Investigation of Intercellular Salicylic Acid Accumulation during Compatible and Incompatible Arabidopsis-Pseudomonas syringae Interactions Using a Fast Neutron-Generated Mutant Allele of EDS5 Identified by Genetic Mapping and Whole-Genome Sequencing

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Abstract

A whole-genome sequencing technique developed to identify fast neutron-induced deletion mutations revealed that iap1-1 is a new allele of EDS5 (eds5-5). RPS2-AvrRpt2-initiated effector-triggered immunity (ETI) was compromised in iap1-1/eds5-5 with respect to in planta bacterial levels and the hypersensitive response, while intra- and intercellular free salicylic acid (SA) accumulation was greatly reduced, suggesting that SA contributes as both an intracellular signaling molecule and an antimicrobial agent in the intercellular space during ETI. During the compatible interaction between wild-type Col-0 and virulent Pseudomonas syringae pv. tomato (Pst), little intercellular free SA accumulated, which led to the hypothesis that Pst suppresses intercellular SA accumulation. When Col-0 was inoculated with a coronatine-deficient strain of Pst, high levels of intercellular SA accumulation were observed, suggesting that Pst suppresses intercellular SA accumulation using its phytotoxin coronatine. This work suggests that accumulation of SA in the intercellular space is an important component of basal/PAMP-triggered immunity as well as ETI to pathogens that colonize the intercellular space.

Introduction

In response to pathogens Arabidopsis relies on various induced defenses. Basal resistance or PTI (PAMP-Triggered Immunity) is a defense response elicited by the recognition of conserved pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by pattern recognition receptors [1]. Some bacteria, for example, certain pathovars of Pseudomonas syringae, are able to suppress PTI by delivering effector proteins into plant cells using a type-three secretion system [1]. Several of these effectors interfere with various stages of PTI, contributing to the pathogen’s ability to cause disease on host plants [2–6]. In response, many plants possess resistance genes (R genes) that recognize effectors either directly or indirectly, leading to R gene-mediated resistance or Effector-Triggered Immunity (ETI) [7]. Some of the defense mechanisms associated with ETI are thought to overlap with those of PTI although they seem to occur more rapidly and with greater strength during ETI [1]. These defenses include extensive transcriptional reprogramming resulting in cellular changes such as expression of defense genes (e.g., PR [PATHOGENESIS-RELATED] genes), production of phytoalexins, salicylic acid (SA) biosynthesis, and cell-wall modifications [8–10]. In addition, ETI is often associated with the hypersensitive response (HR), a form of programmed cell death thought to contribute to inhibition of pathogen spread [11].

Many R genes encode NB-LRR (nucleotide-binding leucin-rich repeat) proteins which can be subdivided into two groups; those possessing N-terminal coiled-coil domains (CC-NB-LRR) and those with N-terminal TIR (Toll and Interleukin-1 receptor) domains (TIR-NB-LRR) [7]. CC- and TIR-type R genes are generally thought to function using separate pathways defined by their requirement for either NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE1) or EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) respectively [12]. For example, RPS2 encodes a CC-NB-LRR resistance protein that confers resistance to strains of P. syringae expressing avrRpt2 [13,14]. Similarly, the TIR-NB-LRR resistance protein RPS4 confers resistance to strains of P. syringae expressing avrRps4 [15,16].
Another form of induced disease resistance is the developmentally regulated Age-Related Resistance (ARR) response (reviewed in [17,19]). In Arabidopsis, ARR results in enhanced resistance to certain pathogens with increasing plant age [19]. Specifically, 6-week-old Col-0 plants grown in short days limit the growth of P. syringae pv. tomato (Pst) to levels that are 10- to 100-fold lower than in 3-week-old plants. Unlike PTI and ETI, the molecular mechanisms underpinning ARR in Arabidopsis are only beginning to be understood.

A common player in many disease resistance pathways is the phytohormone SA (reviewed in [20,21]). Wild-type Arabidopsis accumulates SA in response to inoculation with both virulent and avirulent Pst [22]. The importance of SA accumulation for different disease resistance responses is typically tested using transgenic and mutant plants with a reduced ability to accumulate SA. NahG plants expressing a bacterial salicylate hydroxylase gene convert SA to catechol and consequently accumulate very little SA [23]. ICSI/SID2 (ISOCHORISMATE SYNTHASE1/SALICYLIC ACID INDUCTION DEFICIENT2) encodes a key enzyme in the biosynthetic pathway responsible for most pathogen-responsive SA production in Arabidopsis [24, 25]. ED5/SID1 (ENHANCED DISEASE SUSCEPTIBILITY3) encodes a multidrug and toxin extrusion (MATE) family protein that localizes to the chloroplast envelope and transports SA from its site of synthesis into the cytoplasm [26–29]. Both sid2 and eds5/sid1 mutants accumulate little SA in response to pathogens [22]. NahG, sid2, and eds5 support higher growth of virulent strains of P. syringae compared to wild-type plants suggesting that SA accumulation is important in limiting pathogen growth even in a compatible (susceptible) interaction [22,23,30,31]. ETI is compromised in NahG [8,23,32–34], sid2, and eds5 [22,32,35] when initiated by several R genes (RPS2, RPS4, RPM1) interacting with their corresponding effectors. In experiments using type-three secretion system mutants or PAMPs (flg22) to initiate PTI in wild-type Col-0 or sid2, Tsuda et al. [36] demonstrated that SA accumulation is required for a successful PTI response. Thus, in Arabidopsis, SA accumulation is important in numerous ETI and PTI pathways.

SA accumulation is also required for the Arabidopsis ARR response to Pst as demonstrated by the ARR-defective phenotypes of NahG, sid2-1, and sid1/eds5-3 [19,37]. Examination of mature plants responding to Pst revealed 6-fold higher SA levels in intercellular washing fluids (IWFs) relative to young plants [38]. Anti-microbial activity was often observed in the IWFs of mature intercellular washing fluids (IWFs) relative to young plants [38]. Preventing SA accumulation in the intercellular space by pressure-infiltrating ARR-competent plants with salicylate hydroxylase disrupted their ability to undergo ARR. Conversely, adding exogenous SA to the intercellular space rescued ARR-defective mutants and enhanced ARR in wild-type Col-0 [38]. Taken together this data led to the hypothesis that SA may act as an antimicrobial agent in the intercellular space during ARR.

