 2008; Moschou et al., 2009), *Botrytis cinerea* (Seifi, Zarei, Hsiang, & Shelp, 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 (Mitsuya et al., 2009; Sagor et al., 2012; Seifi & Shelp, 2019; Vilas et al., 2018; Yoda, Yamaguchi, & Sano, 2003). 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, Atanasov, Tiburcio, & Alcázar, 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.

2 | MATERIALS AND METHODS

2.1 | 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 hr light (8:00 a.m. to 4:00 p.m.)/16 hr 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 hr light/16 hr dark cycles at 20–22°C at 100–125 μmol photons m^{-2} s^{-1} of light intensity. The npr1-1 (Cao, Glazebrook, Clarke, Volko, & Dong, 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).

2.2 | 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.

2.3 | RNA-seq analyses

All polyamine treatments were performed at the same time of the day (12:00 p.m.) 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 hr 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 hr 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 (2×75 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 ≤.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 (Carbon et al., 2019; Cheng et al., 2017). 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).

2.4 | 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.

2.5 | 2,7′-Dichlorofluorescein staining

Twelve-day-old wild-type (Col-0) seedlings grown in vitro were transferred to liquid growth media and incubated 24 hr before treatment with the different polyamines at 100 μM, 100 μM Put + 5 mM DMTU or mock (5 mM MES pH 5.7) for 1 hr. 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 hr 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 10 independent plants were visualized for every treatment. Figure captions represent a representative sample from each treatment.

2.6 | 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.

2.7 | Pipecolic acid and salicylic acid quantitation

Leaves from 5-week-old *Arabidopsis* plants were inoculated with 500 μM Put or mock (5 mM MES pH 5.7). Local inoculated (1°) and distal non-inoculated (2°) leaves were collected at 24 and 48 hr 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$_2$O (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 (30 m × 0.25 mm × 0.25 μm) 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).

2.8 | 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 (Alcázar, García-Martínez, Cuevas, Tiburcio, & Altabella, 2005; Marcé, Brown, Capell, Figueras, & Tiburcio, 1995). Analyses were performed in three to four biological replicates per treatment, each including three technical replicates.

2.9 | 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, Chao, Luthin, Goff, & Dangl, 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 (1°) and distal non-inoculated (2°) leaves were harvested at 24 hr post-infiltration for polyamine quantitation. Analyses were performed in three to four biological replicates per treatment, each including three technical replicates.

2.10 | Pathogen inoculation assays

Three local leaves from at least eight independent *Arabidopsis* plants per genotype were inoculated with 100 μl of a *P. 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 (2°) were infiltrated with *P. 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 hr post-inoculation as described (Alcázar et al., 2010) using eight biological replicates per treatment and genotype.

2.11 | Accession numbers

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

3 | RESULTS

### 3.1 | 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 hr. 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 <.05) (Figures 1 and S1; Tables S2.1–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, 11 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.

3.2 | 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 (Angelini et al., 2010; Cona et al., 2006; 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
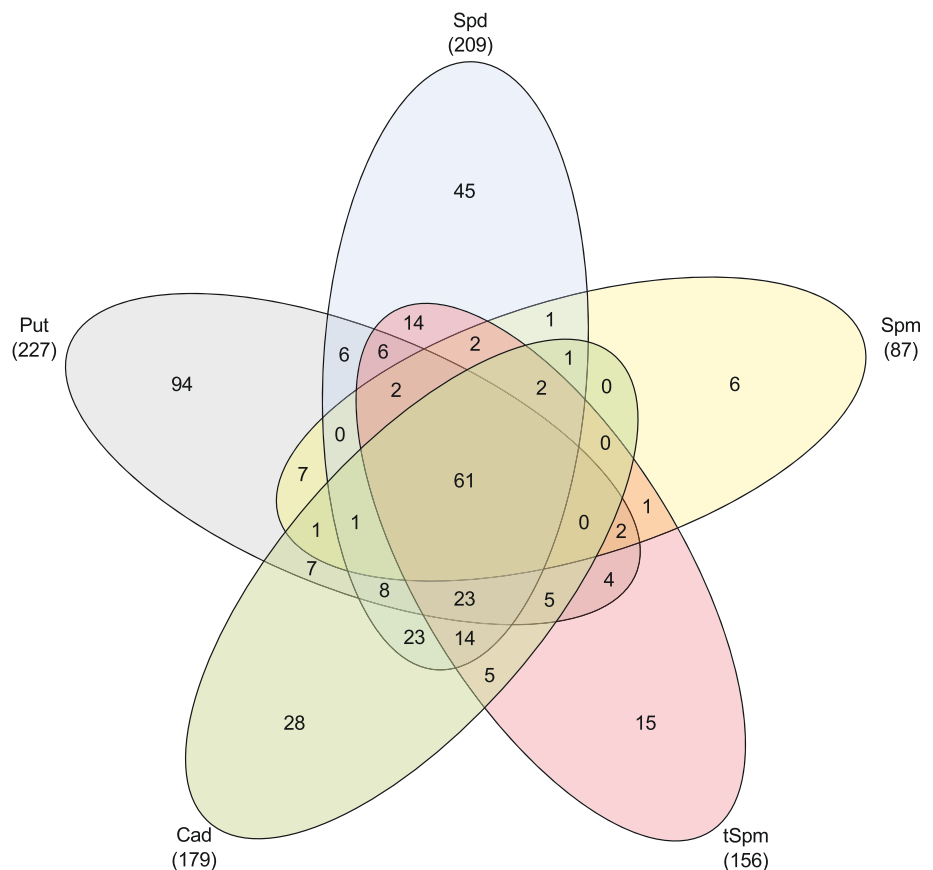
FIGURE 1 Venn diagram of polyamine responsive genes. Venn diagram of unique and shared genes responsive to 100 μM putrescine (Put), 100 μM cadaverine (Cad), 100 μM spermidine (Spd), 100 μM spermine (Spm) and 100 μM thermospermine (tSpm) after 1 hr of treatment (see Tables S2.1–S2.6). RNA-seq analyses were performed in 12-day-old *Arabidopsis* wild-type (Col-0) seedlings [Colour figure can be viewed at wileyonlinelibrary.com]
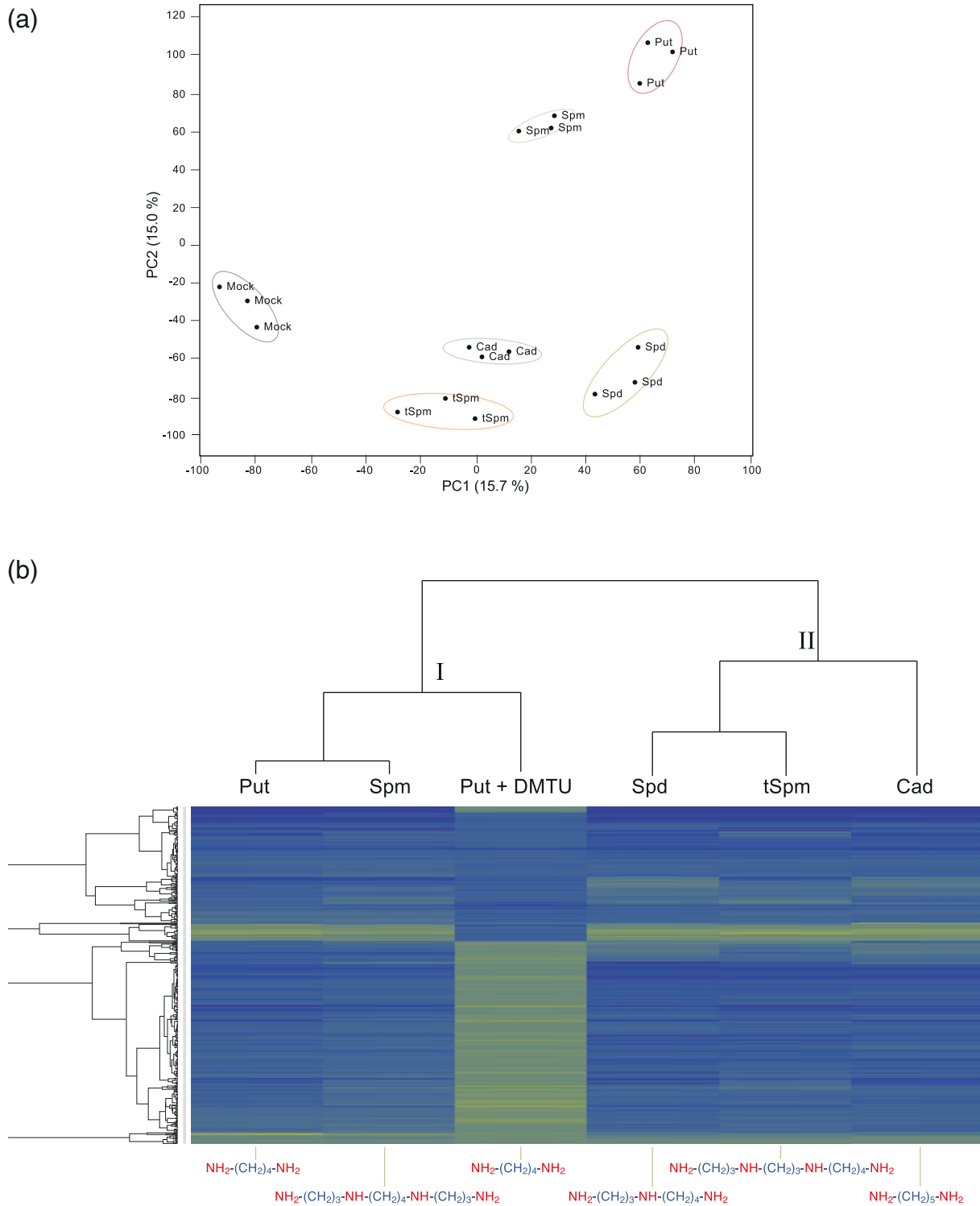
FIGURE 2 (a) Principal component analysis (PCA) and (b) hierarchical clustering analysis (HCA) of transcriptional responses to different polyamines. PCA and HCA of RNA-seq data from the treatments with 100 μM putrescine (Put), 100 μM cadaverine (Cad), 100 μM spermidine (Spd), 100 μM spermine (Spm) and 100 μM thermospermine (tSpm). Ellipses in the PCA indicate the 95% confidence interval. The chemical structure of the different polyamines is shown at the bottom of the HCA clade [Colour figure can be viewed at wileyonlinelibrary.com]

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., 2010). 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.

3.3 | Involvement of SA pathway in putrescine signaling

GO and array similarity analyses of polyamine responsive genes highlighted many categories related to defense (Figures S2 and 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 w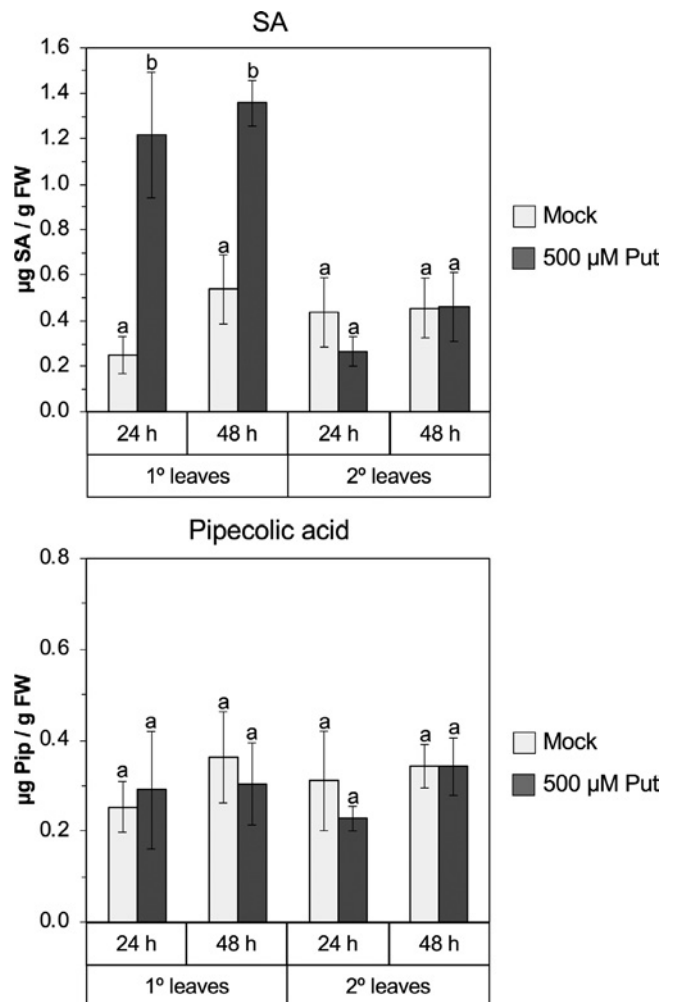ere independent of NPR1, SID2 or EDS1. The majority of EDS1 (66%) and SA (87%) gene expression sectors were also NPR1 dependent (Figure 3 and Table S5). Overall, we concluded that most transcriptional responses to Put require a functional EDS1/SA/NPR1 pathway.

3.4 | 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 (1°) and systemic non-inoculated (2°) leaves at 24 and 48 hr post-treatment. Free SA levels were five-fold higher in 1° leaves infiltrated with Put than mock. Conversely, no differences in SA were detected between Put and mock treatments in 2° 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

FIGURE 3 Dependence of Put-triggered transcriptional responses on EDS1, SA and NPR1. Distribution of Put responsive genes in EDS1, SA and NPR1-dependent gene expression sectors. The data was obtained from RNA-seq analyses in *eds1-2*, *sid2-1* and *npr1-1* mutants treated with 100 μM Put or mock for 1 hr. Expression differences are relative to mock in each genotype (see Table S5) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 Quantitation of SAR-related metabolites salicylic acid (SA) and Pipecolic acid (Pip) in Put-inoculated leaves. Free SA and Pip levels in primary inoculated (1°) and systemic non-inoculated (2°) leaves of 5-week-old *Arabidopsis* wild-type plants at 24 and 48 hr post-treatment with 500 μM Put or mock (5 mM MES pH 5.7). Values are the mean of at least three biological replicates ± standard deviation (SD). Letters indicate values that are significantly different according to Tukey's HSD test at $p < .05$. The levels of the SAR-active Pip derivative *N*-hydroxypipecolic acid (NHP) were below the limit of detection for all the samples under investigation

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.

