An apoplastic peptide signal activates salicylic acid signalling in maize

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Abstract

Control of plant pathogen resistance or susceptibility largely depends on the promotion of either cell survival or cell death. In this context, papain-like cysteine proteases (PLCPs) regulate plant defence to drive cell death and protection against biotrophic pathogens. In maize (*Zea mays*), PLCPs are crucial in the orchestration of salicylic acid (SA)-dependent defence signalling. Despite this central role in immunity, it remains unknown how PLCPs are activated, and which downstream signals they induce to trigger plant immunity. Here, we present the discovery of an immune signalling peptide, *Zea mays* immune signalling peptide 1 (Zip1). A mass spectrometry approach identified the Zip1 peptide being produced after salicylic acid (SA) treatment. *In vitro* studies using recombinant proteins demonstrate that PLCPs are required to release bioactive Zip1 from its propeptide precursor (PROZIP1). Strikingly, Zip1 treatment strongly elicits SA accumulation in maize leaves. Moreover, RNAseq based transcriptome analyses revealed that Zip1 and SA treatments induce highly overlapping transcriptional changes. Consequently, Zip1 promotes the infection of the necrotrophic pathogen *Botrytis cinerea* in maize, while it reduces virulence of the biotrophic fungus *Ustilago maydis*. Together, Zip1 represents the previously missing signal that is released by PLCPs to activate SA defence signalling.

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Introduction

Plants face a wide range of biotic threats including viruses, bacteria, insects and fungi. Protective processes including local and systemic defences are mediated in part by plant proteases that additionally regulate stomatal development, embryogenesis, and cuticle deposition [1]. Importantly, proteases from diverse catalytic classes have been associated with immunity in plants [1]. The apoplastic aspartic protease CDR1 (Constitutive Disease Resistance1), for instance, induces local and systemic defence responses in *Arabidopsis thaliana*. Increased bacterial susceptibility to *Pseudomonas syringae* occurs in *cdr1* mutants whereas *CDR1* overexpression results in enhanced resistance [2]. Another example of proteases involved in plant immunity is the tomato subtilisin-like protease P69 [3]. Out of six characterized isoforms, two (P69B and P69C) are transcriptionally upregulated by the defence hormone salicylic acid (SA) and by infection with *P. syringae*, suggesting that serine proteases are important during pathogenesis [4]. In addition, the *A. thaliana* serine protease SITE-1 PROTEASE (S1P) cleaves RAPID ALKALIZATION FACTOR23 (RALF23) to inhibit plant immunity via the malectin-like receptor kinase FERONIA (FER) [5].

Among the classes of plant proteases, the papain-like cysteine proteases (PLCPs) are central hubs in the regulation of programmed cell death and plant immunity [1,6]. A crucial role of PLCPs in plant immunity is highlighted by the discovery that evolutionary unrelated plant pathogens have independently evolved effector proteins that target PLCPs to promote virulence. For instance, the tomato PLCP RCR3 (Required for *Cf-2*-Dependent Disease Resistance3) is targeted by the Avr2 (Arvirulence-2) effector protein of the fungal pathogen *Passalora fulva* (previously *Cladosporium fulvum*) [7]. In addition, it is inhibited by the cystatin-like effectors EPIC1 (Extracellular Cystatin-like Protease Inhibitor1) and EPIC2B of the oomycete pathogen *Phytopthtora infestans* and the allergen-like effector Gr-VAP1 (Venom Allergen-like effector Protein1) of the nematode *Globodera rostochiensis* [8,9].

Apoplastic PLCPs have significant roles in the activation of diverse plant defence responses. Further, the regulation of plant immunity also commonly involves the fine-tuned interplay of phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Among defence-related phytohormones, SA is a key player that orchestrates responses to both biotic and abiotic stresses [10,11] and extensive studies have detailed the role of SA in innate immune signalling [12]. In general, research in *A. thaliana* and *Nicotiana benthamiana* has revealed that SA signalling promotes efficient defence activation against biotrophic pathogens, whereas necrotrophic pathogens are sensitive to JA/ET-dependent defence signalling. Early publications emphasized the potential for SA-mediated antagonism for the strong inhibition of wound-induced JA signalling [13,14]. Beyond classical phytohormones, endogenous plant peptides can act on different levels of signal amplification relevant to JA/ET dependent defence signalling [10,15]. In *A. thaliana* and maize, small peptides can be released from larger pro-peptides to act as damage-associated molecular patterns (DAMPs) [16-18]. In maize, transcripts encoding the PLANT ELICITOR PEPTIDE 1 (ZmPEP1) precursor protein (*ZmPROPEP1*) display induced expression following JA treatment [16]. In *A. thaliana*, AtPEP1 activates pathogen defence responses and confers disease resistance when ectopically expressed [18]. Likewise in maize, ZmPEP1 promotes the production of JA, ET, and defence gene expression. Consequently, pretreatment of maize with ZmPEP1 leads to enhanced resistance to necrotrophic fungal pathogens. Thus, PEPs from *A. thaliana* and maize are functionally conserved DAMPs regulating JA-associated innate immune responses in diverse plant species [16,17].

