A rust fungus effector directly binds plant pre-mRNA splice site to reprogram alternative splicing and suppress host immunity

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Introduction

Alternative splicing (AS) is a crucial post-transcriptional regulatory mechanism in plant resistance. However, whether and how plant pathogens target splicing in their host remains mostly unknown. For example, although infection by *Puccinia striformis* f. sp. *tritici* (Pst), a pathogenic fungus that severely affects the yield of wheat worldwide, has been shown to significantly influence the levels of alternatively spliced transcripts in the host, the mechanisms that govern this process, and its functional consequence have not been examined. Here, we identified Pst_A23 as a new Pst arginine-rich effector that localizes to host nuclear speckles, nuclear regions enriched in splicing factors. We demonstrated that transient expression of Pst_A23 suppresses plant basal defence dependent on the Pst_A23 nuclear speckle localization and that this protein plays an important role in virulence, stable silencing of which improves wheat stripe rust resistance. Remarkably, RNA-Seq data revealed that AS patterns of 588 wheat genes are altered in Pst_A23-overexpressing lines compared to control plants. To further examine the direct relationship between Pst_A23 and AS, we confirmed direct binding between two RNA motifs predicted from these altered splicing sites and Pst_A23 in vitro. The two RNA motifs we chose occur in the cis-element of TaXa21-H and TaWRKY53, and we validated that Pst_A23 overexpression results in decreased functional transcripts of TaXa21-H and TaWRKY53 while silencing of TaXa21-H and TaWRKY53 impairs wheat resistance to Pst. Overall, this represents formal evidence that plant pathogens produce ‘splicing’ effectors, which regulate host pre-mRNA splicing by direct engagement of the splicing sites, thereby interfering with host immunity.

Keywords: *Puccinia striformis* f. sp. *tritici* (Pst), alternative splicing, effector, plant immunity.

Summary

Alternative splicing (AS) is a crucial post-transcriptional regulatory mechanism in plant resistance. However, whether and how plant pathogens target splicing in their host remains mostly unknown. For example, although infection by *Puccinia striformis* f. sp. *tritici* (Pst), a pathogenic fungus that severely affects the yield of wheat worldwide, has been shown to significantly influence the levels of alternatively spliced transcripts in the host, the mechanisms that govern this process, and its functional consequence have not been examined. Here, we identified Pst_A23 as a new Pst arginine-rich effector that localizes to host nuclear speckles, nuclear regions enriched in splicing factors. We demonstrated that transient expression of Pst_A23 suppresses plant basal defence dependent on the Pst_A23 nuclear speckle localization and that this protein plays an important role in virulence, stable silencing of which improves wheat stripe rust resistance. Remarkably, RNA-Seq data revealed that AS patterns of 588 wheat genes are altered in Pst_A23-overexpressing lines compared to control plants. To further examine the direct relationship between Pst_A23 and AS, we confirmed direct binding between two RNA motifs predicted from these altered splicing sites and Pst_A23 in vitro. The two RNA motifs we chose occur in the cis-element of TaXa21-H and TaWRKY53, and we validated that Pst_A23 overexpression results in decreased functional transcripts of TaXa21-H and TaWRKY53 while silencing of TaXa21-H and TaWRKY53 impairs wheat resistance to Pst. Overall, this represents formal evidence that plant pathogens produce ‘splicing’ effectors, which regulate host pre-mRNA splicing by direct engagement of the splicing sites, thereby interfering with host immunity.

Howard et al., 2013; Huang et al., 2020; Ling et al., 2015; Mandadi and Scholthof, 2015). AS is an efficient mechanism to rapidly and precisely tune plant immunity by ensuring the expression of certain transcripts, such as proteins required for the pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) (Sanabria and Dubery, 2016; Zhang et al., 2014), and many resistance R proteins, which recognize pathogen Avirulence proteins (Avr) (Yang et al., 2014). Given the essential role of AS regulation in plant immunity, pathogens have evolved effectors to reprogram host AS by engaging the spliceosome (Fu et al., 2007; Huang et al., 2017, 2020; Nicaise et al., 2013). However, despite the prevalence, many mechanisms that pathogens use to hijack host AS remain unknown.