3.5 | 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 *P. syringae* pathovar *tomato* (*Pst*) DC3000 carrying *AvrRpm1* (*Pst AvrRpm1*) or mock

(MgCl₂) and determined Put, Spd and Spm concentration at 24 hr post-inoculation (Figure 5). Because *Pst AvrRpm1* is a potent inducer of SAR, these analyses were performed in both local (1°) and systemic (2°) 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 1° leaves, polyamine levels in 2° 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 1° leaves of β-estradiol infiltrated *a11* transgenic plants expressing β-estradiol-inducible *AvrRpm1*, but not in 2° leaves from the same plants. Conversely, Put accumulation was attenuated in
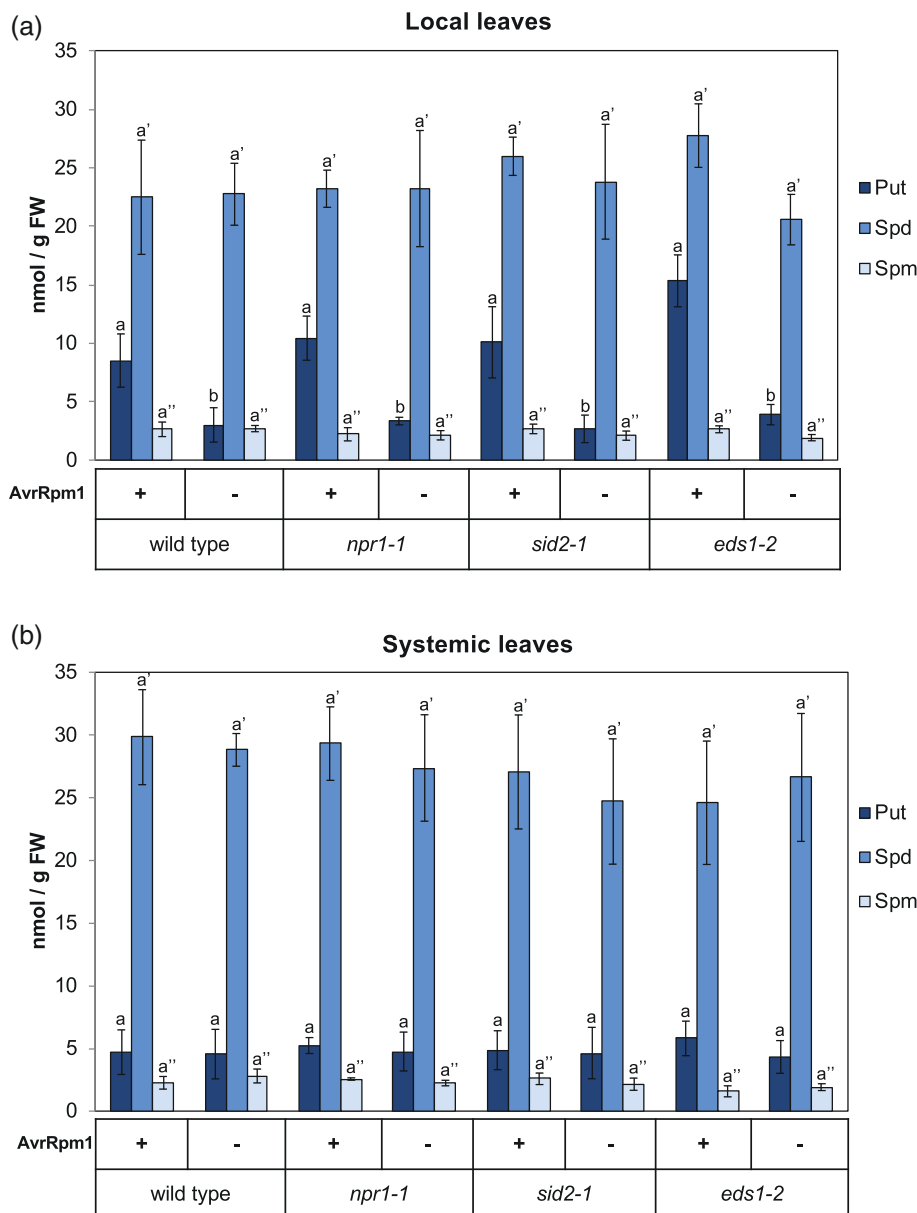
FIGURE 5 Polyamine levels in response to *Pst AvrRpm1*. Levels of putrescine (Put), spermidine (Spd) and spermine (Spm) in local and systemic leaves of five-week-old *Arabidopsis* wild-type, *npr1-1*, *sid2-1* and *eds1-2* plants at 24 hr post-inoculation with ETI and SAR-inducing *Pst AvrRpm1* bacteria (*AvrRpm1*) at $OD_{600\,nm}$ = 0.001 or mock (10 mM MgCl₂). 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 $p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

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.
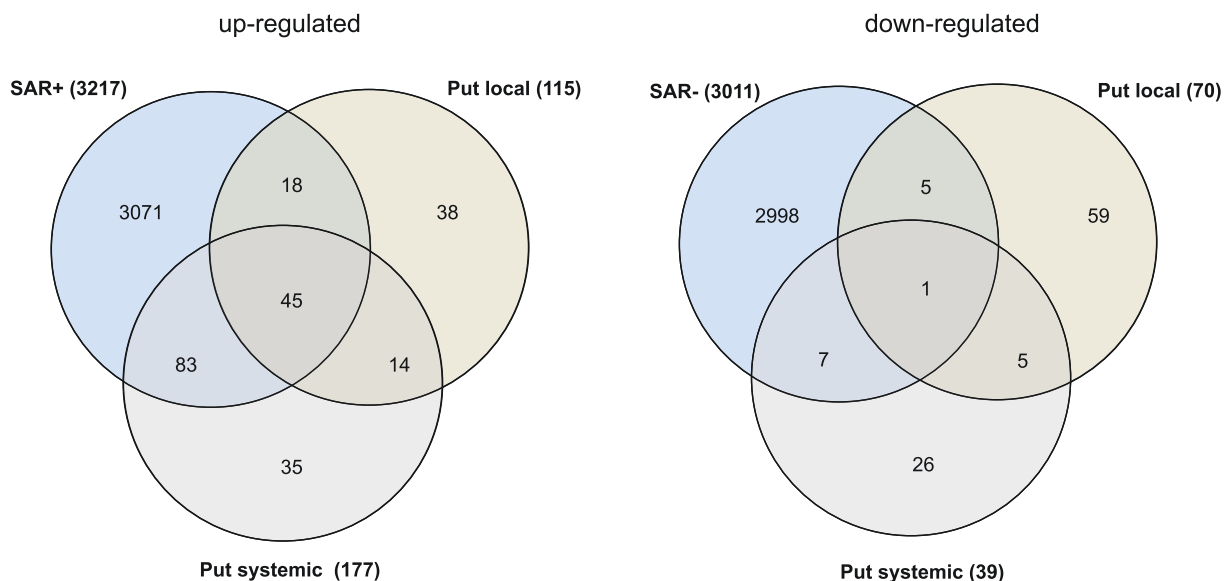
3.6 | Systemic transcriptional responses triggered by Put

Having found that Put accumulates in local leaves during ETI (Figures 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 (1°) and systemic (2°) wild-type leaves at 24 hr post-treatment. A total of 185 and 216 genes were deregulated by Put in 1° and 2° leaves, respectively (Figure 6; Tables S6.1–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 1° leaves and 72.3% of the 177 genes up-regulated by Put in 2° 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 1° but not 2° leaves, as well as the up-regulation of SAR-related genes *FLAVIN-DEPENDENT MONO-OXYGENASE 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 2° 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.

3.7 | 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, 1° leaves of the wild-type were pre-inoculated with 500 µM Put, *Pst AvrRpm1* or mock. After 48 hr, 2° leaves were inoculated with *Pst* DC3000 and bacterial titers determined at 72 hr post-inoculation (Figure 7a). Pre-treatment with Put led to lower *Pst* DC3000 growth in 2° 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.

up-regulated

SAR+ (3217) — Put local (115)
3071 | 18 | 38 | 45 | 83 | 14 | 35
Put systemic (177)

down-regulated

SAR- (3011) — Put local (70)
2998 | 5 | 59 | 1 | 7 | 5 | 26
Put systemic (39)

FIGURE 6 Local and systemic transcriptional responses to Put. Five-week-old *Arabidopsis* wild-type (Col-0) plants were infiltrated with 500 µM Put or mock (5 mM MES pH 5.7). Local inoculated and systemic non-inoculated leaves were harvested at 24 hr post-treatment for global gene expression analyses by RNA-seq. Venn diagram represents the distribution of up- and down-regulated genes in local (Table S6.1) and systemic (Table S6.2) leaves that exhibit significant expression differences in response to Put inoculation compared to mock, and its comparison with previously annotated SAR genes [Colour figure can be viewed at wileyonlinelibrary.com]

(a)

(b)
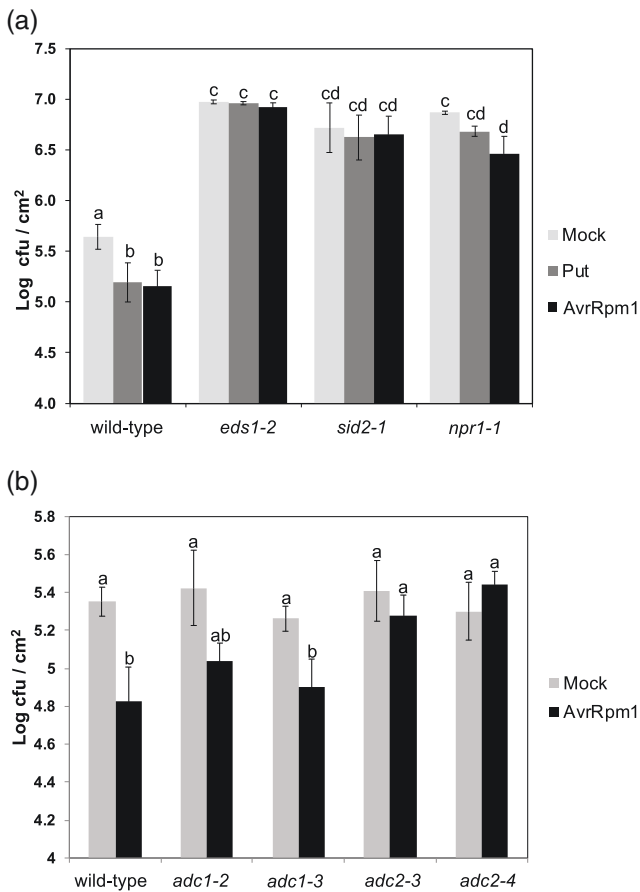
FIGURE 7 Analysis of Put and *Pst AvrRpm1*-triggered systemic resistance in SA pathway and Put deficient mutants. (a) Five-week-old *Arabidopsis* wild-type, *eds1-2*, *sid2-1*, *npr1-1* and (b) *adc1-2*, *adc1-3*, *adc2-3* and *adc2-4* plants were pre-treated with 500 μM Put (Put), *Pst AvrRpm1* ($OD_{600 nm}$ = 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 hr 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 $p < .05$

3.8 | 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*, *A. 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
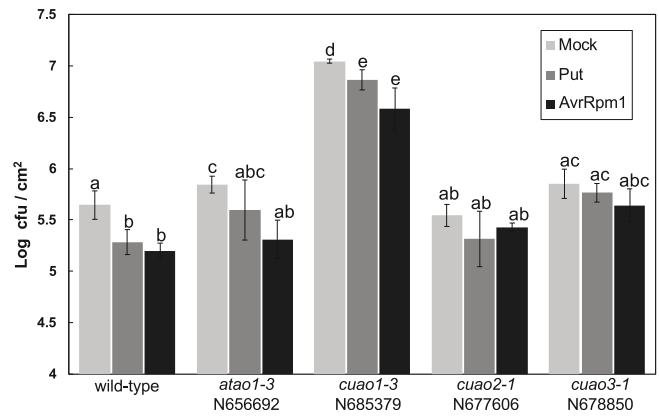
FIGURE 8 Analysis of Put and *Pst AvrRpm1*-triggered systemic resistance in *copper amine oxidase* mutants. Five-week-old *Arabidopsis* wild-type, *atao1-3*, *cuao1-3*, *cuao2-1* and *cuao3-1* mutants were pre-treated with 500 μM Put (Put), *Pst AvrRpm1* ($OD_{600 nm}$ = 0.001) or mock (10 mM $MgCl_2$). Two days later, systemic leaves were inoculated with *Pst* DC3000 (OD_{600nm} = 0.0005). Bacterial numbers were assessed at 72 hr 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 $p < .05$

(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.

4 | 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 et al., 2016). However, this possibility needs further investigation. By focusing on Put, which accumulates in response to a large variety of stresses (Alcázar, Altabella, et al., 2010), 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 (Bindschedler et al., 2006; Daudi et al., 2012; Macho, Boutrot, Rathjen, & Zipfel, 2012; Mammarella et al., 2015; Rojas et al., 2012; Torres, Dangl, & Jones, 2002; Wang et al., 2014).

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 (Liu et al., 2019; Mishina & Zeier, 2007; Zhang & Li, 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, 2015). 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 (Liu et al., 2019; Marina et al., 2008; Moschou et al., 2009; Yamakawa, Kamada, Satoh, & Ohashi, 1998). 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 (Durner, Shah, & Klessig, 1997; León, Lawton, & Raskin, 1995). 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 (Mammarella et al., 2015; Nühse, Bottrill, Jones, & Peck, 2007; O'Brien et al., 2012; Zhang et al., 2007). 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 et al., 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 biphasic (first oxidative and second reductive) redox dynamics (Herrera-Vásquez et al., 2015).

In the context of plant-pathogen co-evolution, the polyamine pathway might be a good target for pathogen effectors or small molecules which are delivered to manipulate host defenses. The observed restriction in *Pseudomonas* growth triggered by Put is consistent with the activity of the TALE-like Bgr11 effector from the plant pathogen *Ralstonia solanacearum* that boost Put biosynthesis to inhibit the growth of microbial niche competitors (Wu et al., 2019). In addition, the *P. syringae* virulence factor phevamine A, which is derived from Spd, suppresses the flg22-induced ROS potentiation of defense responses triggered by Spd in *Arabidopsis* (O'Neill et al., 2018). Overall, we provide new insights into polyamine signaling and defense with a focus on Put. Future research on this topic might help at the rational establishment of plant breeding and/or engineering strategies for plant protection against disease.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

CHAPTER 3

Mutations at *Glucan Synthase-like* (*GSL*) *2* and *GSL10* Suppress Immune-related Hybrid Incompatibility in *Arabidopsis thaliana*

Mutations at *Glucan Synthase-like* (*GSL*) 2 and *GSL10* Suppress Immune-related Hybrid Incompatibility in *Arabidopsis thaliana*

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Abstract

Hybrid incompatibility (HI) is a common phenomenon in plants. The epistatic interaction between the *NUCLEOTIDE BINDING LEUCINE RICH REPEAT* (NB-LRR) *RPP1-like* (*RECOGNITION OF PERONOSPORA PARASITICA 1*) locus, from Landsberg *erecta* (L*er*), and Kas-2 alleles of *STRUBBELIG RECEPTOR KINASE FAMILY 3* (SRF3) leads to immune-related HI, which is associated with fitness loss and constitutive activation of salicylic acid (SA) pathway at 14 -16 °C. Through an EMS mutagenesis screen, here we report the identification of new *suppressors of Ler/Kas-2 incompatibility* (*sulki3-1* and *sulki4-1*) mutants mapping to *GLUCAN SYNTHASE-LIKE 2* (*GSL2*) and *GLUCAN SYNTHASE-LIKE 10* (*GSL10*) genes, respectively. Artificial microRNA (amiRNA) gene silencing of *GSL2* and *GSL10* family members also suppressed dwarfism, cell death, and constitutive SA pathway activation at 14-16 °C. The *sulki3-1*, *sulki4-1* and amiRNA *GSL2/10* lines still exhibited callose deposition in response to the PAMP flg22, wounding and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) inoculation. Furthermore, disease resistance of the L*er*/Kas-2 was restored to parental (L*er* or Kas-2) levels by *GSL2/10* mutations or gene silencing. We concluded that suppression of L*er*/Kas-2 HI by *GSL2* or *GSL10* mutation suppressed HI, without a reduction in basal disease resistance. Our results point to an important role for GSL family and callose deposition in the occurrence of hybrid incompatibility in plants.

INTRODUCTION

Hybrid incompatibilities (HI), as opposed to hybrid vigor, represents a fitness loss generally occurring in F1 or later generations of different species or subspecies, which are separated by reproductive isolation barriers (Bomblies and Weigel, 2007; Rieseberg and Willis, 2007). HI shows reduced viability and fertility, and often involves epistatic interactions which conform to the Bateson–Dobzhansky–Muller (BDM) model (Coyne and Orr, 2004). In the recent decades, the identification of the genetic bases of HI revealed the frequent involvement of one *NUCLEOTIDE BINDING LEUCINE-*

RICH REPEAT (NLR) locus that, in interaction with other disease resistance genes (*R*), or genes with diverse functions, triggers the occurrence of immune-related HI (Bomblies and Weigel, 2007; Alcázar et al., 2010; 2012; Chae et al., 2014). NLR proteins contain a central nucleotide-binding site (NB) domain and a C-terminus leucine-rich repeat domain. They are divided into two subclasses, depending on whether they carry N-terminus Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) domains (Sinapidou et al., 2004). NLR proteins play important roles during the immune response of plants, through recognition of pathogen effectors and activation of defense responses called effector-triggered immunity (ETI). ETI is an accelerated and amplified version of pathogen-associated molecular patterns (PAMPs) - triggered immunity (PTI) (Jones and Dangl, 2006), and both PTI and ETI potentiate each other (Ngou et al., 2020). As such, the study of immune-related HI can be used as a model for the study of ETI in the absence of pathogen challenge.

In Arabidopsis (*Arabidopsis thaliana*), the highly polymorphic TIR-NLR (TNL) *RPP1* (*RECOGNITION OF PERONOSPORA PARASITICA 1*) - *like* locus, also known as *DANGEROUS MIX* locus 2 (*DM2*), is a hotspot for immune-related HI studies (Bomblies et al., 2007; Chae et al., 2014; Stuttmann et al., 2016; Vaid and Laitinen, 2019), Some *RPP1-like* genes are known to recognize effectors from pathogenic oomycete *Hyaloperonospora Arabidopsis*, thus contributing to resistance through activation of ETI. The *DM2* locus was first reported in the accession Uk-1, which in interaction with the *SSI4* (*DM1*) from Uk-3, caused immune-related HI (Bomblies and Weigel, 2007). Such NLR allelic

interactions are expected to lead conformational changes in NLR proteins that lead to constitutive activation of defense. Alcázar et al. (2009; 2010) identified another temperature-dependent incompatible interaction between TNL *RPP1-like* genes (*R1-R8*) of Landsberg *erecta* (L*er*) (overlapping with the *DM2* locus also known as QTL3) and Kashmir-2 (Kas-2) alleles of *STRUBBELIG RECEPTOR KINASE FAMILY 3* (SRF3). A L*er*/Kas-2 near-isogenic line (NIL) carrying a single L*er* *RPP1* locus introgression on QTL 3 in a homogeneous Kas-2 background reconstituted dwarfism, cell death and sterility at 14-16°C (hereafter referred to as low temperature). However, the growth and reproductive loss of the L*er*/Kas-2 NIL could be suppressed at 20-22°C (Alcazar et al., 2009; 2010).