The maize pathogen *Ustilago maydis* is a biotrophic fungus, which induces formation of tumors on all aerial parts of its host plant [19]. At the onset of infection, *U. maydis* transiently induces pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) responses, including *PR*-gene expression. In the compatible interaction with maize, these responses are suppressed upon fungal penetration and accommodation of biotrophic infection structures 24 hours after infection [20]. In incompatible interactions, *U. maydis* induces typical plant immune responses

including the rapid accumulation of reactive oxygen species (ROS), induction of *PR*-gene expression, SA-associated defence responses and programmed cell death [20-22]. Successful *U. maydis* infection depends on the induction of the maize cystatin CC9, which inhibits a set of SA-induced, apoplastic PLCPs [23]. In turn, activity of these apoplastic enzymes can trigger the activation of SA-associated defence signalling [23]. Three maize PLCPs (CP1, CP2 and XCP2) are also inhibited by the *U. maydis* effector Pit2, and the inhibitory activity of this protein is essential for virulence of the pathogen [24]. While these findings demonstrate the important role of apoplastic PLCPs for the regulation of plant immunity, key questions remain unanswered. For example, how do apoplastic PLCPs induce downstream SA signalling? What are the targets of PLCPs? Are signals released by PLCPs? What downstream signalling pathways are involved? Based on previous findings, we hypothesized that the activation of SA-related defences by PLCPs is mediated by the release of apoplastic peptides that in turn act as signals to activate downstream responses. In the present study we describe the identification and functional characterization of a novel peptide which is released by PLCP-activity and induces SA accumulation and signalling in maize.

Results

Peptides present in SA-treated apoplastic fluid induce defence responses

To examine if bioactive maize peptides are released by the activity of PLCPs, leaves were treated with SA to first promote apoplastic protease activity. Confirming previous results [23], apoplastic fluid of SA-infiltrated leaves showed strongly induced PLCP activity compared to mock samples 24h after treatment (Fig. S1). Apoplastic fluids of both SA- and mock-treated leaves were subjected to Amicon® filtration to separate small peptides (<10 kDa) from proteins. Peptide fractions of SA-treated and mock treated leaves were then re-introduced into naïve plants by leaf infiltration to test for activity. After infiltration, transcriptional changes of SA-related *PR*-genes were analysed by qRT-PCR at 24 h (Fig. 1A). Peptide fractions from SA-treated

leaves resulted in a significant induction of the previously identified maize SA marker genes *ZmPR3, ZmPR4* and *ZmPR5*. In contrast to SA-related markers, transcript levels of JA-induced *ZmCC9* [23] were not affected by apoplastic peptides (Fig 1A). This result suggests that activity of SA-induced PLCPs can release peptide(s) into the apoplastic fluid, which in turn activate SA mediated processes.

Identification of *Zea mays* immune signalling peptide 1 (Zip1)

To identify bioactive peptide candidates, fractions (<10 kDa) from apoplastic fluids of SA- and mock treated plants were analysed by liquid chromatography mass spectrometry (LC-MS) (Fig. S2A). MS-identified, SA-induced peptides were synthesized and infiltrated into naïve maize leaves to test their ability to induce *PR*-gene expression *in vivo* 24 h after infiltration. In parallel, plants were treated with 2 mM SA as a positive control (Fig. S2B, S3). qRT-PCR was done for the SA markers *ZmPR3, ZmPR4, ZmPR5*, as well as Zm*PRm6b*, and Zm*PR10* [23,25,26]. Out of four candidates, this assay identified one peptide eliciting the accumulation of *PR*-gene transcripts to a similar level compared to SA (Fig. 1B). This 17 amino acid peptide [$^{+}$EGESELKLATQGASVRR^{-}] was termed *Zea mays* immune signalling peptide 1 (Zip1). To test whether Zip1 induced *PR*-gene expression is sequence specific, a mutated peptide version ($Zip1_{mut}$) was generated, in which the N-terminal charged amino acids Glu and Lys were substituted to neutral Ala (Fig. 1B). In the maize leaf assay for elicited *PR*-gene expression, the $Zip1_{mut}$ peptide is completely inactive (Fig. 1B), indicating that the charged N-terminus is required for the induction of Zip1-induced defence signalling. In contrast to the $Zip1_{mut}$ peptide. a native Zip1 version with a three amino acid N-terminal extension (QPW) triggered *PR*-gene induction similar to the 17aa version (Fig S3), indicating potential variability for the N-terminal boundary of Zip1.

Zip1 is released from a pro-peptide by PLCP activity

A MASCOT algorithm-based maize genome search for Zip1 identified an annotated open reading frame for a precursor protein (AC210027.3_FGP003) that was named PROZIP1. . The 137 aa protein is predicted for unconventional secretion (SecretomeP 2.0; http://www.cbs.dtu.dk/services/SecretomeP/) but does not contain any known domains (Expasy PROSITE, https://prosite.expasy.org/). A qRT-PCR experiment showed that transcript levels for PROZIP1 are neither induced by Zip1, nor by SA, (Fig. S2C) which indicates a post-transcriptional regulation of its activity. To test if Zip1 can be released from PROZIP1 by maize PLCPs, PROZIP1 was cloned and fused to an N-terminal HA-tag for heterologous production in *Escherichia coli* (Fig. S4) and co-incubated with apoplastic fluid from SA treated maize plants. Co-incubation resulted in a time-dependent cleavage of PROZIP1, which can be blocked by the addition of E-64 [27], a specific PLCP inhibitor (Fig 2A). This result indicates that PROZIP1 is a substrate of SA-activated maize PLCPs. To test, if individual maize proteases are capable of PROZIP1 cleavage, co-incubation assays with the previously identified [23] apoplastic maize PLCPs CP1, CP2, CatB and XCP2 were performed. PLCPs were heterologously expressed in *N. benthamiana* and protease activity was normalized and monitored via activity based protein profiling (ABPP) [28] using the fluorescent PLCP-specific probe MV-202 [29] (Fig. 2B: chemical structure Fig. S1A). Co-incubation of equal amounts of active individual PLCPs resulted in cleavage of PROZIP1 by CP1 and CP2, but not by CatB and XCP2 (Fig. 2C). This result shows that the maize PLCPs CP1 and CP2 are required for processing of PROZIP1.