One such pathogen is *Puccinia striformis* f. sp. *tritici* (Pst), a fungus that severely threatens the yield of wheat worldwide. During Pst infection, 10.4% of the wheat multixen genes had AS transcripts (Zhang et al., 2019), but the molecular mechanism by which Pst interfere with host RNA splicing to suppress immunity has not been elucidated. Here, we identified a putative effector, Pst_A23, from the genome of Pst CYR32 (Zhang et al., 2013). Transient expression of Pst_A23 inhibited basal defence triggered by PAMP flg22 and bacteria *Pseudomonas syringae*. Stable overexpression of Pst_A23 significantly promoted Pst pathogenicity, and knockdown of Pst_A23 resulted in host resistance.

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Significantly, we obtained evidence that Pst_A23 is an arginine-rich RNA-binding protein that accumulates in plant nuclear speckles to bind specific RNA motifs in splice sites and impacts the AS. Our analysis of TaXa21-H and TaWRKY53 splicing demonstrated that Pst_A23 alters product ratios of TaXa21-H and TaWRKY53 and that silencing of TaXa21-H and TaWRKY53 severely weakens plant resistance to Pst. Overall, our results show that Pst_A23 acts as a splicing regulator that directly binds the cis-elements of host genes, ultimately resulting in a reduction of the plant defence response.

Results

Pst_A23 localizes in host nuclear speckles dependent on the C-terminal Arg-rich domain

Pst_A23 was predicted to encode a small, secreted, nuclear-localized effector based on the analysis of the genome and secretome of the Pst CYR32 (Zheng et al., 2013). The abundance of Pst_A23 mRNA in wheat plants increased to a peak at 24 h post-inoculation (hpi) with Pst ureidiospores and thereafter declined (Figure S1), indicating that Pst_A23 likely plays a role at the early infection stage. To investigate whether this protein is indeed secreted, the putative signal peptide (Pst_A23SP) was introduced into the yeast signal sequence trap vector, pSUC2t7-M13ori, and transformed into a yeast strain, YTK12, lacking a secreted invertase (Jacobs et al., 1997). The transformants were streaked on CMD-W and YPRAA plates, on which only yeast cells that secrete invertase can grow. The authentic signal peptide of the Avr1b effector of Phytophthora sojae was used as a positive control and the empty vector (EV) as a negative control (Figure S2a). Invertase activity was detected by the reduction of 2,3,5-triphenyltetrazolium chloride. Yeast transformed with the vector encoding either Pst_A23SP or Avr1bSP had high invertase activity, whereas the negative control did not (Figure S2b). To confirm the function of the signal peptide in plants, Pst_A23SP was cloned into pCAMBIA1302 and transiently expressed as a fusion protein with GFP (Pst_A23SP-GFP) in Nicotiana benthamiana leaves. The signal peptide of the wheat protein TaPR1 was used as a positive control (TaPR1SP-GFP) and GFP lacking a signal peptide (GFP) was used as a negative control for secretion. After plasmolysis, the secreted GFP signal of Pst_A23SP-GFP or TaPR1SP-GFP was diffuse in the extracellular space, whereas the negative control GFP was seen mainly in the plant protoplast (Figure S2c). These two assays demonstrate that the signal peptide of Pst_A23 is sufficient to drive the secretion of a fusion protein.