A classical mutant screen for mature plants with defects in ARR was used to identify genes involved in the ARR response, including iap1-1 (important for the ARR pathway1-1). Along with their ARR-defective phenotype, mature iap1-1 plants accumulate little SA [37]. In this work, a combination of genetic mapping, whole-genome sequencing, and complementation analysis was used to identify iap1-1 as a mutant allele of ED55. While mapping the iap1-1 mutation, we investigated the role of IAP1 in RPS2- and RPS4-mediated ETI by measuring bacterial levels and monitoring HR cell death using trypan staining and electrolyte leakage. Intercellular SA accumulation is important during ARR and requires functional IAP1, therefore we investigated both inter- and intracellular SA accumulation during ETI (incompatible interaction) and during a compatible interaction with virulent Pst in young Col-0 and iap1-1. Our resuls suggest that inter- and intracellular SA accumulation is important during both compatible and incompatible interactions.

**Results**

The iap1-1 mutant is partially compromised in resistance to Pst(avrRpt2) and Pst(avrRps4)

The iap1-1 mutant is defective for ARR to virulent Pst [37]. To determine whether IAP1 is also required during NDR1- or EDS1-dependent ETI/incompatible interactions, young plants at 3 weeks post-germination (wpg) were inoculated with 10⁶ cfu (colony-forming units) ml⁻¹ Pst, Pst(avrRpt2), or Pst(avrRps4) and in planta bacterial density was measured 3 days post-inoculation (dpi). Pst(avrRpt2) grew to significantly lower levels than Pst in both Col-0 and iap1-1 indicating that an ETI response occurred (Figure 1A). However, Pst(avrRpt2) levels were significantly higher in iap1-1 compared to Col-0 indicating that NDR1-dependent ETI to Pst(avrRpt2) is partially compromised by the iap1-1 mutation. Similar results were obtained when Col-0 and iap1-1 were inoculated with Pst(avrRps4) which indicates that EDS1-dependent ETI to Pst(avrRps4) is also partially compromised by the iap1-1 mutation (Figure 1B). Therefore, IAP1 is required for a full and robust ETI response to Pst carrying effectors recognized by two distinct classes of resistance proteins.

The hypsersensitive response is partially compromised in iap1-1

Since the hypersensitive response (HR) is a common component of ETI elicited by both AvrRpt2 and AvrRps4 effectors [13,14,16] we investigated whether the iap1-1 ETI defect was accompanied by a reduced HR. Four-week-old Col-0 and iap1-1 were inoculated with 10⁵ cfu ml⁻¹ Pst(avrRpt2) or 10 mM MgCl₂ (mock-inoculated) and leaves were collected at 24 hours post-inoculation (dpi) and stained with trypan blue. Trypan blue does not pass through intact cell membranes of live cells therefore it selectively stains dying or dead cells and can be used to measure HR-associated cell death [47]. Visual analysis revealed little staining in mock-inoculated leaves whereas intense staining was observed in leaves inoculated with Pst(avrRpt2) (Figure S1). There was no obvious difference in the intensity of staining between Col-0 and iap1-1 leaves suggesting that iap1-1 undergoes a wild-type HR. By floating treated leaf tissue in a solution in a measure conductance, electrolyte leakage from dead or damaged cells can be quantified and used to track the progression of HR cell death over time [48]. Electrolyte leakage was monitored in tissue collected from 4-week-old Col-0 and iap1-1 that were either mock-inoculated or inoculated with 10⁵ cfu ml⁻¹ Pst(avrRpt2) (Figure 1C). Mock-inoculated leaves showed little change in electrolyte leakage over time. Electrolyte leakage from leaves inoculated with Pst(avrRpt2) increased substantially between 7 and 13 hpi. By 13 hpi, electrolyte leakage from Col-0 leaves inoculated with Pst(avrRpt2) was approximately 4-fold higher than mock-inoculated tissue, while the increase in electrolyte leakage from iap1-1 leaves was more modest (2.7-fold increase compared to mock-inoculated tissue). Electrolyte leakage from iap1-1 leaves was significantly less than from wild-type leaves between 10 and 13 hpi with Pst(avrRpt2) (T-test P<0.01) suggesting that HR-mediated cell death is compromised in iap1-1.
Intracellular SA accumulation in response to \textit{Pst} and \textit{Pst(\textit{avrRpt2})}

SA is required for RPS2- and RPS4-mediated immunity [8,22,23,32–34]. If young \textit{iap1-1} plants accumulate little SA like mature \textit{iap1-1} [37], this could explain the compromised ETI response observed in young \textit{iap1-1}. To test this hypothesis, an ADPWH\_lux SA biosensor [42] was used to measure SA levels in both intracellular (IWFs) and intercellular (leaves minus IWFs) compartments of young (4 wpg) Col-0 and \textit{iap1-1} plants that were untreated, mock-inoculated, or inoculated with 10^6 cfu ml^{-1} \textit{Pst} or \textit{Pst(\textit{avrRpt2})} (Figure 2). Intracellular SA results are discussed in this section and the next section respectively. Col-0 and \textit{iap1-1} had similar levels of intracellular free SA in both untreated and mock-inoculated tissues at 12, 24, and 48 hpi (<100 ng gfw^{-1}) (Figure 2A). Col-0 inoculated with \textit{Pst} accumulated 199 ng gfw^{-1} intracellular free SA by 48 hpi, significantly more than mock-inoculated controls (T-test P<0.01); whereas intracellular free SA levels in \textit{iap1-1} were similar to mock-inoculated controls at all time points. In response to \textit{Pst(\textit{avrRpt2})}, Col-0 accumulated 546–682 ng gfw^{-1} intracellular free SA at 12, 24, and 48 hpi, whereas \textit{iap1-1} accumulated very little intracellular free SA (109–164 ng gfw^{-1}). This suggests that \textit{iap1-1} accumulates little intracellular free SA in response to \textit{Pst} or \textit{Pst(\textit{avrRpt2})}. In addition, Col-0 accumulated higher levels of intracellular free SA in response to \textit{Pst(\textit{avrRpt2})} compared to \textit{Pst} (T-test P<0.01). Similar results were observed for intracellular total SA (free SA+SA-glucosides) with the exception that \textit{iap1-1} accumulated high levels of total SA at 48 hpi with both \textit{Pst} and \textit{Pst(\textit{avrRpt2})} (Figure 2B).