PROZIP1 contains six RR/FR motifs that are predicted to be potential protease cleavage sites due to their hydrophobic and dibasic properties (Fig 2D) [30,31]. Maize PLCP activity towards these sides was tested with different fluorescent substrates that identified Arg-Arg and Phe-Arg sequence motifs as most efficient cleaved sites (Fig. S5). To test if cleavage at these predicted sites actually releases Zip1, two different PROZIP1 versions with substituted RR/FR motifs were generated and purified from *E. coli* (Fig. 2D and S4). In PROZIP1Mut^{CS} all six di-arginine motifs

were substituted into di-alanines. A second version of the propeptide (PROZIP1Mut^{CS2}) contained only mutations of the two predicted cleavage sites surrounding the Zip1 peptide (Fig. 2D), while the remaining four sites remained unaffected. Apoplastic fluid containing active PLCPs, as well as individual proteases expressed in *N. benthamiana* were co-incubated with PROZIP1mut$^{CS/CS2}$. Unlike the native propeptide, PROZIP1mut^{CS} was not processed upon PLCP treatment, which indicates that the mutated sites are required for PLCP-induced cleavage. For PROZIP1mut^{CS2}, the α-HA immunoblot showed PLCP-dependent processing (Fig. 2C, Fig. S5B), reflecting that this mutant version carries four of the six predicted cleavage sites.

To test if the *in vitro* processed PROZIP1 releases biologically active forms of Zip1, a large-scale cleavage assay with subsequent extraction of peptides of a molecular weight <10kDa was performed. Naïve plants were infiltrated with these peptide fractions of PROZIP1 treated with active proteases or E-64-inhibited proteases as negative control. Subsequent qRT-PCR revealed a significant upregulation of *PR*-genes triggered by PROZIP1 peptide fractions that were incubated with PLCPs (Fig 3A). This induction of *PR*-genes was not observed when PLCPs were inhibited with E-64 prior to co-incubation with PROZIP1, demonstrating a PLCP-dependent release of active Zip1 (Fig 3A). In addition, co-incubation of both PROZIP1mut^{CS} and PROZIP1mut^{CS2} with active PLCPs did not result in release of peptides inducing significant *PR*-gene expression. This confirms that i) the RR/FR motifs in PROZIP1 are crucial for the release of the signalling peptide Zip1, ii) PROZIP1 contains no additional *PR*-gene activating peptides besides Zip1, and iii) the activity observed is most likely not caused by small residual amounts of SA itself (Fig 3A).

Zip1 activates maize PLCPs

To further characterize downstream responses triggered by Zip1, we tested the rapid production of reactive oxygen species (ROS), a typical immune response induced upon perception of PAMPs or damage-associated molecular patterns (DAMPs), such as elf18, flg22, chitin or

AtPEP1 [32-35]. For this, maize leaf discs were treated with 5 µM Zip1. While both 1 µM chitin and 1 µM flg22 elicited typical PAMP-induced ROS bursts, Zip1 treatment did not cause detectable production of ROS (Fig. S6). Next, phosphorylation of maize MAP-kinases was tested by western blotting. However, in contrast to chitin and flg22, Zip1 did not cause any phosphorylation detectable with an α-Phospho p44/p42 antibody (Fig. S6B). Thus, in the context of rapid ROS production and MAPK phosphorylation, Zip1 lacks common overlapping PTI responses in maize.

We previously demonstrated the reciprocal activation of PLCPs and SA signalling in maize [23]. To explore the potential direct influence of Zip1 on PLCPs, ABPP assays were performed on apoplastic extracts from maize leaves 24h after treatment with SA, Zip1 or $Zip1_{mut}$, respectively. While an ABPP of $ZIP1_{mut}$-treated samples showed only weak PLCP activity compared to mock samples, Zip1 treated leaves displayed strong induction of apoplastic PLCP activity, which is similar to samples that were infiltrated with SA (Fig. 3B). A possible explanation for this result could be an exosite activation of PLCPs by direct interaction with the Zip1 peptide [36]. To test if PLCPs are directly activated by the Zip1 peptide, leaf extracts of SA- and mock- treated leaves were incubated with Zip1 and subsequently labelled with DCG-04. Co-incubation with Zip1 *in vitro* did not result in elevated DCG-04 labelling (Fig. 3B) which suggests an indirect Zip1-mediated PLCP activation via a so far unknown signalling cascade. Our results point towards a positive feedback loop in which Zip1 is released from PROZIP1 by SA-activated PLCPs and, in turn, induces the activity of these proteases.