We previously reported the isolation of suppressors of L*er*/Kas-2 incompatibility (*sulki*) mutants from an EMS mutagenesis screen of the incompatible NIL (Atanasov et al., 2018). Although most *sulki* mutants mapped to the *RPP1*-like L*er* cluster (*RPP1*-like L*er* R8 in *sulki1-1* to *sulki1-10* and *RPP1*-like L*er* R3 in *sulki2-1*) (Atanasov et al., 2018), *sulki3-1* and *sulki4-1*, carried wild-type alleles at incompatible loci thus representing extragenic mutations. Here we report the mapping of *sulki3-1* and *sulki4-1* mutants to *GSL* (glucan synthase-like) *2* and *GSL10* genes, respectively.

The β-1,3 -D-glucan callose is synthesized by GSL also known as callose synthase (CalS) proteins (Stone and Clarke, 1992; Xie and Hong, 2011). Callose is an essential component during growth, development, and accumulates during the defense response (Dong et al., 2005; Huang et al., 2009; Ellinger et al., 2013; Shikanai et al.,

2020). Papilla formation in higher plants contributes to innate immunity and associates with global transcriptional changes (Jones and Dangl, 2006; Ellinger et al., 2013). Arabidopsis has twelve glucan synthase family members (Hong et al., 2001), which are divided into two groups based on their biological functions. GSL1, GSL2, GSL6, GSL8 and GSL10 constitute the largest group, which is mainly responsible for callose biosynthesis during pollen development and cell division (Drábková and Honys, 2017). GSL4, GSL5, GSL7 and GSL12 are involved in callose deposition during plugging, barrier formation, and other types of structural reinforcements (Hong et al., 2001; Ellinger and Voigt, 2014). For the rest GSL members, their function is yet unclear. GSL5 is crucial for callose synthesis in response to many external stimuli (Jacobs et al., 2003; Nishimura et al., 2003). GSL2 is responsible for the establishment of the callose wall enclosing pollen mother cells and pollen tubes in Arabidopsis (Dong et al., 2005; Nishikawa et al., 2005). GSL10 mostly localizes to the plasma membrane and is essential for microspore growth, alleviation of cell wall damage and defense responses in Arabidopsis (Töller et al., 2008; Huang et al., 2009; Chen and Kim, 2009; Shikanai et al., 2020). However, the contribution of GSL to hybrid incompatibilities (ETI) or *GSL2* and *GSL10* in defense, have not been reported. In this study, we investigated the involvement of *GSL2* and *GSL10* to L*er*/Kas-2 immune-related HI. Analysis of *sulki3-1*, *sulki4-1* and artificial microRNA (amiRNA) silencing of *GSL2* and *GSL10* in incompatible NIL background demonstrated that these two GSL contribute to L*er*/Kas-2 HI at low temperature. We also analyzed the effect of L*er*/Kas-2 HI

suppressive mutations on defense against pathogenic bacteria. Our results support a role for callose biosynthesis in the occurrence of immune-related hybrid compatibilities (ETI) in plants.

RESULTS

Mapping extragenic suppressors of L*er*/Kas-2 HI

A screen for suppressors of L*er*/Kas-2 HI at 14-16ºC identified two extragenic mutants, *sulki3-1* and *sulki4-1*, which carried dominant mutations that fully suppressed dwarfism and cell death at 14-16ºC, which are hallmarks of HI (**Figure 1 and Supplemental Figure S1**). Both mutants were backcrossed five times with the parental L*er*/Kas-2 NIL and the genome sequences of BC_5F_1 plants were obtained by next generation sequencing. Mapping of *sulki3-1* and *sulki4-1* reads to the L*er*/Kas-2 NIL identified unique G/C to A/T transition mutations that segregated with the suppression of incompatibility. The *sulki3-1* mutant carried a R758Q non-synonymous substitution in exon 21 of the *At2g13680* gene, coding for *GLUCAN SYNTHASE-LIKE 2* (GSL2). This amino acid change is next to a transmembrane domain and mapped to a highly conserved amino acid in the glucan synthase-like (GSL) family (**Figure S2**). The *sulki4-1* mutant carried a R1130C non-synonymous substitution in exon 31 of *At3g07160*, coding for another member of the GSL family (*GLUCAN SYNTHASE-LIKE 10,* GSL10). This amino acid change mapped to a highly conserved residue in a conserved GSL domain (**Figure S2**). These results

pointed to an important contribution of callose synthases to the occurrence of L*er* /

Kas-2 HI and by extension, to ETI.

A

B
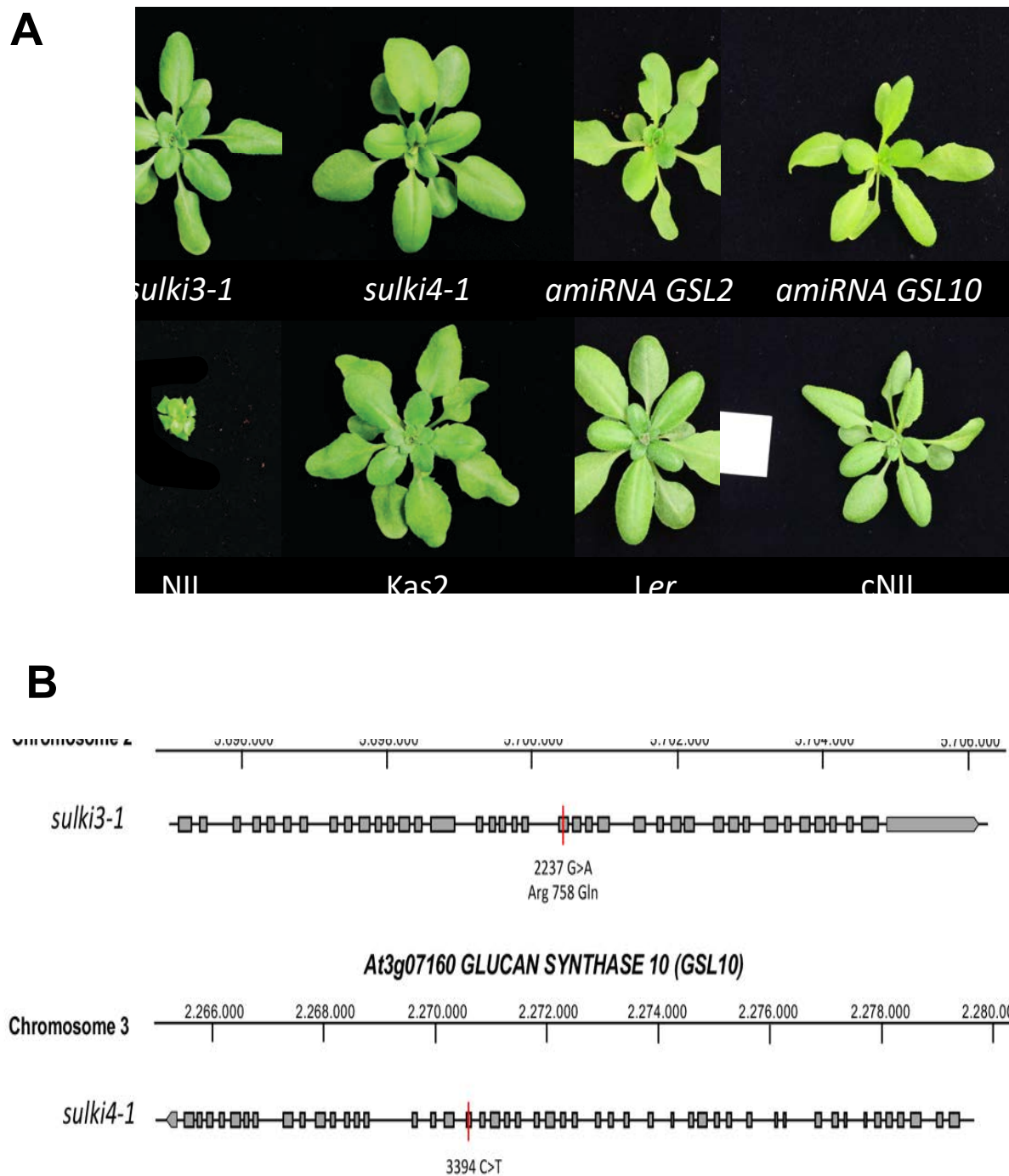
Figure 1. Suppression of L*er*/Kas-2 HI in *sulki3-1*, *sulki4-1*, and amiRNA lines (NIL) at 16 °C.

(A) Growth phenotypes of 5-week-old L*er*, Kas-2, L*er*/Kas-2 NIL, L*er*/Kas-2 NIL complemented w *SRF3* L*er* (cNIL), *sulki3-1*, *sulki4-1*, amiRNA *GSL2* and *GSL10* lines grown on soil at 14-16 °C. **(** Schematic representation of *GSL2* and *GSL10* genes and position of nonsynonymous substitutions *sulki3-1* and *sulki4-1*.

GSL expression in *sulki3-1*, *sulki4-1*, and amiRNA lines (NIL)

The relative expression of the twelve Arabidopsis *GSL* gene members was determined in *sulki3-1*, *sulki4-1*, and amiRNA *GSL2/10* plants by real-time quantitative reverse transcriptase mediated PCR (qRT-PCR) (**Figure 2**). Compared with the NIL, the expression of *GSL2* and *GSL10* was suppressed in *sulki3-1*, *sulki4-1* and amiRNA *GSL2/10* plants (three- to five-times lower), which resembled the parental line L*er* and complemented L*er*/Kas-2 NIL (cNIL) (**Figure 2B and 2F**). Intriguingly, in comparison with the NIL, *GSL5* expression was reduced two- to three-time in *sulki3*-1, *suilki4*-1 and amiRNA *GSL2*/*10* lines (**Figure 2E**). In addition, expression of *GSL8* and *GSL9* were also significantly lower in suppressed L*er*/Kas-2 HI plants. *GSL9* expression was between 2.5 to 6-fold higher in NIL than in *sulki3-1*, *suilki4-1* and amiRNA *GSL2* and *GSL10* (**Figure 2J and 2I**). Collectively, these results indicate that expression of *GSL2* and *GSL10* but also of other *GSL* members is reduced in mutants and amiRNA lines suppressing L*er*/Kas-2 HI.