Intercellular SA accumulation in response to \textit{Pst} and \textit{Pst(\textit{avrRpt2})}

Intercellular SA accumulation is essential for \textit{ARR} to \textit{Pst}, and intercellular SA addition and subtraction experiments suggest that SA acts as an anti-microbial agent in the intercellular space during \textit{ARR} [38]. To determine if SA accumulates in a similar manner in young plants undergoing ETI, SA levels were measured in IWFs collected from leaf tissue of young (4 wpg) Col-0 and \textit{iap1-1} that were untreated, mock-inoculated, or inoculated with 10^6 cfu ml^{-1} \textit{Pst} or \textit{Pst(\textit{avrRpt2})} (Figure 2C,D). IWFs from untreated and mock-inoculated Col-0 and \textit{iap1-1} had similar levels of intercellular free SA at 12, 24, and 48 hpi (<200 ng ml IW^{-1}). Intercellular free SA levels in IWFs collected from Col-0 inoculated with \textit{Pst} were similar to mock-inoculated controls. This is consistent with previous findings that young Col-0 accumulates little intercellular SA in response to \textit{Pst} [38]. IWFs collected from \textit{iap1-1} plants inoculated with \textit{Pst} contained similar free SA levels relative to mock-inoculated controls. IWFs from Col-0 inoculated with \textit{Pst(\textit{avrRpt2})} contained high levels of free SA at 12, 24, and 48 hpi (262 ng ml IW^{-1}), whereas IWFs from \textit{iap1-1} inoculated with \textit{Pst(\textit{avrRpt2})} had similar levels as mock-inoculated controls (<200 ng ml IW^{-1}). Similar results were observed for total SA levels in IWFs with the exception that Col-0 accumulated a modest level of intercellular total SA in response to \textit{Pst} at 48 hpi (822 ng ml IW^{-1}) compared to 50 ng ml IW^{-1} in mock-treated plants T-test P<0.01. Therefore, \textit{iap1-1} accumulated little intercellular SA in response to \textit{Pst} or \textit{Pst(\textit{avrRpt2})}. In addition, wild-type Col-0 accumulated high levels of intercellular SA in response to \textit{Pst(\textit{avrRpt2})}, and modest levels in response to virulent \textit{Pst}.

SA accumulation in response to \textit{Pst(\textit{avrRpt2})} is RPS2-dependent

SA accumulates (3- to 7-fold, Figure 2) both intra- and intercellularly in young Col-0 during ETI to \textit{Pst(\textit{avrRpt2})}. If this response is specific to ETI, SA accumulation should be reduced in the \textit{rps2-201} resistance gene receptor mutant in response to
Figure 2. Salicylic acid accumulation in response to Pst and Pst(avrRpt2). Four-week-old Col-0 and iap1-1 were left untreated, mock-inoculated, or inoculated with $10^6$ cfu ml$^{-1}$ Pseudomonas syringae pv. tomato (Pst) or Pst(avrRpt2). Levels of intracellular free salicylic acid (SA) (A) and total SA (free SA + SA glucosides) (B) were determined in leaves from which intercellular washing fluids (IWFs) had been removed. Intercellular levels of free SA (C) and total SA (D) were determined using IWFs collected from the same leaves as in (A) and (B). The mean ± standard deviation of three
Intercellular SA accumulated concurrently with cell death during ETI

As indicated above, elevated intercellular SA levels were observed by 12 hpi with \( Pst(\text{avrRpt2}) \) in wild-type Col-0 (Figure 2C,D). How SA reaches the intercellular space is not known, however, it is possible that SA leaks from dead or damaged cells during the HR that accompanies ETI. To test this hypothesis, electrolyte leakage was measured in Col-0 and \( rps2-201 \) controls. To make it possible to associate SA accumulation and cell death, plants were inoculated with the same concentration of \( Pst(\text{avrRpt2}) \) that was used for the SA accumulation experiments \((10^6 \text{ cfu ml}^{-1})\), Figure 4A. Use of a smaller and more sensitive conductivity meter made it possible to use small, standardized units of leaf tissue (leaf discs), which increased the accuracy of electrolyte leakage measurements. Since a lower bacterial inoculum concentration was used, fewer cells would undergo the HR, therefore cell death was monitored over 32 hours. If cell death occurs before and/or concurrently with SA accumulation, then SA may access the intercellular space from dying and dead cells. Bacterial density measured at 3 dpi demonstrated that \( Pst(\text{avrRpt2}) \) grew to significantly higher levels in \( rps2-201 \) mutants than in Col-0 indicating that an ETI response occurred in Col-0 and was defective in the \( rps2-201 \) mutant (Figure 4B). Electrolyte leakage from Col-0 inoculated with \( Pst(\text{avrRpt2}) \) was significantly greater than in mock-inoculated controls by 10 hpi \((P<0.05)\) suggesting that cell death was occurring by this time (Figure 4A). Thus, the possibility that SA accesses the intercellular space by leaking out of dead cells during ETI could not be ruled out.

Electrolyte leakage was also measured in \( iap1-1 \) to confirm the results of our first set of electrolyte leakage experiments which were carried out with higher inoculum concentrations and a different technique (see materials and methods). \( Pst(\text{avrRpt2}) \) levels supported by \( iap1-1 \) at 3 dpi were significantly higher than in Col-0, consistent with an ETI defect in \( iap1-1 \) (Figure 4B). In this set of experiments electrolyte leakage from \( iap1-1 \) inoculated with \( Pst(\text{avrRpt2}) \) was lower than Col-0 between 14 and 18 hpi, supporting previous evidence for a less robust HR in \( iap1-1 \) relative to Col-0 (Figure 4A). It is interesting to note that electrolyte leakage was greater in \( iap1-1 \) compared to Col-0 from 26 to 32 hpi with \( Pst(\text{avrRpt2}) \), perhaps due to the fact that \( iap1-1 \) is more susceptible to \( Pst \) than Col-0, and \( Pst \) switches to necrotrophy at the end of the infection cycle \([50]\).