We investigated the subcellular localization of Pst_A23 without signal peptide by expressing it as a fusion protein with GFP (Pst_A23-GFP) in N. benthamiana leaves. The protein contains one nuclear localization sequence, therefore, we expected to find Pst_A23-GFP in the nucleus. Rather than appearing throughout the nucleoplasm, however, the fluorescent signal accumulated in irregular spots in the plant cell nuclei (Figure 1a). We also transiently expressed the fusion protein Pst_A23:GFP in wheat protoplasts, in which the fluorescent fusion proteins localized in irregular spots in nuclei as observed in N. benthamiana (Figure 1b). To see whether these spots reflected Pst_A23 localization in nuclear speckles, we expressed Pst_A23:GFP with the Arabidopsis protein AtSR45, a specific marker of nuclear speckles (Li et al., 2016), fused to RFP (AtSR45:RFP). The results showed that Pst_A23:GFP and AtSR45:RFP co-localized in nuclear speckles (Figure 1c). The Pst_A23:GFP and AtSR45:RFP fusion proteins in N. benthamiana leaves were detected by Western blotting with antibodies against GFP and RFP, indicating that they were stably expressed (Figure S3a). Pst_A23 has two C-terminal Arg-rich sequences, R1-rich domain (138–153) and R2-rich domain (165–181) (Figure 1d). To investigate whether these domains influence Pst_A23 localization, we replaced the R residues in either the R1-rich domain, the R2-rich domain, or both with Ala (Figure 1e). Our results obtained using these mutant proteins indicate that the R1-rich domain is dispensable, whereas the R2-rich domain is required for localization at nuclear speckles (Figures 1e and S3b). We conclude that the R2-rich domain is necessary for the nuclear speckle localization of Pst_A23. Taken together, our evidence thus far supports the role of Pst_A23 as a secreted Pst molecule that localizes to the nuclear speckles in the host’s cells.

Pst_A23 suppresses plant basal defence responses

To examine the effects of Pst_A23 further, we used inhibition of Bax-induced programmed cell death (PCD), an established assay for validating virulence factors and their effect on plant defence reaction (Wang et al., 2011). We expressed the fungal protein in N. benthamiana leaves for 24 h and then infiltrated the leaves with Agrobacterium expressing Bax protein. Similar to Avr1b, an oomycete avirulence effector used as the positive control (Dou et al., 2008), Pst_A23 suppressed PCD triggered by Bax, whereas eGFP, the negative control, did not (Figure S4a). Furthermore, we also examined whether Pst_A23 suppresses cell death induced by P. syringae DC3000 in wheat. The model pathogen, P. syringae DC3000 triggered visible cell death in wheat expressing dsRed, whereas when P. syringae DC3000 was infiltrated with Pst_A23, suppression of cell death was observed (Figure S4b). Taken together, these results indicate that Pst_A23 suppresses cell death induced by Bax or bacteria in plants, suggesting that this protein directly affects plant defence mechanisms.

To exploring the effects of Pst_A23 on plant defences further, Pst_A23:GFP and mutant R1R2A:GFP were transiently expressed in N. benthamiana and the basal defence response was induced by means of the conserved PAMP, Flg22 (Gomezgomez and Boller, 2000). Callose deposition, one of the basal defence responses, was observed by staining the leaves with aniline blue. Fewer callose deposits were seen in leaves expressing Pst_A23:GFP than in leaves expressing GFP alone or expressing R1R2A:GFP (Figure S5a,b). To assess more precisely the ability of Pst_A23 to weaken the Flg22-induced immune response, the expression of three basal defence marker genes was quantified using RT-qPCR. The expression level of NbrPr1a, NbrPr2, and NbrWRKY12 were 3-, 2-, 7-fold lower, respectively, in leaves expressing Pst_A23:GFP than in control leaves expressing GFP (Figure S5c), indicating that the fungal protein suppresses the basal defence response in this system.