Callose formation in response to flg22, wounding and *Pst* DC3000 inoculation

Callose can be detected under an epifluorescence microscope using aniline blue staining (Stone et al., 1984; Ellinger et al., 2013). In control conditions, no evident callose deposits are observed in suppressed L*er*/Kas-2 HI plants (*sulki3-1*, *sulki4-1* and amiRNA *GSL2/10*), or L*er*. Conversely, the NIL and Kas-2 accession exhibited callose deposition at low temperature (**Figure 3**, Mock). Relative callose intensity (RCI) was used to quantify the extent of callose formation (Miché et al., 2018). RCI of the NIL and Kas-2 were 55.1% and 29.4% higher than cNIL in the control condition (Mock) (**Figure 4A**). Indeed, Kas-2 is a naturally occurring spontaneous cell death mutant (**Figure S1**). These results are also consistent with higher expression of *GSL5* in NIL and Kas-2 (**Figure 2E**). Callose deposition was also studied in response to exogenous stimuli, such as the well-studied PAMP flg22 (a peptide from flagellin), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and wounding. Deposited callose could be observed in all genotypesupon these three external stimuli (**Figure 3**). Relative to the cNIL, RCI was significantly higher in all genotypes upon flg22 infiltration and wounding. The L*er*/Kas-2 NIL exhibited higher callosic papillae in response to flg22 and wounding among all genotypes (TukeyHSD test, $P<0.05$, **Figure 4B** and **4C**). Conversely, *sulki3-1*, *sulki4-1* and amiRNA *GSL2/10* lines resembled the L*er* parent in all treatments (TukeyHSD test, $P<0.05$, **Figure 4B and 4C**). No difference of callose deposits was observed in response to *Pst* DC3000 in either genotype (**Figure 4D**). We concluded that *GSL2* and *GSL10* contributed to the suppression of L*er*/Kas-2 HI but did not compromise callose deposition in response PAMP or wounding stimulation.
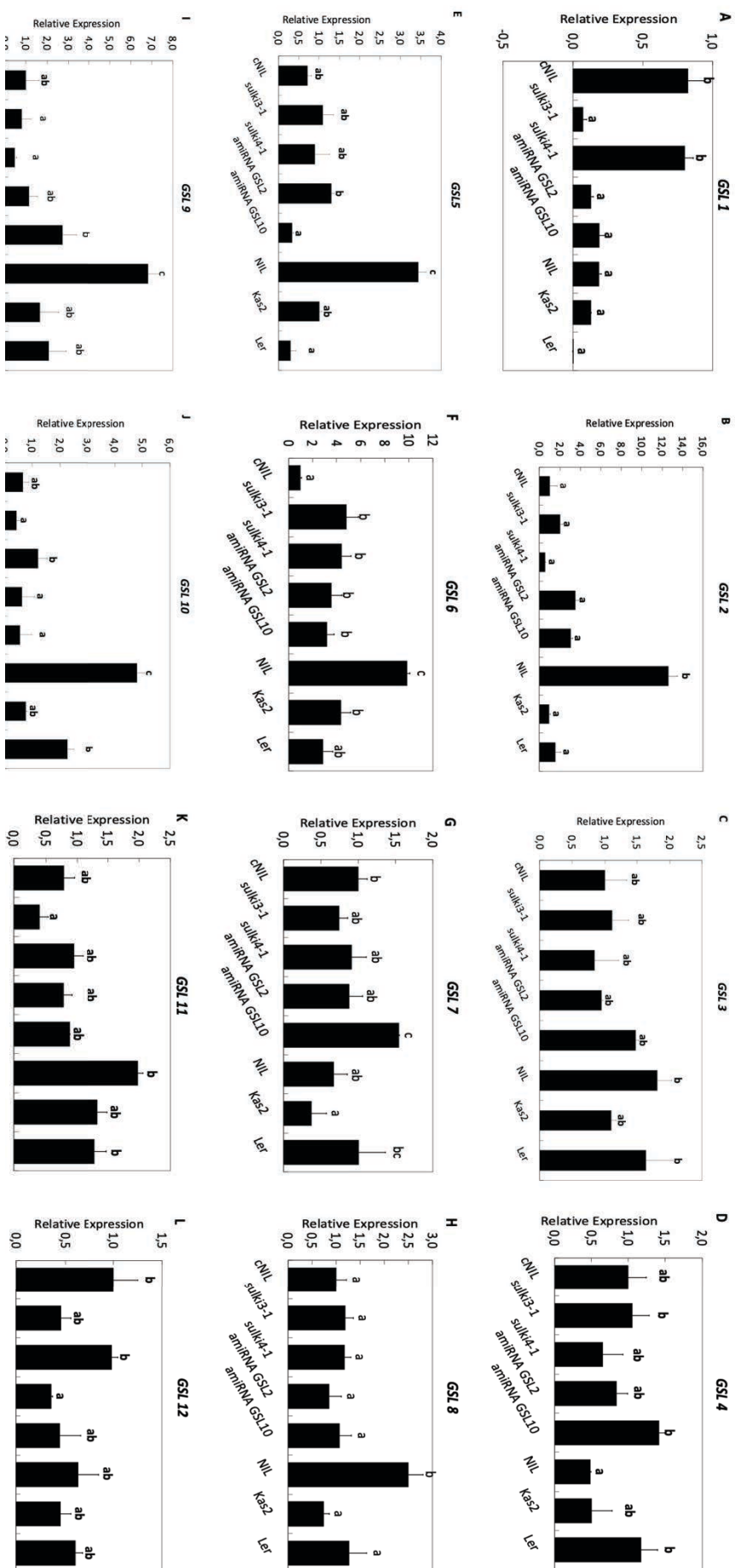
old *Arabidopsis* plants grown at 14-16 °C. Expression differences are relative to the Ler/Kas-2 NIL complemented with *SRF3* (Ler), cNIL. Va are the mean from at least three biological replicates ± SD. Letters indicate values that are significantly different, according to Tukey's HSI at *P* < 0.05.
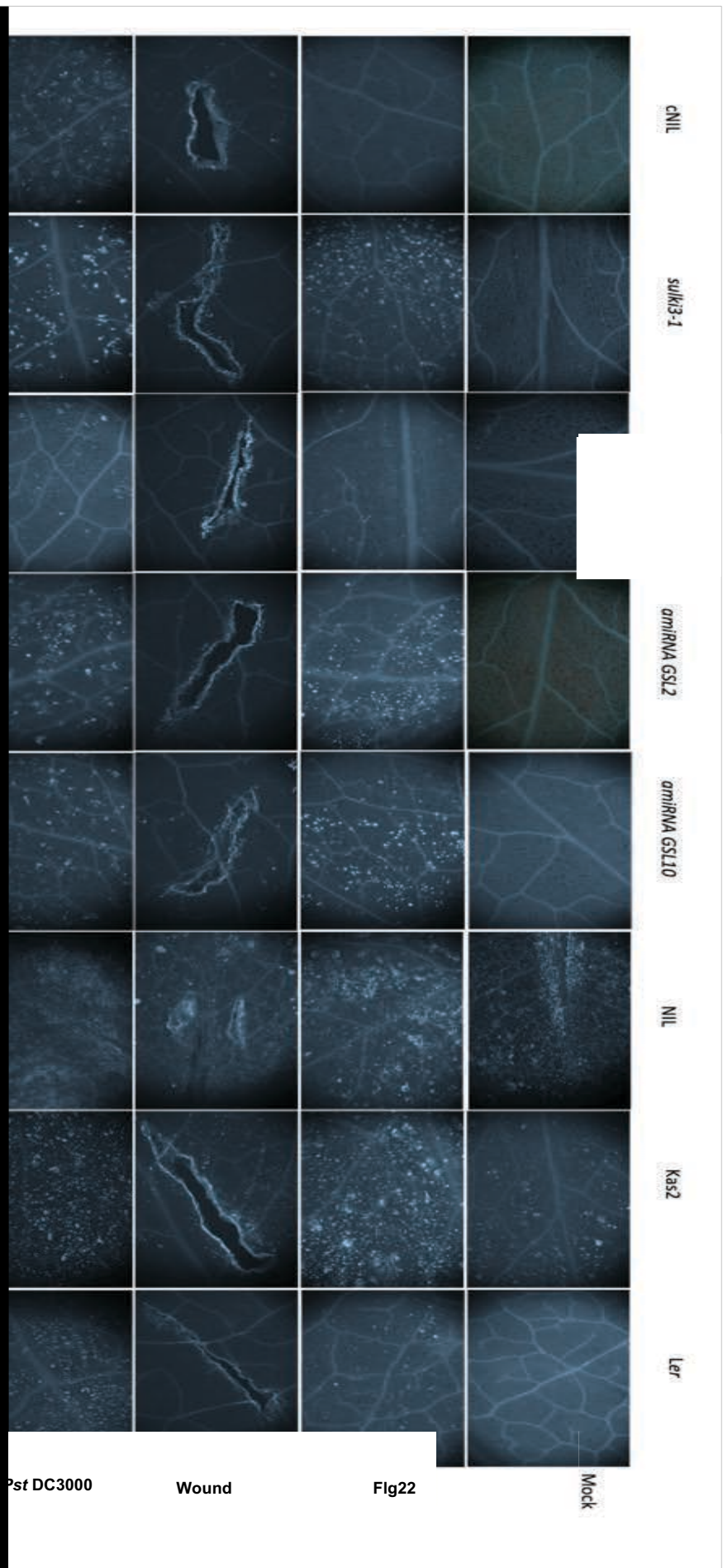
14-16 °C. Leaves of 5-week old *Arabidopsis* plants at grown at 14-16 °C were wounded with a razor blade, inoculated with 1 μM flg22 or spray-inoculated with *Pseudomonas syringae* pv. tomato DC 3000 (*Pst* DC3000) at OD$_{600nm}$ = 0.2. Callose deposits were observed at 24 h post-treatment by staining with aniline blue.
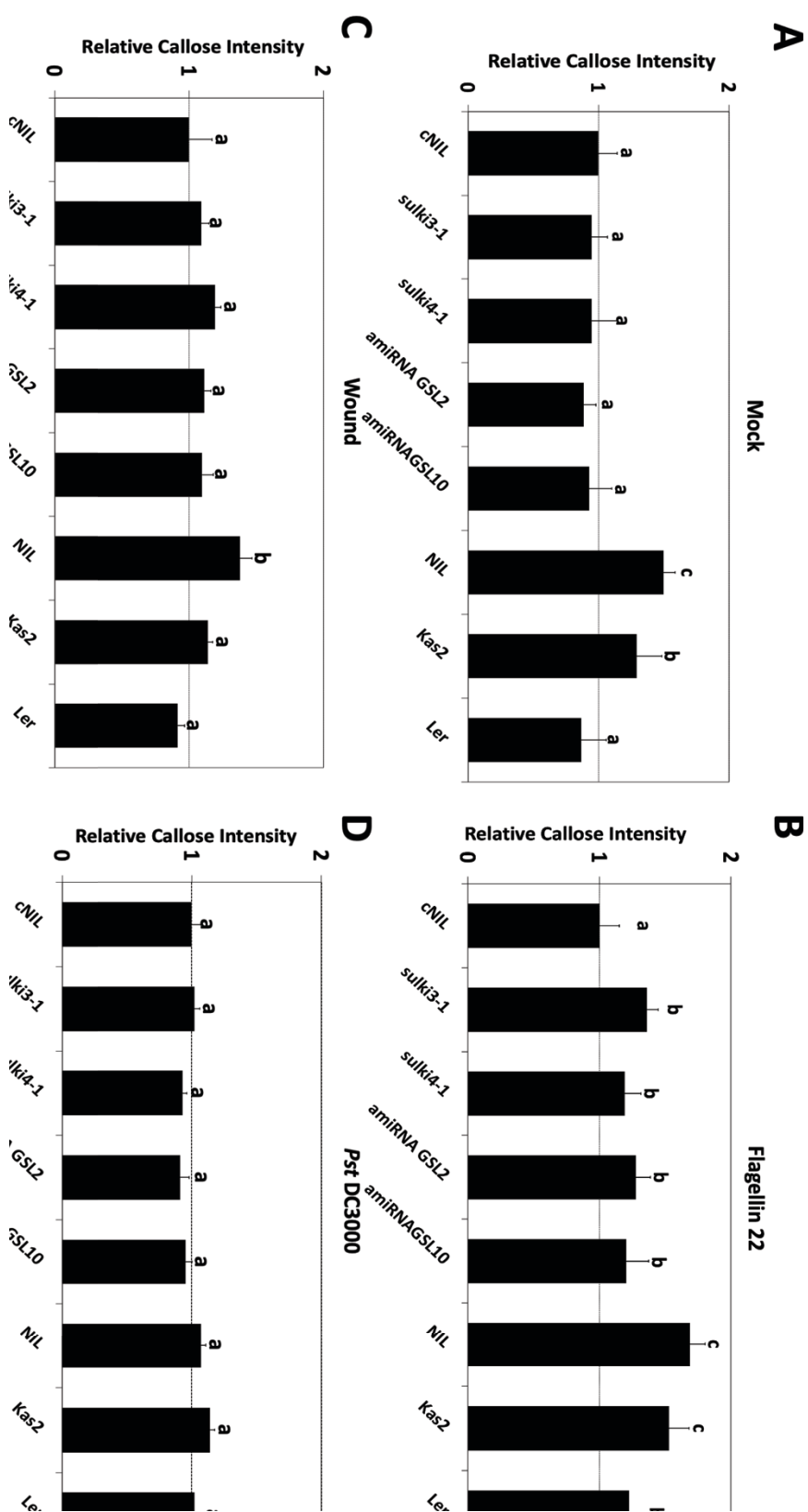
Figure 4. Relative callose intensity (RCI) in *sulki3-1*, *sulki4-1*, and amiRNA lines (NIL) in response to mock treatment (A), flg22 (B), wounding (C) and *Pst* DC3000 (D) at 14-16 °C. Leaves of 5-week old *Arabidopsis* plants at grown at 14-16 °C were, wounded with a razor blade, inoculated with 1 μM flg22 or spray-inoculated with *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000) at OD$_{600nm}$ = 0.2. Callose deposits were observed at 24 h post-treatment by staining with aniline blue. Relative callose intensity (RCI) was calculated according to the method of Luna et al. (2011). Values are relative to the cNIL, and represent the mean from at least twenty replicates + SD. Letters indicate values that are significantly different, according to Tukey's HSD test at *P* < 0.05.

Analysis of SA pathway activation

A previous study revealed that constitutive activation of the SA pathway underlies dwarfism, cell death and sterility of L*er*/Kas incompatible hybrids at low temperature (Alcázar et al., 2009). Given that dwarfism and cell death were alleviated by *GSL2* and *GSL10* mutations or silencing, we performed gene expression analyses and quantified SA levels as a proxy for the determination of SA-pathway status in *sulki3-1*, *sulki4-1* and amiRNA *GSL2/10* lines. The expression of SA pathway marker gene *PR1* (Cao et al., 1997), as well as *EDS1* were significantly lower in suppressed L*er*/Kas-2 HI plants than in the incompatible NIL (**Figure 5**; TukeyHSD test, *P*<0.05). Similarly, free SA was strikingly higher in incompatible NIL than

in *sulki3-1*, *sulki4-1*, amiRNA *GSL2* or amiRNA *GSL10* (**Figure 5D**). *PR1* expression in *sulki3-1*, *sulki4-1*, and amiRNA *GSL2/10* lines was similar to *eds1-1* and L*er* _*NahG*, which carries the bacterial salicylate hydroxylase gene (NahG), involved in the conversion of SA to catechol (Yamamoto et al., 1965). The expression of *GST1* (a marker for oxidative stress) showed no significant differences between suppressed L*er*/Kas-2 HI plants and the parental L*er* (TukeyHSD *P*<0.05, **Figure 5C**), which suggested that oxidative stress is mitigated by *GSL2* and *GSL10* mutation or silencing (Alcázar et al., 2009), except for amiRNA *GSL10* which behaves differently (**Figure S2**). Collectively, these results indicated that *sulki3-1*, *sulki4-1* and amiRNA *GSL2/10* lines suppress the constitutive activation of SA pathway of the NIL.

Effect of *GSL2* and *GSL10* mutations in L*er*/Kas-2 NIL on *Pseudomonas syringae* DC3000 (*Pst* DC3000) disease resistance

To determine whether the enhanced basal disease resistance of L*er*/Kas-2 NIL (Alcázar et al., 2010) was also compromised, the different genotypes were inoculated with *Pst* DC3000 and bacteria titers determined at 0- and 3-days post-inoculation (dpi) (**Figure 6**). The results did not identify significant differences in the bacterial growth between *sulki3-1*, *sulki4-1*, Kas-2 or L*er*. Conversely, amiRNA *GSL2/10* lines resulted to be more susceptible to *Pst* DC3000 than Kas-2 or L*er*. We concluded that suppression of L*er*/Kas-2 HI by *GSL2* or *GSL10* mutation is not at expenses of a reduction in basal disease resistance, although amiRNA lines exhibited enhanced susceptibility.
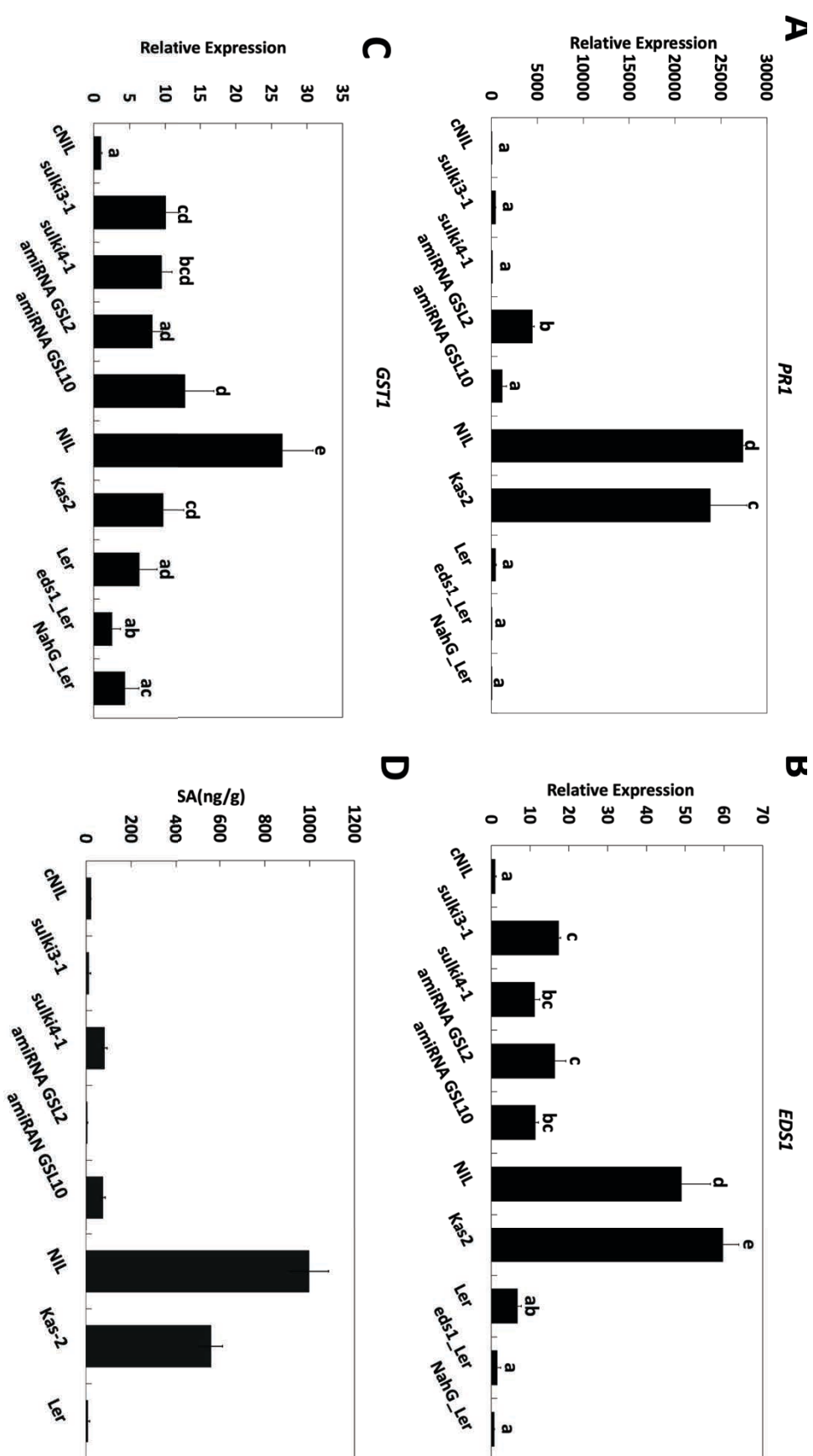
Figure legend text (rotated):

...of expression changes of *PR1* (A), *EDS1* (B), and *GST1* (C) genes, and the SA levels (D), were measured in leaves from 6-week-old *Arabidopsis* plants grown at 14-16°C. Expression differences are relative to the Ler/Kas-2 NIL complemented with *SRF3* (*Ler*), cNIL. Values are the mean from at least three biological replicates + SD. Letters indicate values that are significantly different by Tukey's HSD test at *P* < 0.05.

Panel A — *PR1* (Relative Expression): cNIL (a), sulki3-1 (a), sulki4-1 (a), amiRNA GSL2 (b), amiRNA GSL10 (a), NIL (d), Kas2 (c), Ler (a), eds1_Ler (a), NahG_Ler (a)

Panel B — *EDS1* (Relative Expression): cNIL (a), sulki3-1 (c), sulki4-1 (bc), amiRNA GSL2 (c), amiRNA GSL10 (bc), NIL (d), Kas2 (e), Ler (ab), eds1_Ler (a), NahG_Ler (a)

Panel C — *GST1* (Relative Expression): cNIL (a), sulki3-1 (cd), sulki4-1 (bcd), amiRNA GSL2 (ad), amiRNA GSL10 (d), NIL (e), Kas2 (cd), Ler (ad), eds1_Ler (ab), NahG_Ler (ac)

Panel D — SA(ng/g): cNIL, sulki3-1, sulki4-1, amiRNA GSL2, amiRAN GSL10, NIL, Kas-2, Ler

Figure 6. Analysis of resistance to *Pst* DC3000 in *sulki3-1*, *sulki4-1*, and amiRNA lines (NIL) at 14-16 °C. Leaves of 5-week-old *Arabidopsis* plants grown at 14-16 °C, were inoculated with *Pst* DC3000 (OD$_{600nm}$ = 0.2) by spraying. Bacterial growth was determined at 3 days post-inoculation (DPI). Values are the mean of at least nine biological replicates ± SD. Letters indicate values that are significantly different according to Tukey's HSD test at $P < 0.05$.