Coronatine suppresses SA accumulation during the compatible \textit{Arabidopsis-Pst} interaction

Since young Col-0 accumulates little intercellular SA during the compatible interaction with \( Pst \) \([38]\), Figure 2C,D) we hypothesized that intercellular SA accumulation could be an important part of PTI/basal defense that is suppressed by virulent \( Pst \). Work done by other groups demonstrated that the \textit{Pseudomonas} phytoxin coronatine suppresses SA accumulation in whole leaves \([51,52]\). To test whether coronatine suppresses intra- and/or intercellular SA accumulation, young \((4 \text{ wpg})\) wild-type Col-0 were inoculated with \( Pst \) (strain DC3000) or coronatine-deficient

\( Pst(\text{avrRpt2}) \) \([13]\). Reduced SA accumulation has been observed in whole leaves of \( rps2-201 \) \([49]\), however, inter- and intracellular SA accumulation were not measured. To determine if SA accumulation is specific to the RPS2-AvrRpt2 pathway, intracellular and intercellular SA levels were measured in leaf tissue collected from Col-0 and \( rps2-201 \) mutants that were untreated, mock-inoculated, or inoculated with \( 10^6 \text{ cfu ml}^{-1} \) \( Pst(\text{avrRpt2}) \) (Figure 3). Untreated and mock-inoculated Col-0 and \( rps2-201 \) leaves contained little intracellular SA \((<30 \text{ ng ml}^{-1} \text{ IWF})\), whereas \( rps2-201 \) accumulated 6-fold less \((504 \text{ ng gfw}^{-1})\) than Col-0 inoculated with \( Pst(\text{avrRpt2}) \) accumulated substantial intracellular SA \((3131 \text{ ng gfw}^{-1})\) whereas \( rps2-201 \) accumulated 6-fold less \((504 \text{ ng gfw}^{-1})\). IWFs from untreated and mock-inoculated Col-0 and \( rps2-201 \) contained little SA \((<30 \text{ ng ml IWF}^{-1})\), IWFs from Col-0 inoculated with \( Pst(\text{avrRpt2}) \) contained high levels of SA \((838 \text{ ng ml IWF}^{-1})\) whereas IWFs from \( rps2-201 \) contained 2-fold lower SA \((346 \text{ ng ml IWF}^{-1})\). With respect to both intracellular and intercellular SA levels, \( rps2-201 \) inoculated with \( Pst(\text{avrRpt2}) \) accumulated more SA than mock-inoculated controls but less than Col-0 inoculated with \( Pst(\text{avrRpt2}) \). Little total SA accumulated in \( rps2-201 \) leaves inoculated with \( Pst(\text{avrRpt2}) \) or in Col-0 inoculated with \( Pst \) (Figure 2B,D) at 24 hpi, therefore the majority of intracellular and intercellular SA accumulation induced in response to \( Pst(\text{avrRpt2}) \) is dependent on the RPS2-AvrRpt2 pathway.

Figure 3. Salicylic acid accumulation in response to \( Pst(\text{avrRpt2}) \) is RPS2-dependent. Intracellular total salicylic acid (SA) levels (A) and intercellular total SA levels (B) were measured at 24 hpi in leaves collected from 4-week-old plants that were untreated, mock-inoculated, or inoculated with \( 10^6 \text{ cfu ml}^{-1} \text{ pv. tomato} \) carrying \textit{avrRpt2} \((Pst(\text{avrRpt2}))\). The mean \pm standard deviation of three replicate samples is shown. Asterisks indicate significant differences between means (T-test, * \( P<0.05 \), ** \( P<0.01 \)). This experiment was repeated twice with similar results.

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intercellular SA accumulation in a coronatine-dependent manner. These results suggest that *Pst* cor2 suppresses both intra- and intercellular SA accumulation in a coronatine-dependent manner during the compatible interaction in young plants.

**Map-based cloning and whole-genome sequencing to identify iap1-1**

The iap1-1 mutant was isolated from a screen for ARR-defective plants performed on a population of fast neutron mutants [37]. As demonstrated in this work iap1-1 is partially compromised in ETI. To identify the causal mutation in iap1-1 we began with a typical map-based cloning approach. A mapping population was generated and approximately 160 putative homozygous mutant F2s were used to map the iap1-1 mutation close to marker 461250 (Monsanto Arabidopsis Polymorphism Collection) at 18,087,180 base-pairs (bp) on the long arm of chromosome four. The necessity of screening individual mature plants for an ARR-defective phenotype made the reliable identification of homozygous individuals difficult and time-consuming for fine mapping, therefore we sequenced the iap1-1 genome in order to locate the mutation.

DNA isolated from a homozygous iap1-1 individual was used to create a DNA library that was sequenced using an Illumina HiSeq 1500. This generated roughly 68 million paired-end reads which were then mapped to the Col-0 reference genome (TAIR 10) using BWA software [46] resulting in approximately 50-fold coverage of the genome. Since the majority of fast neutron-generated mutations are deletions [33–35] we reasoned that iap1-1 was likely a deletion mutant. To identify deletions in the iap1-1 genome we compiled a list of positions in the reference genome that had zero coverage by iap1-1 reads. A region of zero coverage might indicate that the corresponding sequence is deleted in iap1-1. Genome-wide, 614 regions of zero coverage were identified, 244 of which were located on chromosome four. Based on the mapping data, zero-coverage regions located near marker 461250 on chromosome four were investigated. Two zero-coverage regions were found within 500 kb of the marker (Table S1), one of which was located in an intergenic region and was therefore unlikely to represent the causal mutation. Intergenic regions are defined as regions between genes not including recognizable promoters or untranslated regions. The other zero-coverage region was 65 bp within the first exon of *EDS3* (Figure 6A), a gene known to be required for SA accumulation [22] and ARR [19]. Gel electrophoresis of *EDS3* RT-PCR products from iap1-1 and Col-0 showed a size difference that was consistent with a deletion in the iap1-1 product (Figure 6B). Sequencing these products confirmed the 65 bp deletion in iap1-1 and also revealed a 6 bp insertion between the nucleotides flanking the deleted region (Figure 6C). When the Col-0 reference genome was modified to contain this insertion/deletion mutation in *EDS3*, the Illumina reads generated from iap1-1 mapped continuously across the modified *EDS3* locus, confirming that iap1-1 harbours the insertion/deletion mutation depicted in Figure 6C. This mutation results in the net loss of 39 nucleotides from the first exon of *EDS3* and causes a frame-shift that produces a premature stop codon after the 32nd amino acid (Figure 6D).