To determine the effect of Pst_A23 on the basal defence response in wheat, we transiently expressed Pst_A23 and mutant R1R2A in wheat using the bacterial type III secretion system from the defective Pseudomonas fluorescens EThAn. Consistent with the experiments described above, we found fewer callose deposits in leaves delivered with Pst_A23 compared to that in leaves expressing dsRed, while expression of R1R2A mutant did not cause significant changes in callose deposition compared to the controls (Figure 2a,b). The expression level of TaPR1 and TaPR2 was reduced by 2.9- and 3.3-fold, respectively, in leaves expressing Pst_A23 than in control leaves expressing dsRed, but not in leaves expressing R1R2A (Figure 2c). These data show that Pst_A23 suppresses the plant basal defence response in the natural host of Pst.
Figure 1  Pst_A23 protein accumulates in plant nuclear speckles. (a) Pst_A23 localizes to the nucleus in N. benthamiana. The fusion protein Pst_A23:GFP and GFP alone were expressed in N. benthamiana, and the epidermis was observed 48 h post-inoculation with Agrobacterium using an Olympus BX-51 microscope. DAPI (4,6-diamidino-2-phenylindole) stained the plant nucleus. Scale bar = 20 µm. (b) Pst_A23:GFP was expressed in wheat protoplasts and localized in the nuclear speckles. Scale bar = 10 µm. (c) Pst_A23: GFP and AtSR45: RFP were co-expressed in N. benthamiana and localized to nuclear speckles. AtSR45: RFP was used as a marker for plant nuclear speckles. N, nucleus. Scale bar = 20 µm. (d) Schematic representation of three mutants of Pst_A23. The red lines indicate arginine (R) and the blue lines indicate lysine (K). Arginine in the R1-rich domain and R2-rich domain was replaced with alanine using a base substitution mutation. (e) The R2-rich domain is necessary for nuclear speckle localization of Pst_A23. Fusion proteins of mutants R1A: GFP, R2A:GFP, and R1R2A:GFP were transiently expressed in N. benthamiana to examine subnuclear localization. DAPI stained plant nuclei. Scale bar = 100 µm.

Pst_A23 contributes to the virulence of Pst

Since the suppressed basal defence in wheat leaves by transient expression of Pst_A23, we further examined whether Pst_A23 contributes to the virulence of Pst. Wheat cultivar Swon11 transiently expressing Pst_A23 and R1R2A mutant was further inoculated with Pst race CYR23, which is avirulent on the wheat cultivar Swon11, and the Pst development is evaluated. Histological observation revealed that transient expression of Pst_A23 resulted in significantly increased hyphae length and infection area of Pst at 48 hpi compared to that in the control leaves expressing dsRed, while no significant changes are observed in leaves expressing R1R2A mutant (Figure 2d–f). These data suggest that transient overexpression of Pst_A23 could promote...
Figure 2  Suppression of plant PTI and enhanced Pst virulence by Pst_A23 in wheat. (a) Suppression of callose deposition induced by bacteria in wheat transiently expressing Pst_A23 or dsRed alone using T3ss. Representative pictures were stained with 0.05% aniline blue and collected using CellSens Entry software 24 h after infiltration with EThAn carrying Pst_A23, R1R2A, and DsRed. Red arrows indicate the callose deposits. Bar = 100 µm. (b) The number of callose deposits was assessed using ImageJ (version 1.50i). DsRed was used as a negative control. Data are presented as mean with SEM from three biological replicates (n = 122). Asterisk indicates significant differences in callose deposition when compared to that in wheat leaves treated with EThAn carrying dsRed (**P < 0.01; ns, not significant using Student’s t-test). (c) RT-qPCR analysis of TaPR1 and TaPR2 expression level in dsRed, Pst_A23, and R1R2A mutant expressing wheat leaves at 24 h after infiltration with the indicated bacteria strains. Data are mean with SEM from three biologically independent samples. Statistical significance was determined by Student’s t-test. **P < 0.01; ns, not significant. (d) Pst development in wheat leaves transiently expressing dsRed, Pst_A23, and R1R2A mutant at 48 h post-infection with Pst CYR23 was detected by staining with wheat germ agglutinin conjugated to Alexa-488 and observed under epifluorescence microscopy. SV, substomatal vesicle; IH, infection hyphae; HMC, haustorial mother cell; H, haustorium. Bar = 20 µm. (e) and (f) Hyphal length and infection areas of Pst at 48 hpi in wheat leaves expressing dsRed, Pst_A23, and R1R2A mutant. Data are mean with SEM from three biological replicates (dsRed, n = 107; R1R2A, n = 122; Pst_A23, and n = 137). Statistical significance was determined by Student’s t-test. *P < 0.05; ns, not significant.
the virulence of Pst CYR23 on wheat cultivar Swon11, and the C-terminal arginine-rich region is essential for its virulence activity.