Zip1 is a functional elicitor of SA signalling

Zip1 is an endogenous maize peptide that induces transcriptional activation of SA marker genes. This finding raises the question, whether Zip1 ultimately has a direct influence on SA levels in maize. To this end, SA contents were determined by LC/MS/MS measurements of maize leaves treated with Zip1. Mock-treated tissue, as well as $Zip1_{mut}$ served as controls. (Fig.

4A). SA levels were significantly elevated in Zip1-treated samples compared to both mock-treated samples and the $Zip1_{mut}$ controls, demonstrating a specific accumulation of SA upon treatment with the Zip1 peptide (Fig. 4A).

Our observation that Zip1 elicits SA accumulation suggests that its perception also causes a much larger transcriptional response beyond the induction of *PR*-genes. We therefore performed whole transcriptome analyses using Illumina-RNA-Sequencing (RNAseq), which revealed 2713 differentially regulated maize genes in response to SA, compared to mock-treated leaf samples at 24 hours after treatment. Zip1 treatment resulted in 2980 differentially regulated genes compared to mock treatment (Table S1). Remarkably, only 56 genes showed significant differential expression between SA and Zip1 treatments. A comparison of Zip1/SA induced genes to the mock-treated control revealed that 21% of the differentially regulated genes are exclusively induced in either SA or Zip1 treated samples, respectively (Fig. 4B). Eighty-nine percent of the top-300 upregulated genes are shared between SA and Zip1 treatment. Similarly, 86% of the top-50 downregulated genes are shared amongst both samples. This surprising and extensive overlap in transcriptional responses induced by both signals demonstrates that Zip1 strongly promotes SA-triggered defence responses in maize. The observed induction of SA accumulation in response to Zip1 (Fig. 4A) is reflected by the transcriptional induction of predicted maize SA biosynthesis key genes *ZmPAL1* (*Phenylalanine Ammonia-Lyase1*) and *ZmPAL4 (*Table S2). GO enrichment analyses of biological processes (BP) further substantiate these findings. Nitrogen metabolic processes and DNA synthesis, as well as genes associated with translation are downregulated by both Zip1 and SA. BPs upregulated by Zip1 and SA treatment include mainly defence responses ranging from response to fungi, bacteria and biotic stress to cell wall organization and biogenesis (Fig. 4C).

As a confirmation of the RNAseq results, *PR*-genes analysed by qPCR for the characterization of Zip1 responses (Fig. 1B) were also predictably up-regulated in both Zip1 and SA treatments (Table S2). Most of the SA and Zip1-upregulated transcripts encode for defence genes including

catalytic and stress protective enzymes like chitinases, β-1,3-glucanases, peroxidases, heat-shock proteins, glutathione S-transferases (GSTs) and other well-known SA markers. In addition, several uncharacterized maize WRKY transcription factors are induced upon SA and Zip1 treatment, whereas two of these are uniquely up-regulated in Zip1-treated samples (Table S2). In summary, RNAseq analyses reveal numerous responses downstream of Zip1, an apoplastic signal that specifically induces SA-dependent gene expression in maize (Fig. 4 and S7). Moreover, Zip1 may also influence ZmPep-mediated defence responses as the ZmPep receptor, Zm*PEPR1* as well as its potential co-receptor *ZmBAK1* are upregulated by Zip1 (Fig. S7) [37,38].

Given that Zip1 activates SA signalling, we hypothesized that Zip1 may trigger overall maize immune responses similar to SA. We therefore pre-treated maize leaves with SA, Zip1, $Zip1_{mut}$ or mock before subsequent infection with the fungal necrotroph *Botrytis cinerea*. Necrotic lesions caused by *B. cinerea* were quantified 4 days after infection to determine the impact of Zip1 as well as SA. SA pre-treated leaves showed about 2.5-fold increase in necrotic lesion area compared to buffer treated control plants (Fig. 5A). Strikingly, the lesion size of Zip1 treated leaves displayed a 4-fold increase compared to mock treatments, while $Zip1_{mut}$ challenged leaves did not show an elevated susceptibility to *B. cinerea* compared to mock controls (Fig. 5A). Complementary to an increased susceptibility towards a necrotroph, the proposed function of Zip1 suggests a negative impact on biotrophic interactions. This was tested via the recently established “Trojan horse” (TH) strategy, which deploys recombinant *U. maydis* strains to deliver bioactive plant peptides into the maize apoplast (van der Linde et al., revised). Strikingly, infection of a *U. maydis* mutant expressing secreted Zip1 during infection resulted in a strongly reduced virulence (Fig 5B), as well as elevated expression of *PR*-genes (Fig 5C). Together, these experiments demonstrate that Zip1 activity closely mirrors SA signalling and predictably promotes disease caused by necrotrophic and biotrophic fungi [10].