DISCUSSION

Hybrid incompatibilities are caused by genetic interactions between gene variants, leading to deleterious epistatic interactions, or parental conflict (Chae et al., 2014; Vaid and Laitinen, 2019). Immune-related HI conditioned by temperature generally involve at least one polymorphic *NLR* locus. In the case of the L*er*/Kas-2 HI, one incompatible locus maps to a RPP1-like TNL cluster in L*er* and the other locus to the receptor-like SRF3 in Kas-2 (Alcazar et al., 2009; 2010). In an attempt to identify suppressors of L*er*/Kas-2 HI, an EMS mutagenesis screen was performed that identified several extragenic mutants not mapping to *RPP1*-like or SRF3. Through NGS sequencing, we mapped two

extragenic mutants (*sulki3-1* and *sulki4-1*) to glucan synthase like members, GSL2 and GSL10, carrying non-synonymous substitutions in conserved GSL domains. The causality of mutations was corroborated by GSL2 and GSL10 silencing in amiRNA lines. Lower *GSL2* and *GSL10* expression (**Figure 2**), suppressed autoimmune response and fitness loss of L*er*/Kas-2 HI at low temperature (**Figure 1A** and **Figure S1**).

Importantly, *GSL2* or *GSL10* expression was not suppressed to parental levels in amiRNA lines (**Figure 2B** and **2J**). This might explain why GSL10 silencing did not reconstitute the dwarfism reported for *GSL10* dsRNAi lines observed at 23°C (Töller et al., 2008). Shikanai et al. (2020) also reported the occurrence of cell death in a *GSL10* loss-of-function mutant (*gsl10-5*)

under low-calcium conditions, but not under optimal calcium levels. These results support the involvement of *GSL10* in triggering cell death/defense under certain environmental conditions. In this study, both *sulki3-1* and amiRNA *GSL2* were able to produce fertile seeds, while *gsl2* (*cals5*) mutant is sterile completely (Dong et al., 2005b; Nishikawa et al., 2005). The data indicated that *sulki3-1* could not mimic amiRNA GSL2 loss-of-function mutation, while which is sufficient to suppress L*er*/Kas-2 HI at low temperature.

Callose deposition upon flg22 and wounding, depends on GSL5 activity. Remarkably, callose deposition in response to these stimuli was not compromised in suppressed L*er*/Kas-2 HI plants but exhibited a similar response to L*er* (**Figure 4B** and **4C**) (Ellinger and Voigt, 2014). *GSL5* and *GSL6* expression are upregulated by inoculation with the *H. arabidopsis* isolate Emco5 (Dong et al., 2008). However, the bacterial type III effector AvrPto was found to restrict callose deposition (Hauck et al., 2003). Effector interference might be considered in the analysis of differences in callose deposition in response to *Pst* DC3000 between genotypes (**Figure 4D**). In addition, wounding is an inherent consequence of pathogen penetration or infiltration (Dong et al., 2008). Therefore, pathogen inoculation activates many different mechanisms leading to callose deposition, which might make physiological differences when stimuli are applied separately. Interestingly, callose was deposited in the vascular buddle cells of the NIL in response to flg22, but not in parental L*er*, which only presented callose as in papilla (**Figure S3**). This might be due to the autoimmune response associated oxidative stress in NIL (**Figure 5C**)

(Alcázar et al., 2009), as callose deposition and ROS occur together in vascular tissue (Kong et al., 2013).

SA pathway was restricted in the *GSL2* and *GSL10* suppressed L*er*/Kas-2 HI (**Figure 5A** and **D**). Shikanai et al. (2020) reported that SA and JA were both synergistically induced in *GSL10* loss-of-function plants (*gsl10-5*) compared with wildtype (Col0) at 22°C. Correspondingly, exogenous SA (2 μM) also induced weak *GSL2* and *GSL10* expression in a *NRP1* independent manner (Dong et al., 2008; Zavaliev et al., 2011). We speculate that the interaction between callose biosynthesis and SA pathway might depend on environmental conditions. Further studies should address this question. In addition, the EMS-generated mutations reported here behave dominant and are not allelic to *GSL* loss-of-function mutations reported elsewhere.

Our study confirmed that the suppression of L*er*/Kas-2 HI in *sulki1* and *sulki2-1* is not at expenses of a reduction in basal resistance, which is similar to parental lines Kas-2 or L*er* (Atanasov et al., 2018). This might be due to the functionality of *GSL5* in *GSL2* and *GSL10* deficient mutants, which contributes to pathogen resistance (**Figure 6**) (Ellinger et al., 2013). Whether callose contributes to disease resistance and ETI in particular is still under debate (Consonni et al., 2010; Ellinger et al., 2013). Here we provide genetic evidence that certain GSL members are required for the establishment of immune-related HI exhibiting constitutive ETI. We speculate that callose deposition is not a mere physiological response to environmental stimuli, but an integrated metabolic response contributing to immune signaling. Overall, our study paves a way to investigate the role of GSL

in contributing to temperature-dependent hybrid incompatibilities and ETI signaling in plants.

MATERIAL AND METHODS

Plant materials and growth conditions

All plants were grown under 12 h dark/12 h light cycles at 14/16 ºC, 100-125 μmol photons m^{-2} s^{-1} of light intensity, and 70% relative humidity. The incompatible L*er*/Kas-2 NIL and cNIL used in this work was described previously (Alcazar et al., 2009; 2010). *eds1-1* and NahG-L*er* plants were kindly provided by Jane Parker.

EMS Mutagenesis

Seeds of L*er*/Kas-2 NIL were soaked in 1 mg/mL KCl at 4°C overnight. After seed imbibition, the solution was discarded and replaced with 0.2% EMS (v/v) and incubated for 16 h before washed 10 times with 50 mL of water, then suspended in 0.1% agarose for sowing on soil. Approximately 25,000 M1 plants were allowed to self at 20-22°C. M2 seeds were collected in pools of 100 to 150 M1 plants. M2 plants were grown at 14-16°C to identify suppressors of Ler/Kas-2 incompatibility (*sulki*) (Atanasov et al., 2018).

Real-Time qPCR expression analyses

Total RNA isolated from 5-week-old Arabidopsis seedlings was extracted with *TRIzol* reagent (Thermo Fisher). Reverse transcription and quantitative real-time PCR was performed as described previously (Liu et al., 2019). Two micrograms of RNA were treated with DNAse I (Invitrogen), and reverse transcription performed with Superscript IV (Invitrogen) and oligo dT were adopted to synthesize first strand cDNA. Semiquantitative real-time PCR using SYBR Green I dye method was performed on Roche LightCycler 480 II detector system following the PCR program: 95 ºC 2 min, 40 cycles (95 ºC, 15 s; 60 ºC, 30 s; 68 ºC, 20 s). qPCR analyses were always performed on at least three biological replicates with three technical replicates each using Actin (*At3g18780*) as the internal control gene. Relative transcript expression was calculated by 2$^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers used for expression analyses are shown in Supplementary Table 1 (**Table S1**).

Free SA levels measurement

Determination of total salicylic acid was performed using *Acinetobacter* sp. ADPWH_lux (Defraia et al., 2008). An overnight culture of *Acinetobacter* sp. ADPWH_lux was diluted in 37°C LB (1:20) and grown for ~3 h at 220 rpm to an OD600 of 0.4. Twenty μl of extract was added to 60 μl room temperature LB in a 96-well black cell culture plate. Using a multi pipet, 50 μl of biosensor culture (β-glucosidase, SIGMA) was added to each well and mixed by pipetting. The plate was incubated at 37°C for 1 h before luminescence was read using a Victor3 Perkin Ellmer Multi-Detection Microplate Reader (PerkinElmer, Waltham, Massachusetts). Each sample was measured in triplicates. Known amounts of SA were dissolved in either LB or acetate buffer, then diluted 10- fold in plant extract. SA

standards were read in parallel with the experimental samples.

Histochemical analyses

For aniline blue staining, seedlings were fixed in a solution of acetic acid: ethanol (1:3) overnight, followed by washing in 150 mM K_2HPO_4 during 30 min for two times, and incubated with 150 mM K_2HPO_4 containing 0.01% aniline-blue (Sigma) for 2 h with shaking. Observations were performed under an epifluorescence microscope and photographs captured with a NIKON microscopy camera coupled to the NIS software 4.45 (NIKON). Callose intensity quantification was performed according to Luna et al. (2011). Briefly, callose was quantified from digital photographs by counting the number of white pixels (callose intensity), using Photoshop 21.1 software. Callose was selected automatically, using the "Color Range" tool. In cases where the contrast settings resulted in significant loss of callose signal due to high autofluorescence from the vasculature, callose was selected manually, using the "Magic Wand" tool. The accuracy of the resulting callose selection was visually verified before proceeding. Callose-corresponding pixels were recorded as the area covered by the total number of measurements using the "Record Measurements" tool. Average callose relative intensity (RCI) measurements were relative to complemented L*er*/Kas-2 HI (cNIL), based on at least 20 pictures from nine different seedlings.

Trypan blue staining for cell death visualization was performed as previously described (Alcázar et al.,2010). Briefly, 5-week-old Arabidopsis leaves were harvested, and stained with lactophenol trypan blue. Leaves were mounted in 60% glycerol before observation under a light microscope (Axioplan; Carl Zeiss), a minimum of nine leaves were visualized per genotype.

Pseudomonas syringae pv. *tomato* DC3000 inoculation assays

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) bacteria was cultivated on solid NYGA medium (5 g/L bactopeptone, 3 g/L yeast extract, and 20 mL/L glycerol, with 15 g/L agar for solid medium) containing 25 µg/mL rifampicin. Single colonies were amplified in solid NYGA plate (25µg/ml rifampicin) and incubated at 28 ºC. Bacteria was suspended with 10 mM $MgCl_2$ to OD_{600}= 0.2. Silwet L-77 was added to a final concentration of 0.04% (v/v) before spray inoculation of 5-week-old *Arabidopsis* plants. Leaves were harvested at 3h and 3 days post pathogen inoculation. Determination of bacterial growth was performed as described by (Alcázar et al., 2010). At least nine biological replicates were determined per genotype and point of analysis.

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General Discussion

Plants have evolved complicated defense systems that enable them to survive in various habitats. In plants, polyamines make vital contributions to growth, development and the stress response, which involve complex genetic and physiological processes (Alcázar et al., 2010a).

The core topic of this Thesis is to investigate the molecular functions of polyamines during defense in Arabidopsis. The polyamine putrescine (Put) is induced during stress. Here we provide evidence that Put induced signaling involves other defense associated pathways.

Polyamines, especially Put, accumulate during PTI induced by *Pst HrcC* (Yuan and He, 1996), the purified PAMP flg22 (*see* **Chapter 1**; **Figure 1** and **Figure 2**), as well as in response to ETI initiated by *Pst AvrRpm1* (*see* **Chapter 2**; **Figure 5** and **ANNEX III Figure S6**) (Vilas et al., 2018). Our data support that effectors from *Pst* DC3000 promote polyamine biosynthesis rather than its suppression, as we observe higher Put levels using virulent than avirulent bacteria (*see* **Chapter 1**; **Figure1**) (Jiménez-Bremont et al., 2014; Vilas et al., 2018). However, it may also well be that the polyamine accumulation depends on bacteria titers. In this regard, avirulent bacteria growth is more restricted. In addition to this, it will be interesting to determine whether living bacteria or the presence of their PAMPs in heat-inactivated bacteria is sufficient to trigger Put biosynthesis. The identification of bacteria elicitors triggering polyamine biosynthesis is attractive to the development of plant protection products.

We confirmed that the main source for polyamine accumulation during pathogen infection is from plants rather than pathogenic bacteria (*see* **ANNEX III**; **Figure S6**), although a small contribution of bacteria cannot be completely excluded. Using bacteria containing Cad might help at these investigations, since Arabidopsis lacks this polyamine. In addition, the ADC2 isoform makes more contribution than ADC1 to Put synthesis in response to PAMP flg22, a result that has also been observed in response to ETI (*see* **Chapter 1 and 2**; **Figure 2**) (Rossi et al., 2018). This is in line with previous investigations in which the different isoforms exhibited a differential response depending on the stress (e.g. drought, wounding or salinity for ADC2, cold stress for ADC1) (Perez-Amador et al., 2002; Alcázar et al., 2006b).

Exogenously supplied Put induced the up-regulation of PTI marker genes (*see* **Chapter 1**; **Figure 4**), and callose deposition, a typical physiological response of PTI (Bigeard et al., 2015) (*see* **Chapter 1**; **Figure 3 and ANNEX II**; **Figure S2**). The process is dependent of hydrogen peroxide, which is likely derived from amine oxidase activity, thus implying that polyamine oxidation plays an important role during Put elicited transcriptional responses (*see* **Chapter 1**; **Figure 5)**. Moreover, plasma membrane NADPH oxidases are required for at least some

transcriptional responses induced by Put (*see* **Chapter 1**; **Figure 6**). We argue that RBOHD/F are downstream of Put or act in a concerted manner with apoplastic CuAOs during PTI. How polyamines and RBOH are connected is unknown. In contrast to tobacco, infiltration with Put does not induce cell death (hypersensitive response) in Arabidopsis, although it produced hydrogen peroxide (H_2O_2) (*see* **ANNEX II**; **Figure S3**). Actually, it has been reported that Put prevents cell death induced by polyamine oxidase-generated hydrogen peroxide (Papadakis and Roubelakis-Angelakis, 2005).

Collectively, Put biosynthesis is stimulated in response to PTI, and in turn, apoplastic Put triggers responses compatible with PTI activation, based on ROS (H_2O_2) and RBOHD/F. Hence, a positive feedback loop is proposed that leads to enhanced basal disease resistance against bacterial pathogens (*see* **Chapter 1**; **Figure 7**).

Considering that Put priming depends on H_2O_2, which is one of the Put catalysis products, we wondered whether Put participated during defense as amine or ROS (H_2O_2) producer. The early transcriptional responses to most abundant polyamines (Put, Spd, Spm, tSpm and Cad) indicated that polyamines, rather than being end-road metabolites in the stress response, are activing participants of stress signaling that exhibit quantitative rather than qualitative differences not correlated with charge (*see* **Chapter 2**; **Figure 2B and Table S2.6**). This finding is important because, so far, differences in charge were thought to underlie polyamine specificities, which ensuring their molecular recognition and subsequently regulation of cellular regulation. However, and at least at signaling level, such differences are only quantitative (Tabor and Tabor, 1984; Wallace et al., 2003).

Polyamine oxidation produces ROS (H_2O_2), which is not only necessary for Put -triggered PTI but also for the establishment of ETI and SAR **(*see* Chapter 2**; **ANNEX III**; **Table S4)** (Cona et al.2006; Del Río, 2015; Mammarella et al., 2015). Put leads to local increases of SA, and potentiates the SA-branch of SAR rather than NHP and Pip-dependent branches **(*see* Chapter 2**; **Figure 4)**. Put induced SAR response exhibits high overlap with EDS1, SA and NPR1-dependent transcriptional reprogramming. The data indicate that, at least in defense signaling, polyamines make use of an already established SA-dependent defense pathway. Because polyamines are sources of both ROS and NO in Arabidopsis, we cannot exclude the potential participation of NO in polyamine signaling (*see* **Chapter 2**; **Figure 3**) (Wimalasekera et al.,

2011; Zhang and Li, 2019). Our data supports that Put oxidation in the apoplast is an important trigger of defense signaling (Marina et al., 2008). Besides NADPH oxidases RBOHD and RBOHF, other CuAO family members (ATAO1, CuAO2 and CuAO3) additively contributed to Put-elicited systemic responses (*see* **Chapter 2**; **Figure 8**) (Cona et al., 2006; Kadota et al., 2015). The detailed analysis of transcriptional responses in *cuao* mutants might shed further light into ROS-dependent polyamine signaling.

Consistent with a priming effect, here we report that pre-treatment with Put of wild-type leaves triggers transcriptional reprogramming of distal tissues overlapping with SAR (*see* **Chapter 2**; **Figure 6**), and enhanced disease resistance to *Pst* DC3000 in systemic tissues via an EDS1, SA and NPR1 dependent manner (*see* **Chapter 2**; **Figure 7A**). However, Put infiltration is not sufficient for full establishment of SAR transcriptional responses in distal leaves. This might prevent activation of defenses in response to local fluctuations of Put levels due to, for example, transient dehydration, low temperature, wounding or UV radiation.

Based on our results, we hypothesize that apoplastic Put could act similarly to damage-associated molecular patterns (DAMPs) triggering a ROS-dependent defense response (Choi and Klessig, 2016; Versluys et al., 2017). In this regard, it seems very intriguing the activation of Put export to the apoplast during response to pathogenic bacteria (Liu et al., 2019).