**The iap1-1 and eds5-3 mutations are allelic**

Both iap1-1 and eds5-3 mutants have been shown to be required for ARR and pathogen-induced SA accumulation [19,22]. To confirm that iap1-1 is an allele of *EDS3*, homozygous iap1-1 mutants carrying the gl1 marker were crossed with eds5-3 and the F1 progeny were tested for ARR competence at 6 wpf. If the two mutations are not allelic then ARR should be restored in the F1 generation (complementation) but if iap1-1 and eds5-3 are allelic then the F1 generation should remain ARR-defective. Wild-type Col-0 supported low *Pst* levels (5 × 10⁷ cfu ml⁻¹) characteristic of ARR whereas iap1-1, eds5-3, and iap1-1×eds5-3 F1 plants supported high *Pst* levels (at least 50-fold higher than Col-0) indicating that they are compromised for the ARR response (Figure 6E). Therefore, iap1-1 and eds5-3 failed to complement each other indicating that these two mutations are allelic and iap1-1 is a new mutant allele of *EDS3*.
Discussion

During ARR IAP1 is required for SA accumulation in the intercellular space where SA is thought to act as an antimicrobial agent [37]. This led us to investigate the role of IAP1 and intercellular SA accumulation during ETI. At the same time, map-based cloning, whole-genome sequencing, and complementation analysis identified \textit{iap1-1} as a mutant allele of \textit{EDS5}. In examining the literature, four \textit{eds5} alleles already exist, therefore we propose that \textit{iap1-1} be referred to as \textit{eds5-5} in subsequent publications.

To avoid the time-consuming process of fine mapping, several approaches have been developed that combine the principles of genetic mapping with next-generation sequencing to identify EMS-generated mutations in \textit{Arabidopsis} [56–61]. Most of these approaches involve sequencing pools of homozygous mutants selected from an F2 mapping population (reviewed in [62]). This step can be problematic if the mutant phenotype is difficult to score and homozygous individuals cannot be selected reliably. For example, heterozygous \textit{iap1-1/eds5-5} plants display an ARR phenotype that is intermediate to that of homozygous mutants and wild-type plants, and can occasionally be misclassified as homozygous [37]. An alternative approach to avoid this issue is to directly sequence the mutant genome. Unfortunately, EMS mutants usually possess thousands of mutations making it difficult or impossible to identify the causal mutation by direct sequencing alone [62]. This problem can be solved by rough mapping the

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**Figure 5.** Coronatine suppresses salicylic acid accumulation. Intracellular (A) and intercellular (B) free and total salicylic acid (SA) levels were measured in leaves collected from 4-week-old Col-0 that were untreated or inoculated with 10^6 cfu ml^{-1} \textit{Pseudomonas syringae pv. tomato} (Pst) strain DC3000 or the coronatine-deficient strain DC3661 (\textit{Pst cor\textsuperscript{-}}). The mean ± standard deviation of three replicate samples is shown. Asterisks indicate a significant difference compared to plants inoculated with Pst at the corresponding time point (T-test, * \textit{P}<0.05, ** \textit{P}<0.01). (C) Bacterial density (colony-forming units per leaf disc [cfu ld\textsuperscript{-1}]) measured at 3 days post-inoculation. Values represent the mean ± standard deviation of three sample replicates. The asterisk indicates a significant difference (T-test \textit{P}<0.01). These experiments were repeated once with similar results. doi:10.1371/journal.pone.0088608.g005
mutation (to exclude most irrelevant mutations by determining the approximate position of the causal mutation) or backcrossing (to physically remove irrelevant mutations). Ashelford et al. [63] used both techniques followed by whole-genome sequencing to identify the early bird (ebi-1) EMS mutant. While successful, this effort was complicated by more than 30 non-synonymous mutations that were linked to the causal ebi-1 mutation that were not removed by backcrossing [63]. We used a similar approach combining genetic mapping and whole-genome sequencing to identify the fast neutron mutant iap1-1/eds5-5. Ashelford et al. [63] performed four backcrosses to eliminate irrelevant mutations from the ebi-1 mutant, whereas iap1-1/eds5-5 was backcrossed only twice since the mutation load in fast neutron mutants is typically lower than in EMS mutants [64–66]. Therefore, fewer irrelevant mutations will be linked to the causal mutation, making it easier to identify. Based on this information we suggest that identifying fast neutron mutants using a combination of rough mapping and direct sequencing can be done rapidly and efficiently in comparison to EMS mutant identification.

Once the genome sequence data for iap1-1/eds5-5 was obtained and the reads were aligned to the Col-0 reference genome, identification of potential deletion mutations was straightforward. Instead of generating a list of SNPs as would be done for an EMS-generated mutant, we compiled and assessed regions of the reference genome that were not covered by reads generated from the iap1-1/eds5-5 mutant genome. A region of zero coverage could indicate that the corresponding sequence is deleted or highly polymorphic in iap1-1/eds5-5 or could represent an error in the reference genome [67]. Regions of low coverage also occur for other reasons, including the difficulty of sequencing some genomic regions (e.g., low GC areas). Since we did not sequence the wild-type parent of iap1-1/eds5-5 we cannot differentiate between these possibilities. While 814 regions of zero coverage were identified, this is probably an overestimate of the fast neutron-induced mutations in iap1-1/eds5-5. Fast neutron mutant genomes are estimated to harbour deletions in approximately 10 genes [64]. Our bioinformatics strategy did not detect the co-localized insertion mutation present in iap1-1/eds5-5. The insertion was discovered later by Sanger sequencing demonstrating the necessity of confirming the molecular basis of fast neutron-generated mutations. Although not as common as deletions, other instances of co-localized insertion-deletion mutations resulting from fast neutron mutagenesis have been described [68,69].

To identifyiap1, we began by genetically mapping it to chromosome four and turned to whole-genome sequencing because it became available and additional mapping became unfeasible. Based on this experience, we propose a more rapid approach for gene identification in which rough mapping and sequencing are performed at the same time. As soon as the causal mutation is mapped to one chromosomal region, the sequencing data can be used to obtain a list of zero-coverage regions in this area. Zero-coverage regions in introns or intergenic sequences can be excluded as they are unlikely to affect gene function. The list of candidate zero-coverage regions in exons, promoters, and untranslated regions would be assessed to determine if mutations in any of these genes makes sense based on the mutant phenotype. If there are too many zero-coverage regions (>10), then further mapping may be required to reduce the number of candidate genes. If as expected for fast neutron-induced mutants, there are only a few zero-coverage regions in candidate genes, complementation analysis can be performed to confirm the identity of the gene. If whole-genome/next-generation sequencing had been available when we started to map iap1-1/eds5-5, we would have mapped it near one or two markers on chromosome four, then realized that there was just one zero-coverage region in one gene near these markers making us confident that this was the mutation responsible for the iap1-1/eds5-5 phenotype. Moreover, this gene encoded EDS5, a SA transporter important for defense and therefore made sense with respect to the ARR-defective phenotype of iap1-1/eds5-5. Many years of challenging mapping would have been avoided if this method (simultaneous mapping and whole-genome sequencing) had been available.