Discussion

The activation and re-localization of plant proteases during pathogen attack has been observed in a wide variety of plant species [6]. We previously demonstrated that apoplastic PLCPs can activate SA-mediated defence signalling in maize and inhibition of these proteases is a crucial step in suppressing immunity and enabling successful infection by biotrophic fungi [23,24]. Within this framework, we proposed two mechanistic scenarios for PLCP action, (a) proteolytic shedding of extracellular receptor domains [39,40], and (b) activation of peptide hormone signalling by proteolysis of a precursor peptide [5,41,42]. Our current work provides strong support for the second hypothesis, namely SA-induced PLCPs activate the production of peptide signals that further amplify SA production and SA-associated defence responses. Specifically, we identified Zip1 as a signalling peptide mediating SA-dependent immunity, which is released by SA activated PLCPs and, in turn, results in a positive feedback loop amplifying SA-related defence responses in maize (Fig. 5B). It was previously shown that exogenously applied SA mediates activation of five apoplastic PLCPs. Upon activation PLCPs promote SA-dependent *PR*-gene expression when infiltrated into naïve plants [23]. Through PROZIP1 cleavage studies, we demonstrate that the mixture of apoplastic PLCPs, as well as active form of two recombinant apoplastic PLCPs, namely CP1 and CP2, cleave the propeptide PROZIP1. This event releases bioactive peptides that act as signals to induce SA-associated defence responses which include the reciprocal activation of PLCP activity similar to action of free SA. Using mass spectrometry we were able to detect the 17aa Zip1 peptide as biologically active component in apoplastic fluids of maize leaves. Biological assays however indicated that also a 20aa Zip1 version with three additional N-terminal residues has similar biological activity. This indicates variability of the Zip1 N-terminus, which might result from secondary cleavage by yet unknown proteases. The role of Zip1 in signal amplification explains why apoplastic maize PLCPs are important effector targets. The previously characterized *U. maydis* effector Pit2, as well as the endogenous JA-induced protein ZmCC9 are secreted to the apoplast to establish biotrophic interactions by

blocking apoplastic PLCPs. Thereby the immune response amplifier Zip1 cannot be released from the PROZIP1 precursor protein. In turn, reduced levels of Zip1 impair further SA production and ultimately SA-mediated immunity is dampened [24]. Future work will aim to specify the exact cleavage process of PROZIP1 by generating several cleavage site mutants and test them in cleavage assays with maize PLCPs. Recently, substrate specificity for two PLCPs of *Nicotiana benthamiana* (NbCysP6, NbCysP7) was analysed in detail [43]. For NbCysP6, which is closely related to maize CP1 a substrate preference for P2-position was identified (L,V or F). While this is in agreement with the predicted N-terminal cleavage site of Zip1, the C-terminal cleavage site (R104 of PROZIP1) is rather unexpected. One possible explanation for this would be that additional plant proteases (e.g. subtilases), which might be activated by the PLCPs, are also involved in the release of the Zip1 peptide.

How precisely Zip1 promotes SA production remains unknown. In the context of pathway regulation, the majority of pathogen-induced SA is synthesized from isochorismate produced by isochorismate synthase (ICS) and partially from cinnamate produced by phenylalanine lyase (PAL) [44]. In line with this is a previous finding that *U. maydis* secretes a chorismate mutase (Cmu1) into maize cells where it re-channels metabolism to lower the substrate availability for SA synthesis [45]. Activity of Cmu1 might also be the reason for a non-complete loss-of-virulence of Zip1-expressing *U. maydis* strain. A possible scenario would be that Cmu1 activity counteracts the Zip1-induced SA-accumulation allowing a residual level of infection.

RNAseq analyses revealed the transcriptional induction of two genes encoding for *ZmPAL1* and *ZmPAL4* by Zip1 (Table S1,S2). Additionally, *ZmPEPR1*, a component of peptide induced immune amplification and its potential co-receptor *ZmBAK1* are upregulated by SA as well as Zip1 (Table S1, S2) [37]. In contrast to Pep/PEPR signal amplification, Zip1 not only promotes strong SA signalling but downregulates the expression of an essential enzyme involved in maize JA biosynthesis, namely lipoxygenase 8/tassel seed 1 (Table S1) [46]. In the context of candidate biochemical defences, a terpene synthase homolog, *ZmTPS21*, is exclusively induced by Zip1

(Table S1). Related terpene synthases in maize, such as ZmTps6/11 are β-macrocarpene synthases predictably responsible for the production of antifungal phytoalexins, termed zealexins [47]. Silencing of *ZmTps6/11* promotes increased susceptibility towards *U. maydis* supporting a role in biochemical immunity [48]. Additionally, two WRKY transcription factors are induced by Zip1 that might be involved in immune signalling (Table S2).

Collectively, we have identified a peptide, termed Zip1, which activates salicylic acid mediated defenses. Given that SA-dependent immune signalling is a conserved mechanism in plants, it is surprising that Zip1 has little or no sequence homologs in other plant species.