Hybrid incompatibilities are models for the study of ETI in the absence of pathogen challenge (Alcazar et al., 2009; 2010b). Through an EMS mutagenesis screen of the L*er*/Kas-2 NIL, we identified the requirement of callose deposition for the full establishment of ETI in L*er*/Kas-2 incompatible hybrids. Two extragenic suppressors of L*er*/Kas-2 HI mapped to *GSL2* and *GSL10* genes, involved in callose biosynthesis. The GSL family is very variable in gene structure and function. So far, unlike *GSL5* plays vital role in resistance, *GSL2* and *GSL10* genes have not been associated with immune-related HI. Consistent with the involvement of *GSL2* and *GSL10* in L*er*/Kas-2 HI, lower *GSL2* and *GSL10* expression (*see* **Chapter 3**; **Figure 2**), suppressed autoimmune response and fitness loss of L*er*/Kas-2 HI at low temperature (*see* **Chapter 3**; **Figure 1A** and **Figure S1**). Callose deposition was not compromised in suppressed L*er*/Kas-2 HI plants upon flg22 and wounding (*see* **Chapter 3**; **Figure 3A** and **3B**). In these mutants, GSL5 activity was functional and responded for callose deposition in response to pathogens or PAMPs (*see* **Chapter 3**; **Figure 2E**) (Ellinger and Voigt, 2014).

The interaction between GSL charged callose biosynthesis and SA pathway might depend on environmental conditions (Shikanai et al., 2020). SA pathway and JA were both restricted in the *GSL2* and *GSL10* suppressed L*er*/Kas-2 HI (*see* **Chapter 3**; **Figure 5**, and **ANNEX IV**; **Figure S3**) (Dong et al., 2008; Zavaliev et al., 2011). This might benefit from the *GSL5* expression, which contributes to pathogen resistance (*see* **Chapter 3**; **Figure 6**) (Ellinger et al., 2013). Whether callose contributes to disease resistance is still debated, although our data suggests that it is required for the full establishment of ETI (Consonni et al., 2010; Ellinger et al., 2013).

Conclusions

The main objective of this Thesis work has been the investigation of the defense signaling mechanisms induced by polyamines, particularly Put in *Arabidopsis thaliana* (*see* **Chapters 1 and 2**). Also, mapping of extragenic suppressors of immune-related HI which identified glucan synthase-like genes contributing to the establishment of ETI (*see* **Chapters 3**).

Based on the results reported in the three Chapters the main conclusions are listed below:

Chapter 1

IV. Putrescine accumulates in response to PTI-inducing bacteria and the purified PAMP flg22. In this response, ADC2 isoform makes a higher contribution to Put biosynthesis than ADC1.

V. Exogenous application of Put triggers GSL5-dependent callose deposition, H_2O_2 and RBOHD/F dependent expression up-regulation of PTI marker genes. We conclude that Put, which accumulates during PTI, also contributes to PTI activation. We suggest the occurrence of a positive feedforward loop contributed by ROS-derived Put, which amplifies defense responses. Importantly, such responses are not associated with the occurrence of cell death.

VI. Pathogen inoculation assays demonstrate that Put acts as a priming agent that enhances basal resistance in local tissues, thus providing biological relevance for the accumulation of this polyamine during defense.

Chapter 2

VI. RNA-seq analyses indicate that transcriptional responses to different polyamines exhibit quantitative rather than qualitative differences, which are not correlated with charge.

VII. Polyamines are triggers of stress signaling, including biotic responses which, at least in the case of Put, are dependent on H_2O_2, EDS1, SA and NPR1.

VIII. Consistent with activation of SA-pathway, Put treatment leads to local but not systemic SA biosynthesis. However, local Put treatment triggers transcriptional reprogramming in systemic tissues overlapping with SAR, thus suggesting the occurrence of a systemic signal triggered by Put is transmitted throughout the plant.

IX. Consistent with a role for Put and Put oxidation in the establishment of SAR, local Put treatment enhances systemic disease resistance to hemibiotrophic bacteria *Pst* DC3000.

This effect is suppressed in *adc2* mutants, impaired in pathogen-induced Put biosynthesis, and *cuao* mutants impaired in Put oxidation.

X. We propose that Put oxidation in the apoplast contributes to the establishment of systemic defenses.

Chapter 3

VI. Two extragenic suppressors of L*er*/Kas-2 hybrid incompatibility (HI) have been mapped to *GSL* (glucan synthase-like) *2* and *GSL10*.

VII. Artificial microRNA silencing of *GSL2* and *GSL10* genes also suppresses L*er*/Kas-2 HI, thus confirming the genes are mapped.

VIII. Suppression of L*er*/Kas-2 HI by *GSL2* and *GSL10* mutation or silencing associates with suppression of salicylic acid pathway, but not enhanced susceptibility to pathogenic bacteria in *sulki3-1* and *sulki4-1*. We conclude that suppression of L*er*/Kas-2 HI is not at expenses of reduced disease resistance.

IX. Callose deposition in response to environmental stimuli (wounding, flg22 and treatment of bacteria inoculation) is not compromised in *sulki3-1* and *sulki4-1* mutants but *gsl5*, thus highlighting that *GSL2* and *GSL10* members are not redundant to *GSL5*.

X. Suppression of L*er*/Kas-2 HI by *GSL2* and *GSL10* mutation suggests that callose synthesis is required for the full establishment of ETI independent of *GSL5*.

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ANNEX I

Supplemental Material for Introduction

NO	Glucan Synthase like	Callose synthase	Locus Tag
1	GSL1	CALS 11	AT4G04970
2	GSL2	CALS 5	AT2G13680
3	GSL3	CALS 6	AT2G31960
4	GSL4	CALS 8	AT2G36850
5	GSL5	CALS 12	AT4G03550
6	GSL6	CALS 1	AT1G05570
7	GSL7	CALS 7	AT1G06490
8	GSL8	CALS 10	AT2G36850
9	GSL9	CALS 4	AT5G36870
10	GSL10	CALS 9	AT3G07160
11	GSL11	CALS 2	AT3G59100
12	GSL12	CALS 3	AT5G13000

Table S1. GSL (Glucan synthase-like) genes, also known as CALS (callose synthase) in *Arabidopsis thaliana.*

ANNEX II

Supplemental Material for Chapter 1
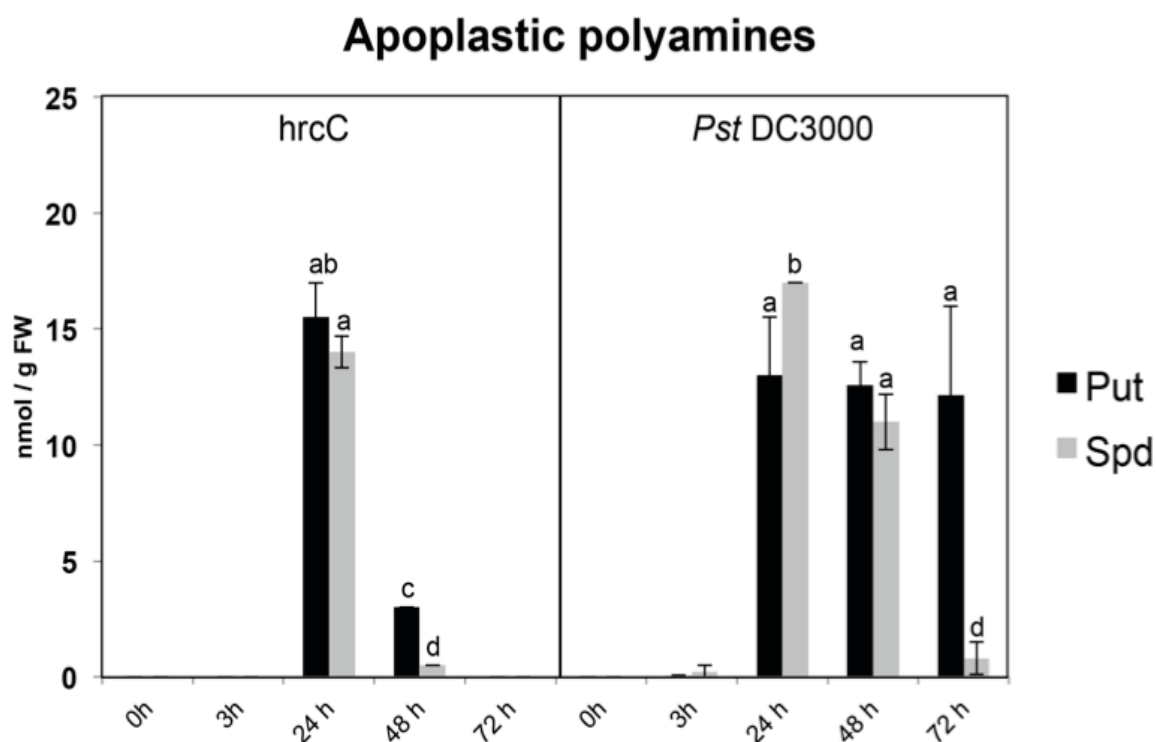
Figure S1. Levels of polyamines in three-week old wild-type plants spray inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 or *hrcC*. Samples for polyamine content determination were collected after 0 h, 3 h, 24 h, 48 h and 72 h of treatment. Values are the mean of three biological replicates ± SD (standard deviation). Letters indicate values that are significantly different according to Student-Newman-Keuls test at *P* value < 0.05. FW, fresh weight.
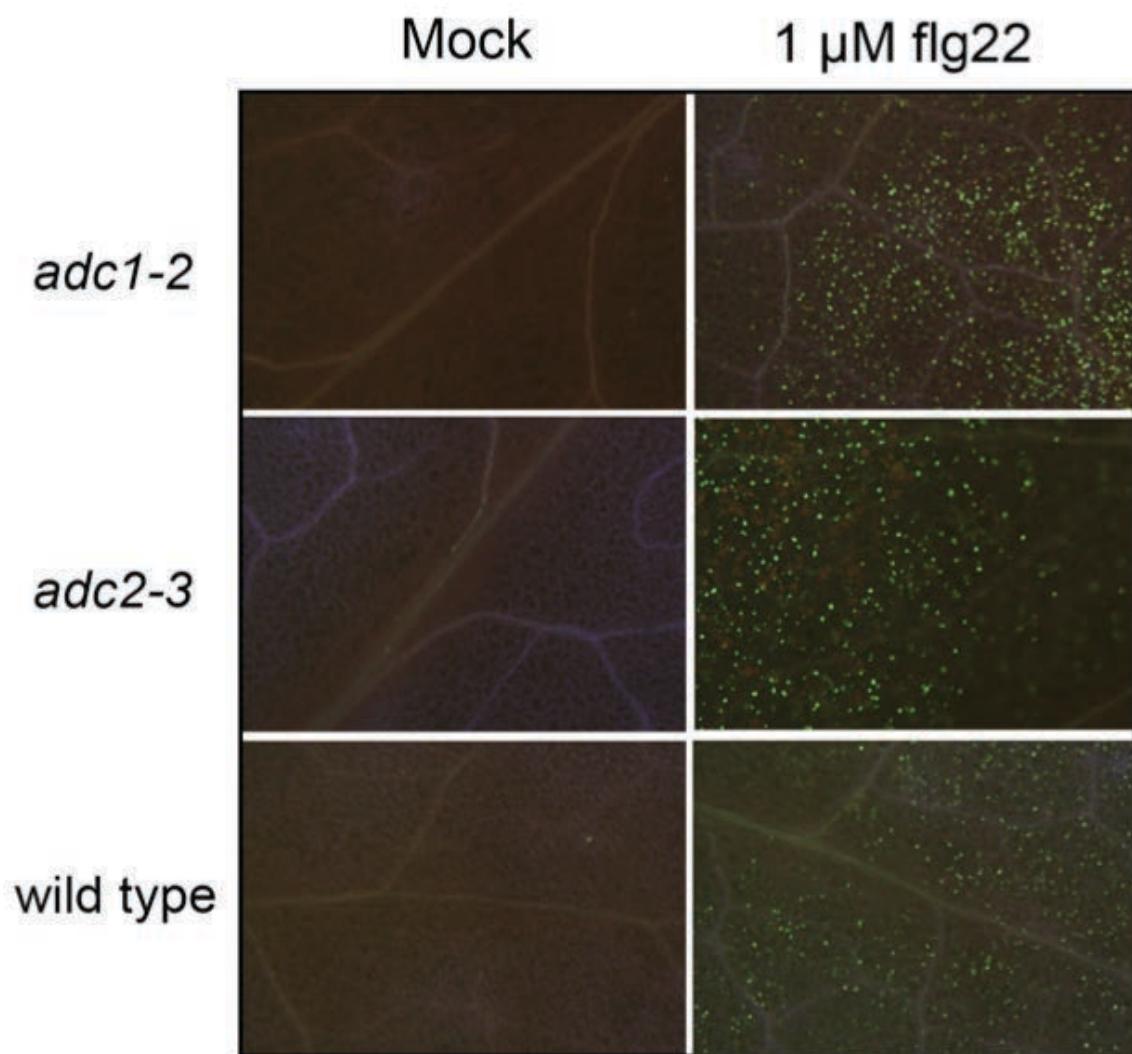
Figure S2. Aniline blue staining of callose deposits in *adc1-1 adc 2-3* and wild-tye seedlings treated with 1 µM flg22 or mock for 24 h. Treatments were performed as described in Figure 3.
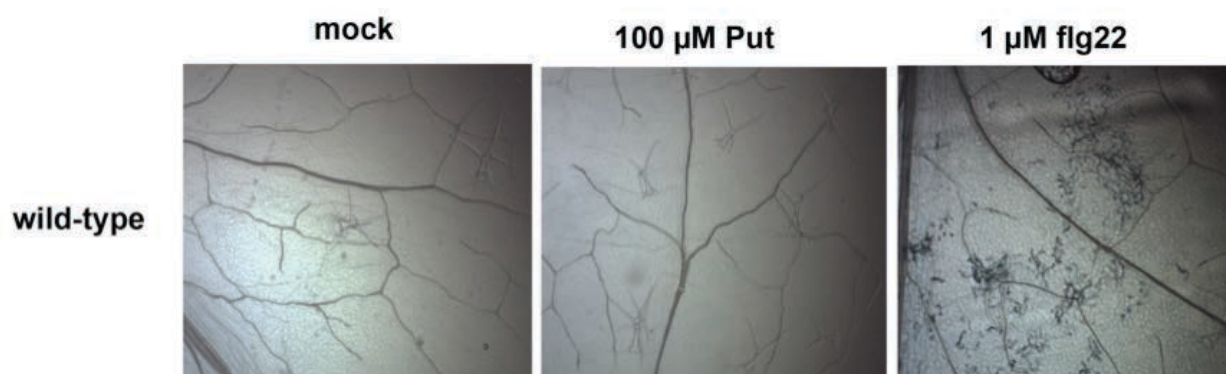
Figure S3. Trypan blue staining of 10-day-old wild-type and *fls2* seedlings after 24 h of infiltration with 100 µM Put or 1 µM flg22. Treatments were performed in at least 12 different seedlings per genotype tested with similar results.

Gene	Name	Forward (5' to 3')	Reverse (5' to 3')
At3g18780	Actin2	GATTCAGATGCCCAGAAGTCTTGT	TGGATTCCAGCAGCTTCC
AT5g64890	PROPEP2	AGAAAAGCCTAGTTCAGGTCGTC	CTCCTTATAAACTTGTATTGCCGC
At5g64905	PROPEP3	GTTCCGGTCTCGAAAGTTCATC	TCTTCCTCGCTGTGTGATGAC
At4g01250	WRKY22	CGTCCTCTTTCTCTCTCTGCTTCTTC	CCATGCCCAGACATCGGAGTTTA
AT4g23550	WRKY29	TTTCACCTTCGTTTTGCCTACC	CGAGCTCATCTAAGCCACTTGTC
AT4g23810	WRKY53	GGAGAAGCGACAAGACACCAGA	TATCCTTTGGCTTTTGGGTAATGG
AT2g19190	FRK1	GCCAACGGAGACATTAGAG	CCATAACGACCTGACTCATC
AT5g57220	CYP81F2	AAATGGAGAGAGCAACACAATG	ATCGCCCATTCCAATGTTAC
AT5g26920	CBP60g	AAGAAGAATTGTCCGAGAGGAG	GGCGAGTTTATGAAGCACAG
At2g35980	NHL10	TTCCTGTCCGTAACCCAAAC	CCCTCGTAGTAGGCATGAGC

Supplementary Table 1. List of oligonucleotides and sequences used in this work.