Plants that are heterozygous for the iap1-1/eds5-5 mutation display an ARR phenotype that is intermediate to that of homozygous mutants and wild-type plants, suggesting that iap1-1/eds5-5 is a semidominant mutation [37]. Other mutant alleles of eds3 have been classified as recessive (eds5-1 [30] and eds5-3 [22]). We believe this difference reflects the nature of assessing dominance in disease resistance mutants. For example, small changes in humidity may affect the growth of Pst in plants [70] such that heterozygotes may exhibit phenotypes that are similar to homozygous mutants or wild-type plants. Therefore during heterozygote analysis, if enough heterozygotes are classified with a wild-type phenotype, a semidominant mutation will be classified as recessive. It is also possible that iap1-1/eds5-5 is a unique semidominant allele. Since EDS5 transcripts are detectable in iap1-1/eds5-5 mutants, a truncated EDS5 protein may be produced in iap1-1/eds5-5 as 52 amino acids precede the premature stop codon in the iap1-1/eds5-5 mutant. If the mutant peptide has a dominant negative effect on the wild-type protein this could explain the semidominant phenotype observed in heterozygous iap1-1/eds5-5. Other eds5 mutant alleles that have been studied have been classified as recessive and also have premature stop codons and may produce truncated proteins [27]. However, the stop codons in eds3-1, eds3-3, and iap1-1/eds5-5 occur at different positions suggesting that distinct peptides could be produced. Future studies to investigate whether these mutant alleles produce different peptides may shed light on the importance of the functional domains of the EDS5 MATE transporter. It is also possible that the semidominant phenotype of iap1-1/eds5-5 is the result of an additional, unknown mutation.

We do not usually observe a difference in disease susceptibility between young Col-0 and iap1-1/eds5-5 during the compatible interaction with virulent Pst [37, this study]. In some of our experiments young iap1-1 is slightly more susceptible than Col-0, however, the difference is not always statistically significant. This observation conflicts with other studies of eds5 mutants [22,30,31]. We propose that this difference may result from our use of a 10-
fold higher inoculum concentration (10⁶ cfu ml⁻¹) during bacterial growth assays. Indeed, Glazebrook et al. [30] indicate that the enhanced disease susceptibility phenotype of eds mutants is more easily observed when lower inoculum concentrations are used.

Although IAP1 is EDS5, a gene already known to be required for SA-mediated defense responses, both confirmatory and novel results were obtained during our investigation of compatible and incompatible (ETI) responses to *P. syringae* in *iap1-1/eds5-5*. Bacterial levels were modestly enhanced in *iap1-1/eds5-5* compared to Col-0 in response to both *Pst*(*avrRpt2*) and *Pst*(*avrRps4*) indicating that the ETI response was reduced, but not abolished in *iap1-1/eds5-5*, suggesting that IAP1/EDS5 contributes to ETI. Similar results were also observed in *eds5-3/ids1* in response to *Pst*(*avrRpt2*) [22]. In addition, Venugopal and colleagues [35] found that SA-deficient *sid2* mutants are also partially compromised for ETI. ETI was fully compromised in *sid2 eds1* double mutants suggesting that SA and EDS1 function redundantly during ETI. A macroscopic HR was observed in NahG, other *eds5* alleles [3,32], and *iap1-1/eds5-5*, and trypan blue staining detected similar levels of cell death in *iap1-1/eds5-5* and Col-0 (this study). Neither of these techniques is sensitive or quantitative, therefore electrolyte leakage assays were used to carefully examine the HR in *iap1-1/eds5-5*. Electrolyte leakage was modestly reduced in *iap1-1/eds5-5* compared to Col-0 inoculated with 10⁶ or 10⁷ cfu ml⁻¹ *Pst*(*avrRpt2*) at several time points. Like the trypan blue assays, the electrolyte leakage assays confirm that the HR cell death occurs in *iap1-1/eds5-5*, however, this sensitive assay indicates that the HR response was modestly reduced. We confirm that IAP1/EDS5 contributes to bacterial growth restriction during ETI and demonstrate that the HR is also partially dependent on functional IAP1/EDS5. In other words, it appears that IAP1/EDS5-dependent SA accumulation is required for a full HR and the bacterial growth restriction that takes place during RPS2-AvrRpt2-mediated ETI. In addition, electrolyte leakage assays revealed that cell death and intercellular SA accumulation occurred concurrently, suggesting that SA may gain access to the intercellular space from dead and dying cells during the HR. Consistent with this idea, the highest levels of intercellular SA accumulation were typically observed at 24 or 48 hpi with *Pst*(*avrRpt2*), once extensive cell death had occurred. The timing of maximal intercellular SA accumulation varied between experiments, potentially because the timing and strength of the HR can be affected by variations in humidity that occur even within growth chambers.

SA accumulates in the intercellular space during ARR where it is thought to function as an antimicrobial agent. The idea that SA might act as an antimicrobial agent in the intercellular space in other defense responses has not been examined, therefore we measured both inter- and intracellular SA accumulation during ETI initiated by *Pst*(*avrRpt2*) and in response to virulent *Pst* (compatible interaction). Wild-type Col-0 accumulated intercellular SA in response to *Pst*(*avrRpt2*) in the same range as observed during ARR (153 to 400 mg ml⁻¹ IWF⁻¹) [37,38] providing evidence for the importance of intercellular SA accumulation during ETI. Additionally, SA accumulation was reduced in *nps2-20I* mutants indicating that the intercellular SA produced was specific to the RPS2-AvrRpt2 ETI pathway. In comparison, intercellular SA levels in *iap1-1/eds5-5* inoculated with *Pst*(*avrRpt2*) were similar to background levels observed in untreated and mock-inoculated controls demonstrating that *iap1-1/eds5-5* accumulates little SA during ETI as would be expected of an *eds5* mutant [22]. Although intercellular SA accumulation was reduced to background levels in *iap1-1/eds5-5*, RPS2-AvrRpt2-initiated ETI modestly reduced bacterial levels, suggesting that inter- and intracellular SA accumulation, as well as SA-independent constituents contribute to ETI.