We speculate that a widely conserved Zip1 sequence in plants would create an accessible evolutionary target for necrotrophic pathogen effectors and manipulation. Importance of Zip1 for induction of pathogen induced immunity might also be reflected by an additional copy of the *PROZIP1* gene on maize chromosome 8 (GRMZM2G140153; *PROZIP2*), carrying a single conservative amino acid difference in the coding region (PROZIP1 Ala100 to Val; Fig S7). Presence of an expressed backup copy on a different chromosome further supports the functional importance of Zip1 (Fig S7). Given this potential “Achilles heel” be used by necrotrophs to promote susceptibility, Zip1 function rather than sequence may be conserved as it has been shown for tomato systemin and hydroxyproline-rich glycopeptide systemins (HypSys) [49-51]. Sytemin and HypSys do not share sequence similarities but are both involved in JA-dependent signalling against herbivorous and pathogen attack including systemic synthesis of protease inhibitors and defensins [50,52]. Similar to the systemin-related peptides, additional research is required to determine how Zip1 is perceived by plant cells and to elucidate key signalling nodes responsible for Zip1-induced SA production. Collectively, our current study fills an important conceptual and mechanistic gap in the understanding of how plant apoplastic proteases promote SA signalling. Based on these findings, we are proposing a model on Zip1-mediated defenes signalling in maize (Fig 5D). In this scenario, an initial SA burst leads to the activation of apoplastic PLCPs, whichresults in processing of the precursor PROZIP1 to release

the Zip1 peptide signal acting as an amplifier of defense responses to further promote SA production. With predictably important roles in balancing effective defences against biotrophs with susceptibility to necrotrophs, endogenous peptide signals that amplify SA-responses are likely to await discovery in numerous plants. The current discovery of Zip1 provides an important conceptual example of the previously missing intermediate signal that links the activation of apoplastic PLCPs to amplified SA signalling and ultimately inducible plant immune responses.

Materials and Methods

Plant treatments

For all experiments maize plants (*Zea mays cv* Early Golden Bantam) were grown in a walk-in Phytochamber at 28°C during a light period of 12h with one hour of twilight, and 22°C during a dark period of 11 h. For each experiment the 2nd and 4th leaf of 10-14 days old plants were taken for analyses. Plants were syringe infiltrated with 2 mM salicylic acid or mock (0.1% of EtOH in H_2O). Treated leaf areas were excised 24 h after treatment and apoplastic fluid was collected from leaves through centrifugation. Protein content was adjusted to 4.5 mg ml^{-1}. For subsequent qRT-PCR analyses, SA treated leaf tissue was collected 3-4 cm distant from site of infiltration. Individual peptides were synthesized by Genscript Biotech Incorporation (Nanjing, China) and dissolved in H_2O. Leaf infiltration treatments were performed using a blunt needless syringe. Briefly the 2nd and 4th leaves of 1-2 week old plants were infiltrated with either mock solution or 5 µM peptide solutions at the base of the leaf and harvested 24 h later. Twelve leaves were pooled per sample and treatment for each of five independent biological replicates.

Identification of *Z. mays* immune signaling peptides and protein precursors

To identify maize peptide signal candidates by mass spectrometry, leaf apolastic fluid of SA or mock treated plants was extracted. Peptide fractions were enriched by filtration using a 10 kDa

Amicon Centrifugation Filter (EMD Millipore, Darmstadt, Germany) and the application of 5 ml samples of apoplastic fluid, corresponding to 4.5 mg total protein. The <10kDa apoplastic fraction was adjusted to a final concentration of 0.5% formic acid (FA) and 5% acetonitrile (ACN). The acidified peptide solution was passed in 150 µL steps over pre-equilibrated C18 spin columns. Next, the columns were washed with 4× 0.5% FA, 5% ACN to remove excess salts. Finally the bound peptides were eluted with 2× 50 µL 0.1% FA, 70% ACN and concentrated until <5 µL liquid remained. The resulting volume was then adjusted to 20 µL by adding 0.1% FA. LC-MS/MS-experiments were performed on a Thermo LTQ Velos mass spectrometer coupled to a Proxeon EASY-nLC. Peptides were separated on a single reverse phase C18 column (inner diameter 75 mm, packed with 12-cm ReproSil- Pur C18-AQ [3 µm]) using an acetonitrile gradient (120 min 5 to 80%; 20 min 80%), at a flow rate of 300 nl min^{-1}. Peptides were fragmented by collision-induced decay in a data-dependent fashion, fragmenting the 20 most intense multiply charged precursors in each MS scan. MS^2 spectra data were searched using the MASCOT algorithm (version 2.3.02) first against a database of known contaminants (as incorporated in MASCOT) followed by searching against the maize sequences from the database ZmB73_5b_FGS_translations_20110205.fasta (www.maizesequence.org/index.html).

Expression and purification of PROZIP1/PROZIP1mut^{CS}/ PROZIP1mut^{CS2}

For heterologous protein expression followed by purification, PROZIP1 was amplified from Early Golden Bantam cDNA using oligonucleotides PROZIP1-f and PROZIP1-r (see Table S3). Putative cleavage sites were substituted to alanine *in silico* and resulting gene was synthesized by Genscript Biotech Incorporation (Nanjing, China). The PROZIP1/ PROZIP1mut^{CS}/PROZIP1mut^{CS2} proteins were purified via glutathione resin and cleavage of GST-tag was performed as described previously [24]. Further purification of PROZIP1/PROZIP1mut^{CS} was achieved by gel filtration chromatography on an ÄKTA sytem

(GE Healthcare Life Science, Buckinghamshire Great Britain) using a Superdex 75 16/600 column equilibrated with storage buffer containing 300 mM NaCl, 100 mM Tris-HCl, pH 8.5.