To further investigate the function of Pst_A23 in the pathogenicity of Pst, we generated transgenic wheat lines overexpressing Pst_A23 (Figure 3a). Flag-tagged Pst_A23 was detected by Western blotting with an antibody against Flag, suggesting the successful expression of Pst_A23 (Figure 3b). The second-generation Pst_A23-overexpressing lines (Pst_A23-OE# L5 and # L7) were inoculated with Pst race CYR31, which is virulent in the common wheat cultivar Fielder. When compared to the
Fielder, *Pst_A23*-overexpressing plants exhibited higher levels of urediniospore pustules, indicative of their increased susceptibility (Figure 3c). At 24 and 48 hpi, the fungal hyphae length and infection area are greater in *Pst_A23*-OE#L5 and #L7 than in wild-type Fielder (Figure 3d–f). RT-qPCR revealed decreased *TaPR1* and *TaPR2* expression levels in *Pst* infected *Pst_A23*-OE# L5 and # L7 at 24 hpi compared to that in WT plants (Figure 3g–h). Taken together, these data suggest that *Pst_A23* positively contributes to the virulence of *Pst*.

Host-induced silencing of *Pst_A23* enhances wheat resistance to *Pst*

Since the specific suppressive role of *Pst_A23* in plant defence and positive contribution to *Pst* virulence, we generated...
transgenic wheat plants expressing an RNAi fragment that is specific for \textit{Pst \_A23} silencing to examine its potential use in improving wheat resistance. Positive transgenic plants were detected by GUS reporter activity assay and using primers specific for the fragment of \textit{Pst \_A23} (Figures 4a,b). Upon inoculation with \textit{Pst} race CYR32, the third generation of transgenic plant lines \textit{Pst \_A23}-RNAi\#L2 and \#L10 showed significantly enhanced resistance with fewer urediniospore pustules (Figures 4c and S6). We observed that relative expression of \textit{Pst \_A23} in \textit{Pst \_A23}-RNAi plants is decreased by 80\%–90\% at 24, 48 and 120 hpi (Figure 4d). Compared to WT plants, the development of \textit{Pst} was also affected, with hyphal length, the number of haustoria, and infected areas all decreased at 24 and 48 hpi in \textit{Pst \_A23}-RNAi plants (Figure 4e,g). The areas of cell death at infection sites were increased in \textit{Pst \_A23}-RNAi plants 24 and 48 hpi (Figure 4h–i), indicating that \textit{Pst \_A23} silencing resulted in the elevation of the plant’s resistance response. The improved wheat resistance by \textit{Pst \_A23} silencing implies the opportunity for engineering wheat resistance using the pathogen effector gene.

\textbf{Pst \_A23} regulates specific gene expression and splicing events

The sub-localization of \textit{Pst \_A23} in nuclear speckles, which is rich in splicing factors (Hasenson and Shav-Tal, 2020), suggested that \textit{Pst \_A23} may interfere with the host’s AS process. Hence, we sequenced the transcriptome of a transgenic plant line overexpressing \textit{Pst \_A23} and compared it to that of WT wheat. A total of 11,241 differentially expressed genes (DEGs) were obtained, among which 6437 were upregulated and 4804 were downregulated (Figure S7a and Table S1). KEGG analysis highlighted that DEGs belong to biological pathways involved in the metabolic pathway, biosynthesis of secondary metabolites, plant-pathogen interactions, protein processing in the endoplasmic reticulum, and so on (Figure S7b). Expression of the genes encoding the pathogenesis-related protein (TraesCS7A02G198900) and Thaumatin-like protein (TraesCS5A02G17900) were significantly
decreased in Pst_A23-OE lines compared to WT, which was confirmed by RT-qPCR analysis (Figure S7c).