During the response to virulent *Pst* over 48 hpi, *iap1-1/eds5-5* accumulated low levels of inter- (free and total) and intracellular (free) SA, similar to untreated or mock-inoculated plants, demonstrating that both intra- and intercellular SA accumulation is reduced by the *iap1-1/eds5-5* mutation. We also observed that total intracellular SA accumulation in Col-0 and *iap1-1* was low at early times after inoculation with virulent *Pst* (12 and 24 hpi), however, by 48 hpi, total intracellular SA levels increased to ~1000 ng g⁻¹ IWF⁻¹ in *iap1-1/eds5-5* and to ~1000 ng g⁻¹ IWF⁻¹ in Col-0, similar to SA levels induced by *Pst*(*avrRpt2*). In Col-0 responding to virulent *Pst*, both intracellular and intercellular free SA levels were similar to background levels (untreated or mock-inoculated plants) with the exception of a modest increase in intracellular free SA levels at 48 hpi. One explanation for these observations is that PTI-associated SA accumulation is suppressed by virulent *Pst*. Support for this hypothesis comes from two studies in which the *Pseudomonas* phytopathogen coronatine was shown to inhibit whole-leaf SA accumulation in *Arabidopsis* in response to virulent *Pst* and *P. maculicola* [51,52]. In addition, SA accumulates during PTI/basal resistance in response to the PAMP flg22 [36]. These investigations suggest that coronatine is important in suppressing SA accumulation during the PTI response and this produces a compatible environment for *Pseudomonas* infection. In this study we demonstrated that coronatine-producing *Pst* suppress both intracellular and intercellular SA accumulation. These data support the idea that intercellular SA accumulation is an important component of the PTI response.

Serrano et al. [28] speculate that *eds5* mutants do not accumulate SA because the EDS5 MATE transporter is not functional such that SA remains trapped in the chloroplast leading to high SA levels and feedback inhibition of SA biosynthesis. It is interesting to note that at the later 48 hpi time point, *iap1-1/eds5-5* accumulated elevated levels of total intracellular SA (free + conjugated) in response to both virulent and avirulent *Pst*. Since little intracellular free SA accumulated at 48 hpi, the conjugated form of SA must be accumulating. We speculate that free SA is quickly converted to its conjugated form at 48 hpi with *Pst* and therefore little free SA is present for feedback inhibition of SA biosynthesis, allowing intracellular conjugated SA levels to rise. Also of note, both *iap1-1/eds5-5* and Col-0 were similarly susceptible to *Pst* even though *iap1-1/eds5-5* accumulated ~500 ng g⁻¹ IWF⁻¹ less intracellular total SA at 48 hpi compared to Col-0. Although free SA is generally regarded as the active form, studies on mutants in which enhanced disease susceptibility corresponds primarily with a reduced capacity to accumulate conjugated SA suggest that conjugated SA could be an important part of a successful defense response [71–74]. However, we speculate that by 48 hpi, suppression of PTI defense by *Pst* is waning such that intracellular conjugated SA accumulating in wild-type Col-0, however it is too late to mount a successful defense as bacteria have multiplied to high levels. It is also possible that the absence of accumulation of free SA in the intercellular space is responsible for the unsuccessful defense response in Col-0 and *iap1-1/eds5-5* to virulent *Pst*.

**Conclusions**

By developing a whole-genome-next-generation sequencing technique to identify deletion mutations, we identified a new mutant allele of *EDS5*. This technique will be useful for other researchers as it allows rapid identification of a deletion mutant by whole-genome sequencing once the mutation is roughly mapped.
Our studies of *iap1-1/eds5-5* revealed that SA accumulates in both the inter- and intracellular spaces during the RPS2-AvrRpt2-initiated ETI response. This suggests that SA contributes as both an intracellular signaling molecule and an antimicrobial agent in the intercellular space. We also demonstrated that intercellular SA accumulation is suppressed in a coronary-dependent manner by virulent *Pst*. Therefore, SA may act as an antimicrobial agent in the intercellular space during PTI/basal resistance in *Arabidopsis*.

Materials and Methods

Plant growth conditions

The *iap1-1* [37], *rps2-201* [13], and *eds5-3/sid1* [22] mutants were used in conjunction with wild-type Columbia (Col-0). *iap1-1* was isolated from 5000 M2 fast neutron-mutagenized seeds (Lebre Seeds, Texas, USA) [37]. Seeds were surface-sterilized in 70% ethanol for 2 minutes and in sterilization solution (1.6% bleach, 0.1% Tween 20) for 10 minutes then washed with sterile water 5 times. Seeds were stratified at 4°C for at least 2 days before they were left to germinate on Murashige and Skoog (1962) medium for 5–7 days under continuous light. Seedlings were transferred to soil at the cotyledon stage (Sunshine Mix No. 1 [JVK]) and watered with 1 g L⁻¹ 20-20-20 fertilizer. Temperature was maintained between 22°C and 24°C during a 9-hour photoperiod with an average light intensity of 150 μE m⁻² sec⁻¹. Humidity ranged between 75% (winter) and 85% (summer).

Disease resistance assays

*Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 (rifampicin and kanamycin resistant) and *Pst*(*avrRpt2*) were obtained from Dr. A. Bent (University of Wisconsin at Madison; [39]). *Pst*(*avrRp4*) was obtained from Dr. R. Subramaniam (Agriculture Canada). *Pst* cor⁻ (strain DC3661) was obtained from Dr. D. Cuppels (Agriculture Canada; [40]). Bacteria were shaken overnight at room temperature (22–24°C) in King’s B media and kanamycin (50 μg ml⁻¹) to mid-log phase then resuspended in 10 mM MgCl₂ at a concentration of 10⁻⁵ or 10⁻⁴ cfu ml⁻¹. Plants were inoculated by pressure infiltration and in planta bacterial levels were determined as previously described [19,41].