Protease activity assays, ABPP and protease cleavage assays

To analyze the activity of different cysteine protease, apoplastic fluid from SA treated plants was extracted as described previously [23] in the presence or absence of E-64 (Sigma-Aldrich, St. Louis, MO, USA) using 10 µM of the following substrates: Z-Phe-Arg-7-amido-4-methylcoumarin (AMC), Z-Arg-Arg-AMC, Boc-Gln-Ala-Arg-AMC, N-Succinyl-Leu-Leu-Val-Tyr-AMC (Sigma-Aldrich, St. Louis, MO, USA). For activity based protein profiling, leaf tissue treated with either Zip1 or SA was used for total protein extraction in H_2O + 1 mM DTT. Protein concentration was adjusted to 0.2 mg ml^{-1} with 15 mM sodium phosphate buffer, pH 6.0, 0.2 mM DTT and pre-incubated with 5 µM E-64 or control buffer in a total volume of 200 µL for 30 min at room temperature prior to the addition of 0.2 µL of 2 mM DCG-04. After incubation for 3 h at room temperature, proteins were precipitated with acetone and resolved in 2x Laemmli loading buffer. 15 µL of dissolved proteins were subjected to SDS-PAGE. Immunoblotting and detection of DCG-04 labeled proteins was performed as described in previously [23]. Biotinylated proteins were detected by strep-HRP (1:3000) (Sigma-Aldrich, St. Louis, MO, USA).

For the *in vitro* cleavage assays 5 µM of purified PROZIP1/PROZIP1mut^{CS}/PROZIP1mut^{CS2} protein was either incubated with apoplastic fluid from SA treated maize leaves containing active PLCPs, or with apoplastic fluid from *N. benthamiana* leaves transiently expressing individual proteases CP1, CP2, XCP2 or CatB according to [24].

Data availability

Mass spectrometry and RNA sequencing data availability. Raw read sequences have been deposited in the Sequence Read Archive (SRA) under the BioProject ID PRJNA379074

(https://www.ncbi.nlm.nih.gov/bioproject/PRJNA379074). Data can be accessed under the following collaborator link :

Study SRP101910: RNA-seq of Zea mays treated with SA, Zip1 or mock: ftp://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP101910_20170711_152605_b1659515b9d1a59ebbc790e01084a8f0

The detailed experimental protocols and methods applied in this study can be found in the *Supplementary information.*

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Authors contribution

S.Z, K.L., and G.D. designed the experiments and analysed the data. S.Z., K.L. and B.A. performed the functional analysis Zip1 / PROZIP1; N.H. and CZ designed and analysed ROS and MAPK assays; Y.D., A.H., and E.S. designed, performed and analysed salicylic acid

measurements; U.L. analysed the transcriptome data; F.K., T.C. and M.K. performed MS experiments and MS related data analysis; S.Z. and G.D. wrote the manuscript with input from all authors.

Competing financial interests.

No financial interests are declared.

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Figure Legends

Fig. 1. Induction of SA-associated *PR*-gene expression by apoplastic peptide fraction as well as by Zip1 [A] qRT-PCR analyses of maize leaves treated with apoplastic peptide fractions from SA-treated leave samples show induction of SA-associated *PR*-gene expression (*PR3*, *PR4* and *PR5*; black bars) compared to peptides of mock treated samples (grey bars). *CC9* as a control for JA-marker genes is not induced. [B] Maize leaves were treated with 5 µM Zip1 (dark grey) and 5 µM Zip1mut (light grey) as well as with 2 mM SA (black). Peptide treatment and subsequent qRT-PCR analyses reveals Zip1 to be capable to induce SA-associated *PR*-gene expression in maize leaves 24 hours after treatment. Charged N-terminal amino acids (red) are essential to maintain biological activity of Zip1 as Zip1mut is not inducing *PR*-gene expression. Experiments shown in this figure were done in five independent biological replicates with two technical replicates in each measurement; error bars represent SEM; *p*-values were calculated by an unpaired *t*-test. *$P<0.05$; **$P<0.005$; ***$P<0.0005$

Fig. 2. Active PLCPs are required for processing of PROZIP1. [A] Heterologously expressed PROZIP1 (5 µM) was co-incubated with AF of SA-treated maize leaves containing active PLCPs. 0, 5 and 15 min timepoints were analysed using α-HA western blot. Activity of PLCPs was monitored by ABPP using DCG-04, a specific probe for the detection of active PLCPs. PLCPs efficiently process PROZIP1 over time, which can be inhibited by E-64. PROZIP1mut^{CS} with putative cleavage sites mutated is not cleaved anymore. [B] Individual PLCPs were heterologously expressed in *N. benthamiana* via *A. tumefaciens*-mediated transformation. Activity of CP1, CP2, CatB and XCP2 was normalized and examined by ABPP using MV-202 as fluorescent probe. [C] PROZIP1, PROZIP1mut^{CS} as well as PROZIP1mut^{CS2} carrying an N-terminal HA epitope were tested in *in vitro* cleavage assays with individual proteases. α-HA

immunoblotting shows that CP2 and CP1, but not CatB and XCP2 are responsible for PROZIP1 cleavage. PROZIP1^{mutCS} with all RR motifs mutated is not processed whereas PROZIP1mut^{CS2} is cleaved although slightly less than wild type PROZIP1. [D] Alignment of PROZIP1 and PROZIP1 variants that were generated in this study. In PROZIP1mut^{CS}/PROZIP1mut^{CS2} different sets of putative cleavage sites (red) were substituted by Alanine (blue). Zip1 is highlighted in green.