ANNEX III

Supplemental Material for Chapter 2
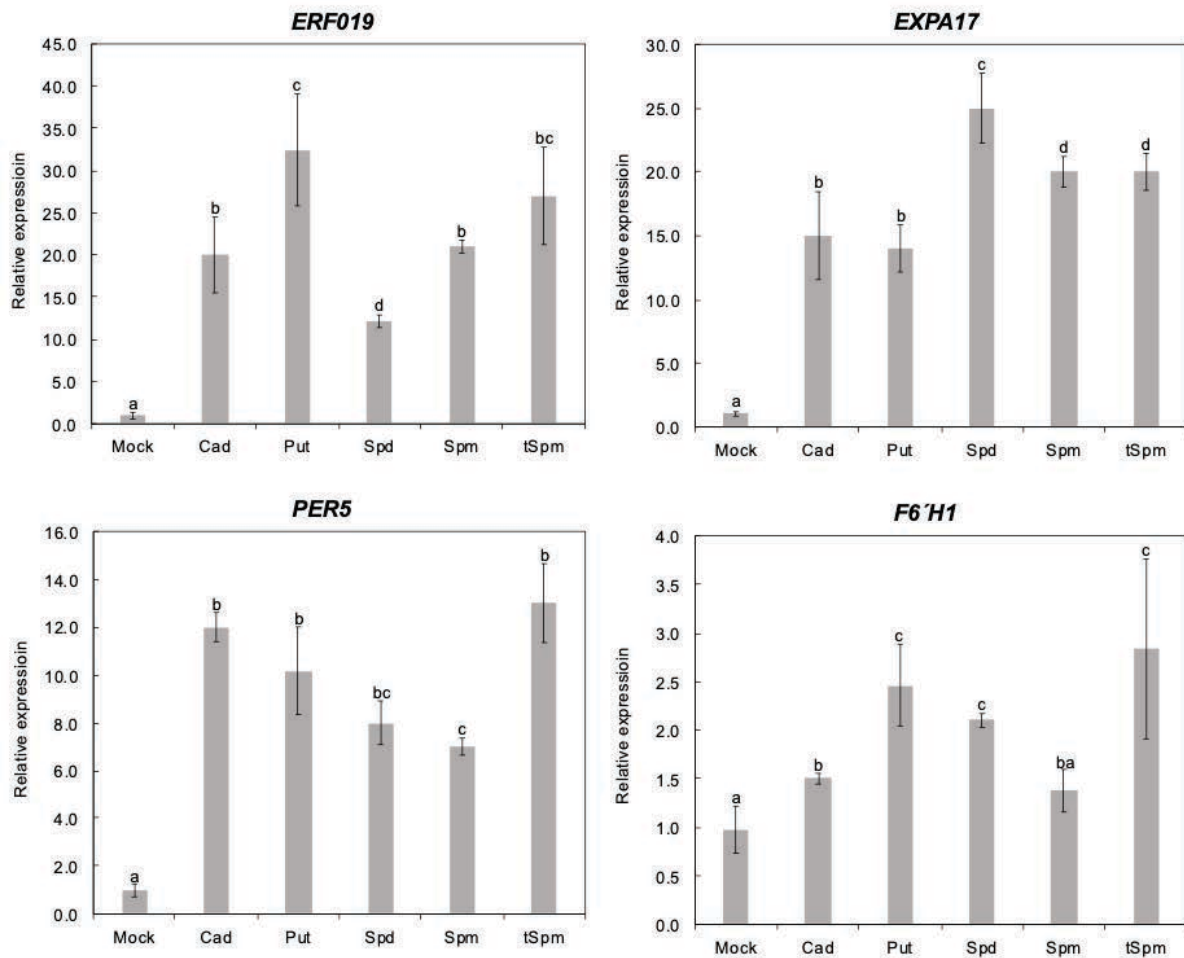
Figure S1. Quantitative RT-PCR expression analyses of *ERF019, EXPA17, PER5* and *F6'H1* genes for RNA-seq validation. Analyses were performed in 12-day-old wild-type (Col-0) *Arabidopsis* seedlings treated with 100 μM Cad, 100 μM Put, 100 μM Spd, 100 μM Spm, 100 μM tSpm or mock for 1 h. Values are the mean from three independent biological replicates ± SD. Letters indicate values that are significantly different according to Tukey's HSD test at $P < 0.05$.
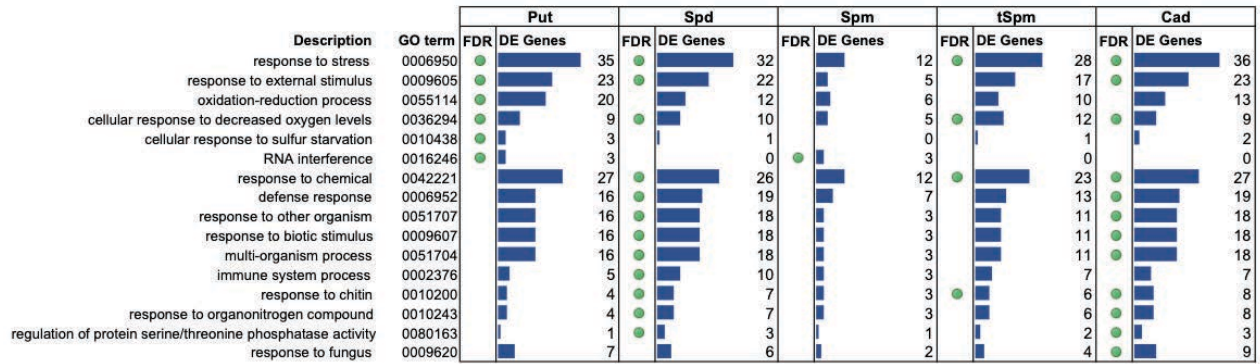
Figure S2. Gene ontology (GO) analyses of polyamine responsive genes. For each GO category, the number of differentially expressed genes (DE Genes) is shown. Green spots indicate significant GO associations (FDR<0.05).
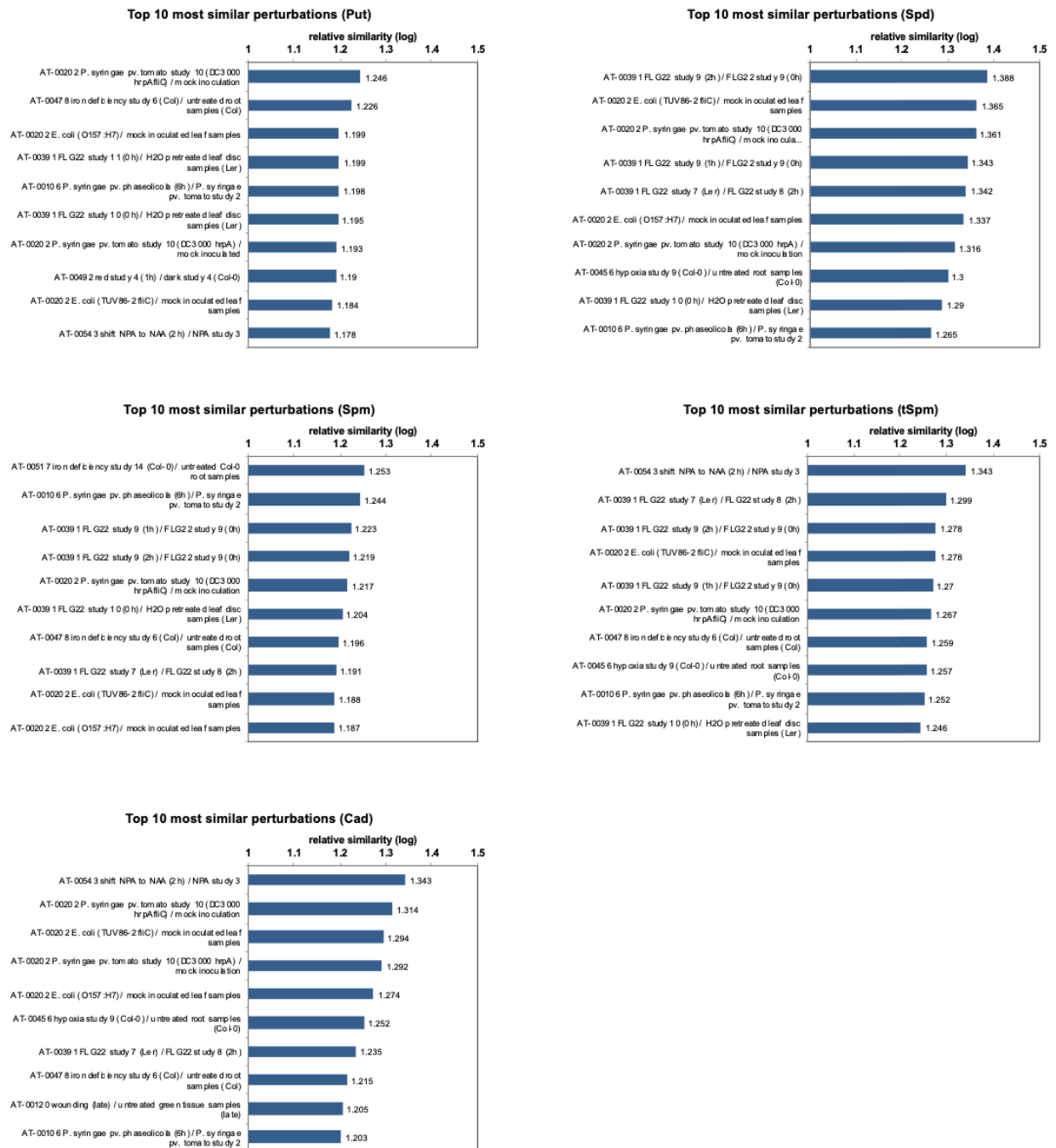
Figure S3. Comparison analysis of the polyamine transcriptome with other perturbation conditions. Gene expression data from the different polyamine treatments was used to identify similar expression signatures in 2,799 publicly available perturbation assays (Genevestigator) obtained from wild-type genotypes in a minimum of three biological replicates. Log similarity values are relative to the average similarity.
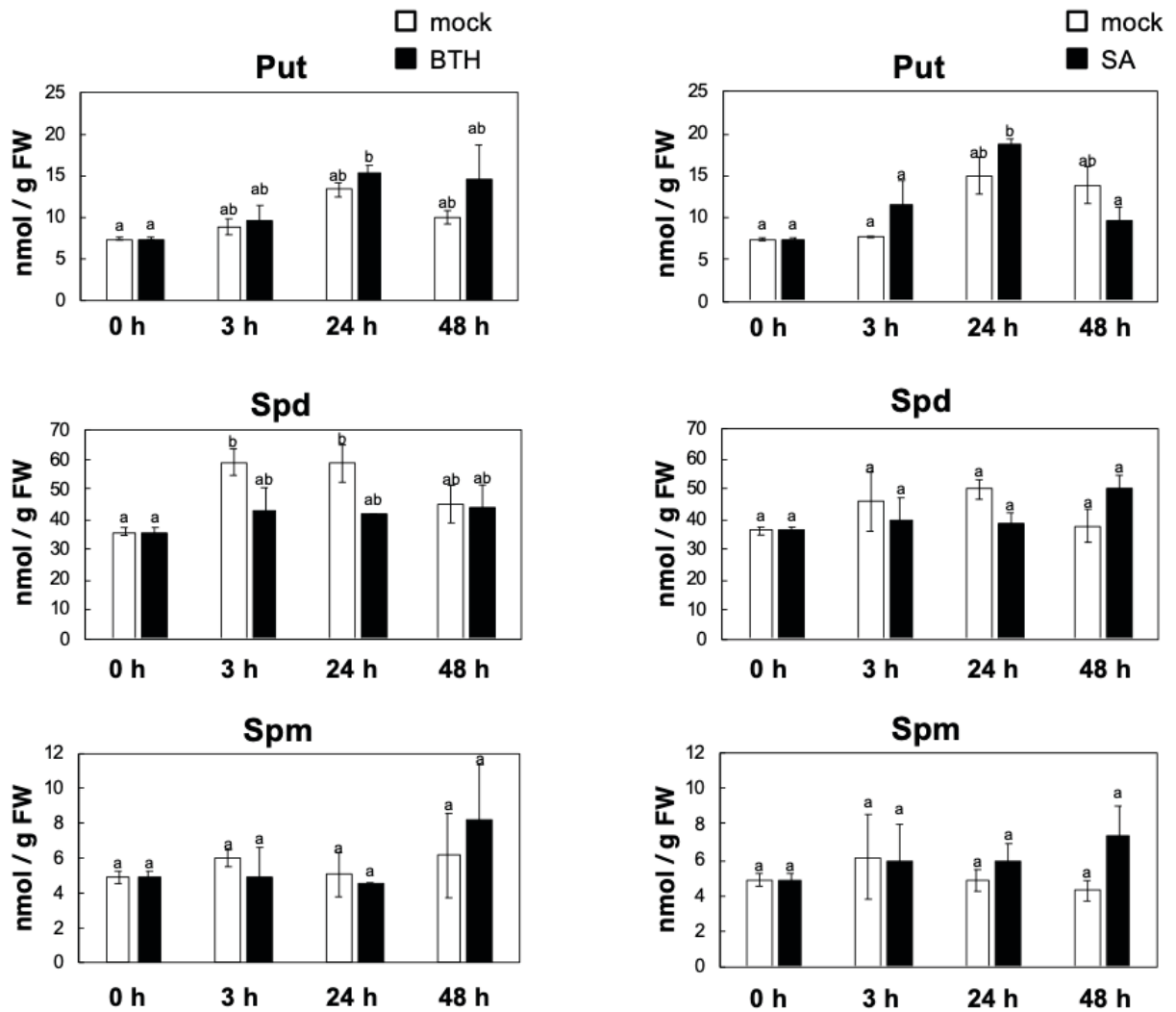
Figure S4. ROS detection in seedlings treated with exogenous polyamines. ROS was visualized by staining with **(A)** 2',7'-dichlorofluorescin diacetate (DCFDA), **(B)** 3,3'-diaminobenzidine (DAB) and **(C)** Nitroblue Tetrazolium (NBT) in 12-day-old *Arabidopsis* wild- type (Col-0) seedlings treated 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.