Trypan staining and electrolyte leakage assays

Plants were inoculated with *Pst*(*avrRpt2*) (10⁵ or 10⁶ cfu ml⁻¹) or mock-inoculated with 10 mM MgCl₂. For trypan staining leaves were harvested at 24 hours post-inoculation (hpi) and immediately submersed in staining solution (0.02 g trypan blue, 8% phenol, 8% glycerol, 8% lactic acid, 8% water, 67% 95% ethanol). Leaves were boiled for 1 minute in the staining solution and left overnight followed by destaining in 70% ethanol. Images were captured using a digital camera (Nikon DS-M1200F) mounted on a Nikon Eclipse TE2000-S microscope at 10× magnification. For the first set of electrolyte leakage assays (Figure 1C) leaves were collected at 2 hpi, weighed, and rinsed in distilled water. 48 leaves per treatment were added to 80 ml of distilled water in triplicate and conductance was measured using a YSI environmental model 556 conductivity meter. For the second set of electrolyte leakage assays (Figure 4A) leaf discs were collected at 1 hpi, rinsed in nanopure water for 1 hour, then transferred to vials containing 10 ml nanopure water (9 plants per treatment, 10 leaf discs per vial, 3 vials per treatment). Conductance was measured periodically using a Jenway model 4070 portable conductivity meter.

IWF collection and SA measurement

IWF (intercellular washing fluid) collections were performed as described previously [19,38]. Briefly, Col-0 and *iap1-1* (4 weeks old) were untreated, mock-inoculated (10 mM MgCl₂) or inoculated with 10⁶ cfu ml⁻¹ virulent *Pst*, avirulent *Pst*(*avrRpt2*), or coronatine-deficient *Pst*. Leaves were harvested at 12, 24, and 48 hpi then surface-sterilized (50% ethanol and 0.05% bleach) and vacuum infiltrated with sterile water. Leaves were then blotted dry and centrifuged at 1000 x g for 30 minutes in 50 ml syringes fitted into microcentrifuge tubes. The IWFs were filter-sterilized to remove *Pst*. ADPWH₅₇₅ is a non-pathogenic soil bacterium that has been modified to produce luciferase proportional to the amount of SA present [42] and was used to measure free SA and total SA (free SA plus SA-glucosides) as described previously [43]. Briefly, ADPWH₅₇₅ was grown overnight in LB media with shaking at 37°C and then diluted to an OD₆₅₀ of 0.4 with fresh LB followed by incubation with IWFs or leaf tissue minus IWFs for 1 hour at 37°C in an opaque 96-well plate (Corning no.3915). Luminescence was measured on a Biotek plate reader at 490 nm and used to calculate SA concentrations.

Map-based cloning

*iap1-1* (Col-0 background) was crossed to Landsberg erecta (Ler) and the cross was confirmed in the F1 generation using InDel molecular markers [44]. Screening for homozygous *iap1-1* or *iap1* in the F2 generation was done by inoculating mature plants with virulent *Pst* (10⁶ cfu ml⁻¹) and determining in planta bacterial levels after 3 days. Plants were classified ARR-incompetent if bacterial levels were ≥10⁷ cfu ld⁻¹ and ARR-competent if bacterial levels were ≤10⁶ cfu ld⁻¹. InDel and CAPS molecular markers [44] were used to map the *iap1-1* mutation to the long arm of chromosome four [45]. Primers for significantly linked markers include AGAGGCACAGTGAATCCAGCT, ACTTC-GCAGCTCTCTGGTTTGG (461250); GGGGCAAATTTAGTCGTCT, GCTTCTATAACTCCGTACCTGG (461260); and GGACAAGGGAGAGATCAATTTGG (460755). Primers for CAPS markers include GGATGGTGTTGAATACCTGCT, GGCATATGCTAATTGCTTCTT (18,288,815 bp); and TGCCGA-GAAATCGAGACA, GCAGCTTGAAGAGTATAATCC (18,111,371 bp) digested with *PstI* and *RsaI*, respectively.

Whole-genome sequencing

DNA was isolated from leaf tissue of an *iap1-1* plant using a modified phenol-chloroform extraction method. Briefly, tissue was ground in liquid nitrogen, incubated with DNA extraction buffer (200 mM Tris-HCl [pH 7.5], 250 mM NaCl, 23 mM EDTA, 0.5% SDS [w/v]), extracted with phenol-chloroform, and precipitated in isopropanol. The pellet was re-suspended and treated with RNase Cocktail (Life Technologies) followed by extraction with phenol-chloroform and again with chloroform alone. The DNA was then precipitated in ethanol. Library preparation was carried out according to manufacturer instructions (Nextera) and sequencing was performed on an Illumina HiSeq 1500 (high output mode) using approximately one third of a lane. Reads were aligned to the *Arabidopsis* Col-0 reference genome (TAIR 10) using BWA software [46].

RT-PCR

Leaf tissue was harvested, flash-frozen in liquid nitrogen, and stored at −80°C. RNA was isolated using Sigma TRI Reagent according to the manufacturer’s instructions. Residual DNA was degraded using TURBO DNA-free (Life Technologies) prior to
RNA quantification. First-strand cDNA synthesis was carried out using M-MLV Reverse Transcriptase (Life Technologies). PCR primers used to amplify the \textit{ipt1-1} polymorphism were: 5‘- ATGGCTAAATCAAATCCCAAGATTGAC (F) and 5‘- CA- GCGAGGGTCATGGAGGAC (R) at 29 cycles. Products were visualized on a 2% agarose gel stained with ethidium bromide.

**Supporting Information**

**Figure S1** Trypan blue staining for hypersensitive response cell death. Four-week-old Col-0 and \textit{ipt1-1} were inoculated with 10 mM MgCl\textsubscript{2} (mock) or 10\textsuperscript{7} cfu ml\textsuperscript{-1} \textit{Pseudomona}s \textit{pyrroc}\textit{oma pv. tomato} (\textit{Pto}) carrying \textit{avrRpt2}. Leaves were stained with trypan blue at 24 hours post-inoculation. (TIF)

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**Table S1** Positions on the Col-0 reference genome near marker 461250 (18,087,180 bp) that were not covered by \textit{ipt1-1} Illumina sequencing reads. (PDF)

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**Author Contributions**

Conceived and designed the experiments: RCC, JD. Performed the experiments: JC, DW, MP. Analyzed the data: RCC, JD, MI, PC, VC. Contributed reagents/materials/analysis tools: RCC, EAW, BG. Wrote the paper: RCC, JD. Performed mapping and optimized SA biosensor assay: JC. Performed bioinformatics: VC.

**Intercellular SA and Whole-Genome Sequencing**
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