Fig. 3. *In vitro* released Zip1 is active *in vivo.* [A] PROZIP (10 µM), PROZIP1mut^{CS} (10 µM) and PROZIP1mut^{CS2} (10µM) were co-incubated with AF fractions containing active PLCPs monitored by ABPP. Subsequently peptide fractions were separated from protein fractions. Maize leaves were treated with each fraction, respectively. 24 hpi qRT-PCR analyses show a significant induction of *PR*-gene expression with peptide fractions of PROZIP1 cleavage reactions. This effect can be abolished by blocking PLCPs activity with E-64 prior to PROZIP1 incubation. PROZIP1mut^{CS} and PROZIP1mut^{CS2} peptide fractions do not induce a significant SA-associated defense gene expression. Protein fractions of all PROZIP cleavage reactions do not induce *PR*-gene expression. The experiments were done in three independent biological replicates; error bars represent SEM; *P*-values were calculated by an unpaired t-test. *$P<0.05$; **$P<0.005$. [B] Zip1 induces PLCP activity. Maize leaves were treated with 5 µM Zip1 and Zip1mut as well as 2 mM SA. 24 hpi PLCP activity was monitored via APBB using DCG-04 probe. Zip1 induces the activation of PLCPs same as SA does (left panel). To ascertain if Zip1 induces PLCP activation by direct interaction, leaf extract of treated plants was co-incubated with Zip1 before ABPP showing no activation of PLCPs by direct interaction with Zip1 (right panel).

Fig. 4. Zip1 induced accumulation of SA in maize leaves and RNA-sequencing analyses of Zip1 and SA treated maize leaves. [A] Maize leaves were treated with 5 µM Zip1 and

Zip1mut. 24 hpi total free SA was measured in mock, Zip1mut and Zip1 treated samples using LC/MS-MS. Zip1 causes a 20-fold accumulation of SA compared to mock. SA induction induced by Zip1 is statistically significant compared to Zip1mut. [B] To identify additional responses mediated by Zip1 whole transcriptome analyses was performed at 24 h using RNAseq. The up- and downregulated genes in SA and Zip1 (compared to mock control) were compared against each other. For this, we took the strongest differentially regulated genes above/below a logFC threshold of ~ +/- 1.6. 266 (89%) of the 300 strongest upregulated genes in SA are also upregulated in Zip1 and 43 (86%) of the 50 strongest downregulated genes in SA are also downregulated in Zip1. *Vice versa*, 268 (89%) of the 300 strongest upregulated genes in Zip1 are also upregulated in SA and 36 (72%) of the 50 strongest downregulated genes in Zip1 are also downregulated in SA. For all comparisons a significant threshold (adj.P) of <0.05 was applied. [C] Differential gene expression of GO-term categories between Zip1/Mock and SA/Mock was calculated with R/DESeq2. With all genes differentially regulated under an FDR-adjusted significance cutoff level of 0.05, parametric analysis of gene set enrichment (PAGE) was applied with agriGO, *Zea mays* AGPv3.30 and the complete GO list. Gene ontologies important in immune response signalling were manually selected and the corresponding Z-score from the PAGE analysis was visualized in a heatmap. Asterisks (*) denote values with an adj. *P* ≥ 0.05.

Fig. 5. Zip1 confers increased susceptibility of maize towards the necrotrophic pathogen *Botrytis* cinerea but mitigates infection by the biotrophic fungus *Ustilago maydis*. [A] Maize leaves were pre-treated with 5 µM Zip1mut or Zip1 and 2 mM SA, respectivley. 24 hpi pre-treated leaves were detached and infected with 10 µL droplets of *B. cinerea* spore solution containing $1x10^6$ spores mL^{-1}. In line with SA measurements Zip1 pre-treatment causes higher susceptibility to *B. cinerea.* [B] Maize seedling were infected with biotrophic *Ustilago maydis* wildtype strain (SG200) and a *U. maydis* mutant that expresses secreted Zip1. *U. maydis* Zip1

expressing strain shows strongly reduced tumor formation at 12 dpi in three independent biological replicates. n=number of plants infected. *P*-values were calculated by an unpaired t-test. *$P<0.05$. [C] qRT-PCR of *U. maydis* infected maize leaves proves that Zip1 secretion by *U.maydis* induces the expression of SA-associated *PR*-genes *PR3 and PR5* at 2 dpi. The experiments were done in three independent biological replicates; error bars represent SEM; *P*-values were calculated by an unpaired t-test. *$P<0.05$. [D] Model of Zip1-mediated defense signalling in maize. Upon infection biotrophic pathogens such as *U. maydis* trigger JA-associated defense responses by so far unknown mechanisms. By that, maize endogenous CC9 as well as the *U. maydis* effector protein Pit2 are induced to inhibit PLCP activity. Likewise, SA signalling is directly suppressed by Cmu1, an effector protein that suppresses SA synthesis. In contrast, induced SA signalling leads to the activation of PLCPs. Thus, PROZIP1 is processed by CP1 and CP2 which releases active Zip1. Zip1 signalling induces several SA-associated downstream signalling events and PLCP activation. Together with Zip1-induced accumulation of SA, the newly discovered peptide Zip1 amplifies SA-associated defense responses.