**Continued 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.

Continued 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 $P < 0.05$.
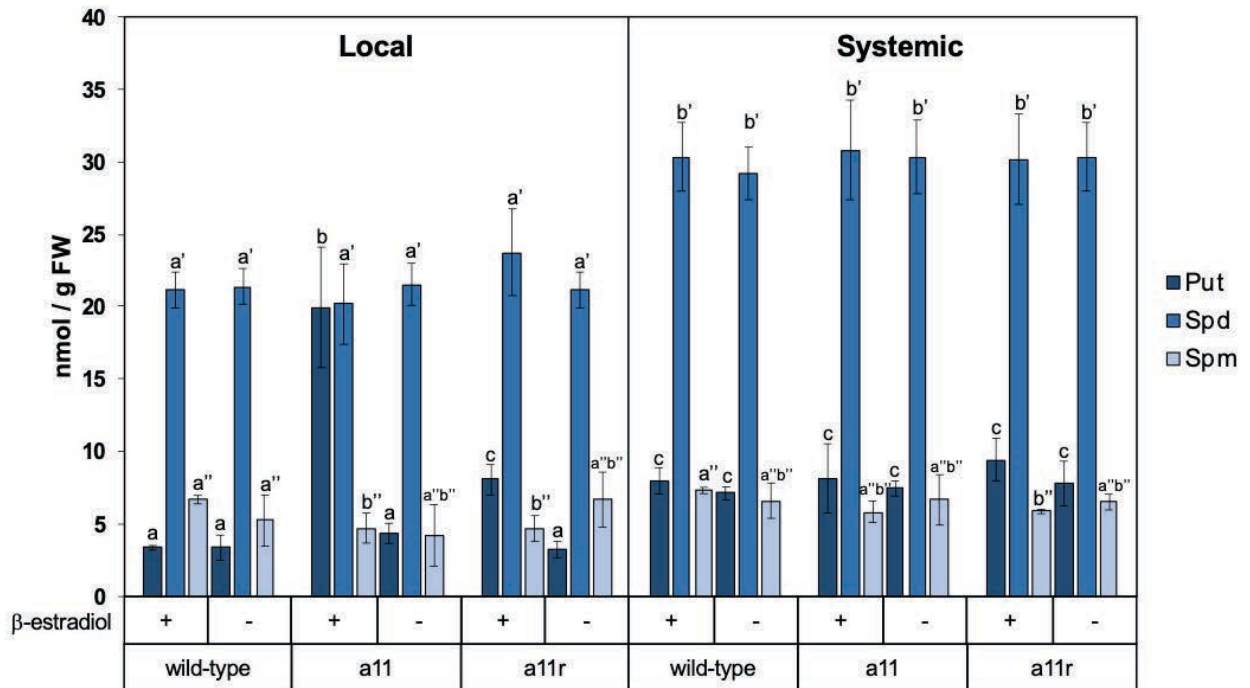
Figure S6. Polyamine levels in local and systemic leaves at 24 h post-inoculation with 10 μM β -estradiol or mock in wild-type, β -estradiol inducible *AvrRpm1* (a11) and β - estradiol inducible *AvrRpm1 rpm1-1* (a11r). Values are the mean from at least four biological replicates ± SD. Letters indicate values that are significantly different according to Tukey's HSD test at *P* < 0.05.
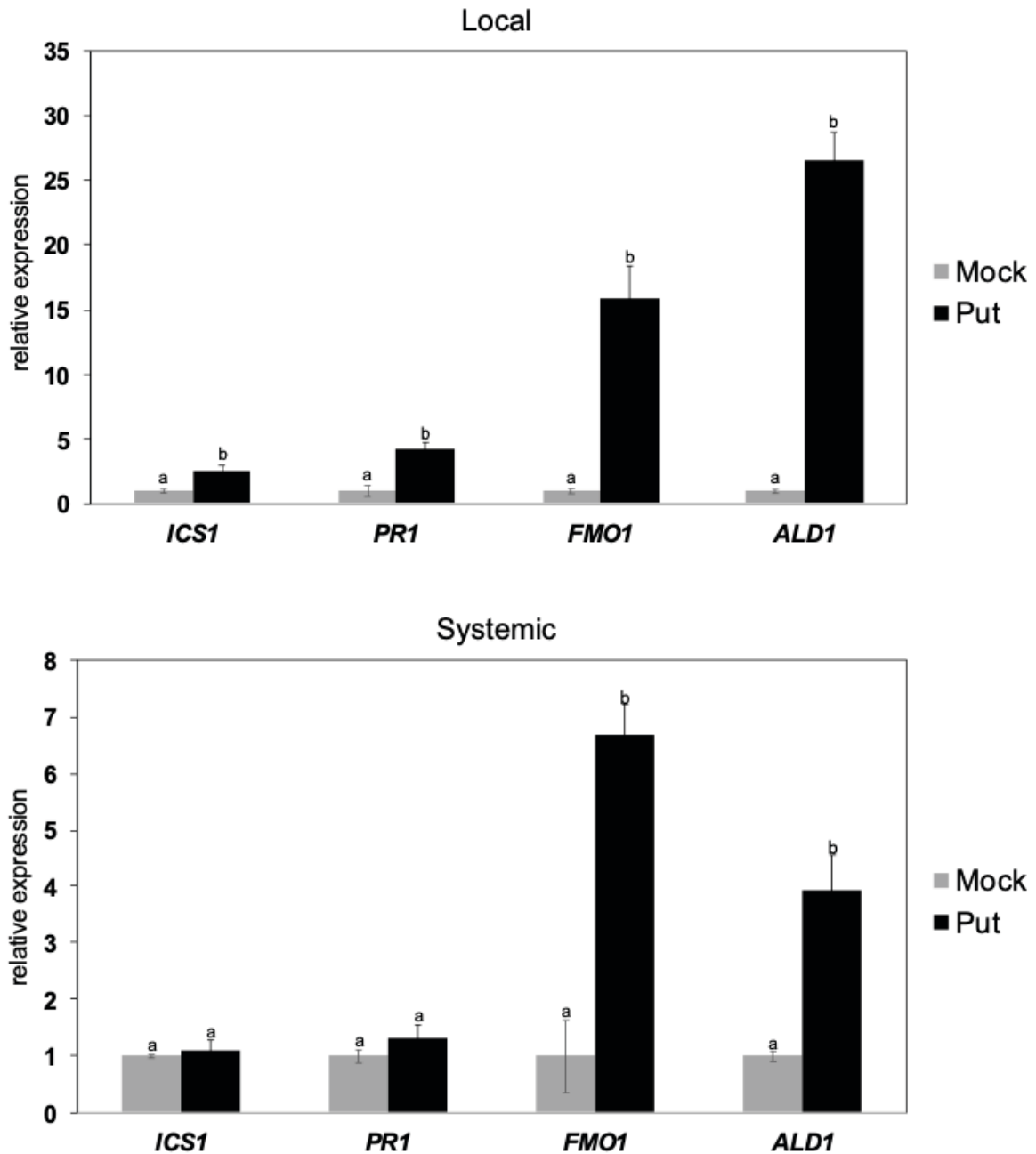
Figure S7. Quantitative RT-PCR expression analyses of *ICS1*, *PR1*, *FMO1* and *ALD1* genes. Analyses were performed at 24 hours post-inoculation in local and systemic leaves of wild-type plants treated with 500 μM Put or mock (5 mM MES pH 5.7). Values are the mean from three independent biological replicates ± SD. For each gene, letters indicate values that are significantly different according to Tukey's HSD test at *P* < 0.05.

SUPPLEMENTAL TABLES

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

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

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

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

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

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

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

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

Table S3. Gene ontology (GO) analysis of polyamine responsive genes. Enrichment of GO terms in the set of differentially expressed (DE) genes in the treatments with 100 μM Put, 100 μM Spd, 100 μM Spm, 100 μM tSpm or 100 μM Cad (related to **Tables S2.1 to S2.7**).

Table S4. Dependence of Put responsiveness on H_2O_2 production. Gene expression changes of Put responsive genes in the presence of 5 mM DMTU + 100 μM Put after 1 h of treatment. Expression changes are relative to treatment with 5 mM DMTU.

Table S5. Expression of Put responsive genes after 1 h of treatment with 100 μM Put in *eds1-2*, *sid2-1* and *npr1-1* mutants. Expression differences are relative to mock treatment.

Tables S6.1 to S6.5 List of genes significantly deregulated after 24 h of 500 μM Put infiltration in local (**Table S6.1**) and systemic (**Table S6.2**) leaves of 5-week-old wild-type *Arabidopsis*. Full list of genes is shown in **Table S6.3**. Expression differences are relative to mock treatment. Gene ontology analyses of Put responsive genes in local (**Table S6.4**) and systemic (**Table S6.5**) tissues.
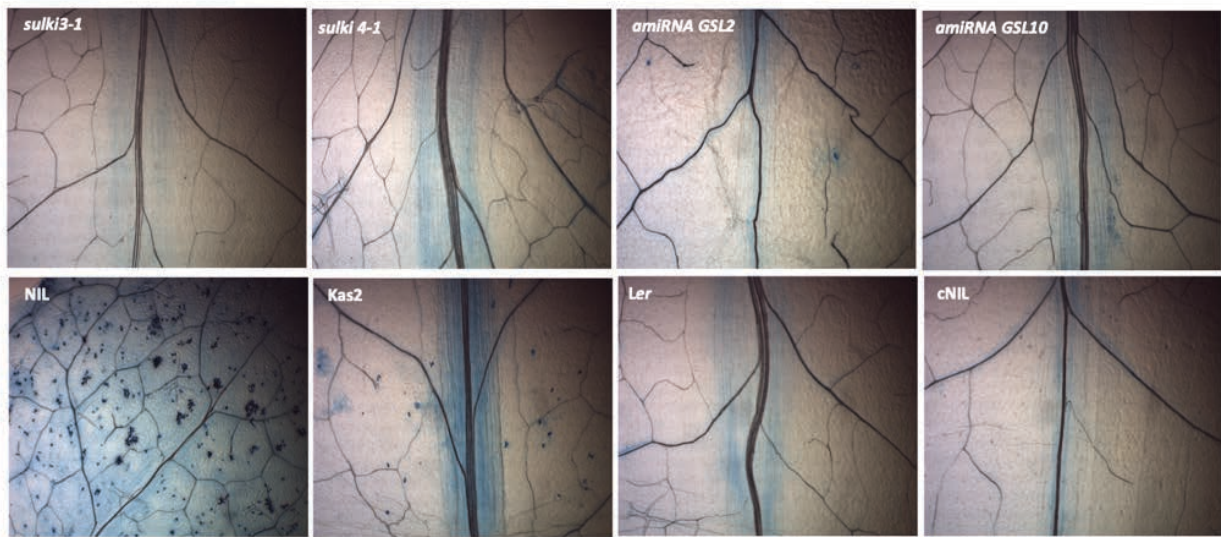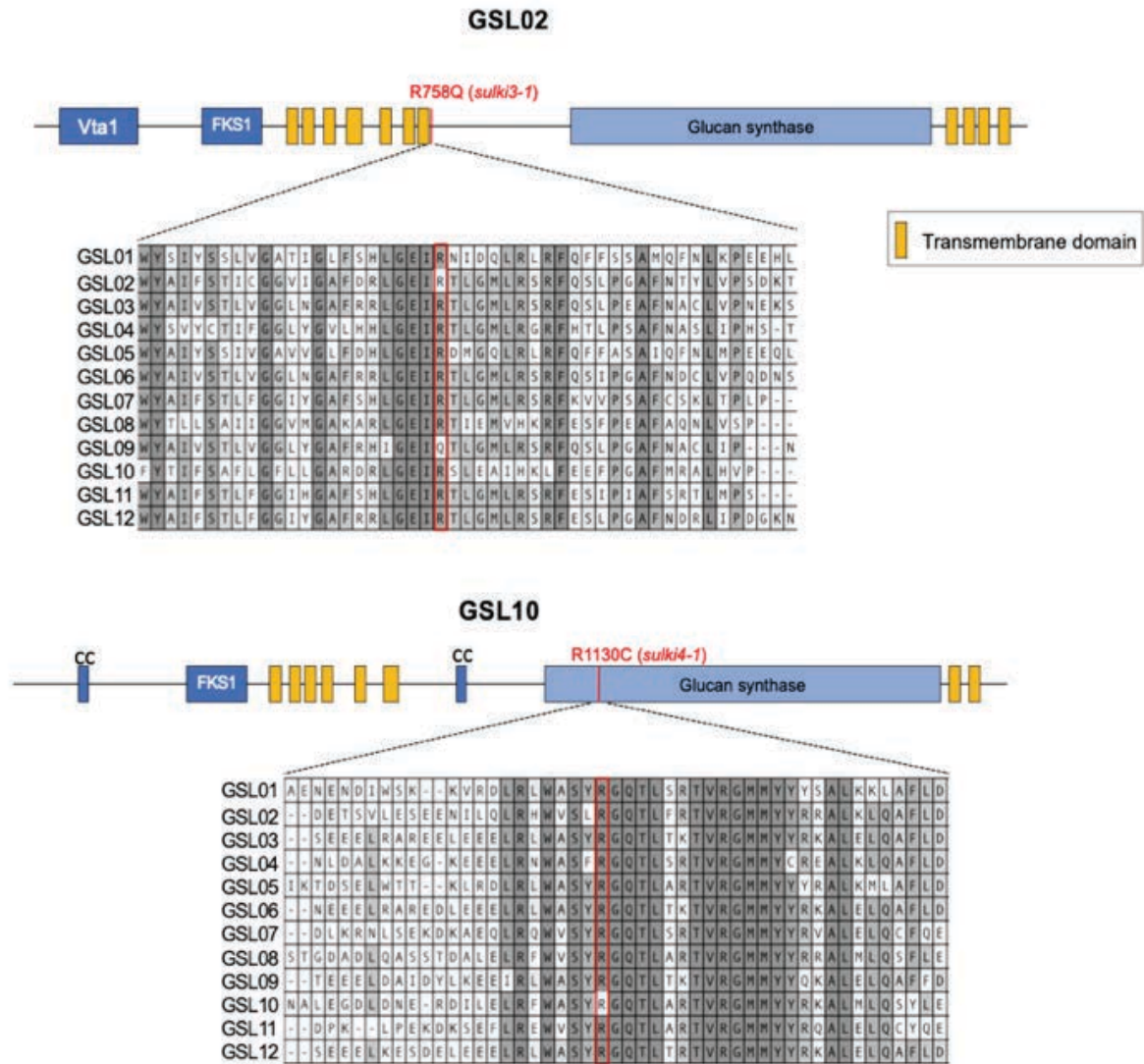
Supplementary tables:

https://onlinelibrary.wiley.com/doi/epdf/10.1111/pce.13874

ANNEX IV

Supplemental Material for Chapter 3

Figure S1. Cell death in *sulki3-1*, *sulki4-1*, and amiRNA *GSL2/10* lines (NIL) grown at 14-16 °C. Microscope visualization of leaves from 5-week-old Arabidopsis plants grown at 14-16°C stained with trypan blue.

Figure S2. Alignment of GSL proteins and nonsynonymous substitution sites *in sulki3-1* and *sulki4-1* mutants. Conserved domains of GSL proteins are indicated. Vta1 (VPS20-associated protein 1-like domain). FKS1 (Alternate catalytic subunit of the yeast 1,3-beta-glucan synthase) CC (coiled-coil).
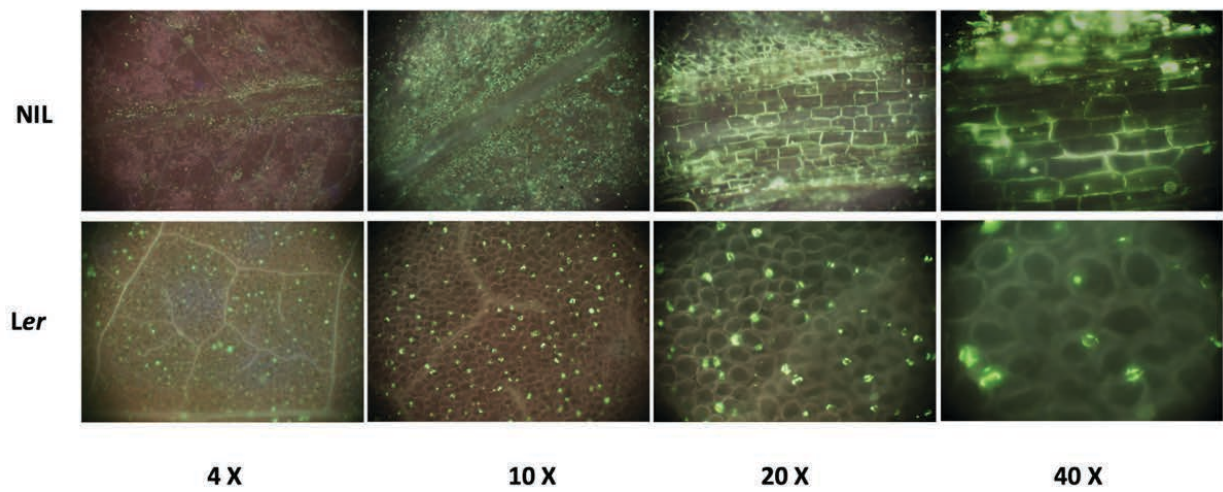
Figure S3. Callose deposition in L*er* and L*er*/Kas-2 NIL at 14-16 ℃. Callose deposition in 5-week-old Arabidopsis L*er* and L*er*/Kas-2 NIL at 14-16 ℃, with 4x, 10x, 20x and 40x under epifluorescence microscope.

No.	Gene	Name	Forward (5' to 3')	Reverse (5' to 3')
1	AT3G18780	Actin2	GATTCAGATGCCCAGAAGTCTTGT	TGGATTCCAGCAGCTTCC
2	AT4G04970	AtGSL1	ACCGTTTTGTGGCATTGCTC	CAACTGGTGCAGTGTGAAGC
3	AT2G13680	AtGSL2	GAAGCCAAGGTTGCTTGTGG	CCTTTCGCTGCTGCAAACTT
4	AT2G31960	AtGSL3	ATGCTCGCTTTCATGCCAAC	GAACGCTACCGGAGTGAACA
5	AT3G14570	AtGSL4	GCTTTCTTGCCCACTGGTTG	GCAAACAGAACAACGCCCAT
6	AT4G03550	AtGSL5	CCCTGATTCGAGACGAGAGC	CCCCCACAGCGTAAAGGAAT
7	AT1G05570	AtGSL6	GACCCAGCAGCTTCCAAAAAT	GGAGAGAGAAAAGGCCGGAG
8	AT1G06490	AtGSL7	AGGATTCTGGGACTCGGTGA	GCGGTTTGAGCCTCACTACT
9	AT2G36850	AtGSL8	GGGAGTGGCTTCGATCACAT	AGCTTGGTTGAAGAGGAGCC
10	AT5G36870	AtGSL9	TGACGTTGGCTTGGGTCTAC	TGCCTGCAAATAGGGTTCTCA
11	AT3G07160	AtGSL10	CCAGTCGATAGTCGTGGCTC	CCTGCCGGTTTTGAGCTTTT
12	AT3G59100	AtGSL11	GCACTTCTCTTCCTCGGCTT	GCTCTTTCACCGAGTCCCAA
13	AT5G13000	AtGSL12	GGTTGGAAGGCGGAGATTCA	TTGCGCAATCTGCAAACACA

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