To examine whether Pst_A23 affects splicing in wheat, we analyzed our RNA-seq data for spliced isoforms of RNA and found 600 differentially AS events in 588 alternatively spliced genes in Pst_A23-OE lines compared to WT plants (Table S2). Among the five major types of AS, alternative 3' splice site usage was seen most often (181 events), followed by changes in intron retention (160 events), alternative 5' splice site usage (142 events), exon skipping (80 events), AS in the first exon (33 events) and last exon (3 events), and shifts in splicing around mutually exclusive exons (1 event). These findings were validated by semiquantitative RT-PCR of transcripts related to the host defence response. In Figure 5, we use several examples to illustrate different splicing outcomes in Pst_A23-OE lines compared to WT: NADPH-cytochrome P450 reductase RNA (TraesCS2A02G505800), the oxidoreductase gene (TraesCS1D02G241000), rust resistance kinase Lr10 (TraesCS1B02G022300), and glycerol-3-phosphate dehydrogenase gene (TraesCS4B02G286700). We observed that different genes are affected differently by Pst_A23 overexpression. Collectively, we determined that Pst_A23 overexpression causes major perturbations to splicing patterns of a subset of transcripts, with functions related to the host defence response, in agreement with the proposed function for Pst_A23 as an effector that regulates the host’s immune response by affecting AS processes.

Pst_A23 binds directly to splice site motifs in RNA

In nuclear speckles, the RNA-binding proteins are usually rich in serine/arginine or glycine (Galganski et al., 2017). The arginine-rich feature of Pst_A23 suggests that this protein engages RNA directly. To investigate the molecular mechanism by which Pst_A23 regulates splicing, we used MEME (motif-based sequence analysis tools) to search for RNA motifs within splice sites of genes whose AS was altered upon Pst_A23 overexpression (Figure S8a). We tested the binding of Pst_A23 to two RNA motifs M1(GA_GAA) and M2 (UUCUUU) in vitro that is highly over-represented or of high confidence (Figure 6a). Interestingly,
we found that Pst_A23 is bound to M1 and M2 (Figure 6b). The negative control, GST, did not bind to M1 or M2 (Figure 6b). To test the specificity of Pst_A23 for these RNA motifs, we mutated the M1 and M2 motifs (Figure 6a) and assayed their binding to Pst_A23 protein by RNA electrophoretic mobility shift assay (RNA-EMSA). With the detected Pst_A23-M1 and Pst_A23-M2 binding complex, no RNA–protein complex was detected when Pst_A23 was incubated with the mutated sequences (Figure 6c). To further eliminate the possibility of non-specific binding, we used polycytidylic acid–agarose, polyuridylic acid–agarose, and single-stranded deoxyribonucleic acid–cellulose, none of which pulled down Pst_A23 protein in vitro (Figure S8b).

The M1 and M2 motifs were found to be enriched at the cis-elements at the splice sites of an LRR receptor-like serine/threonine-protein kinase mRNA (TaXa21-HOE lines) and a WRKY transcription factor TaWRKY53 mRNA (TraesCS1A02G070400). To examine whether Pst_A23 could bind these cis-elements, we used EMSA and observed that the amount of the RNA–protein complex increased proportionally with the increased amount of Pst_A23 protein, whereas the negative control, GST, did not bind to these RNAs (Figure 7a). Overall, we conclude that the fungal effector Pst_A23 binds to specific RNA motifs at splice sites in target mRNAs to regulate their splicing. We used RT-qPCR to analyze the ratios of the splice forms of RNAs produced from these genes using isoform-specific primers. The splicing efficiency of TaXa21-H and TaWRKY53 were both significantly reduced in Pst_A23-overexpressing plants than on the control plants (Figure 7b). In wheat leaves transiently expressing Pst_A23, the splicing ratio of TaXa21-H and TaWRKY53 was also lower than that in control leaves expressing dsRed (Figure 7c). In contrast, transient overexpression of the R1R2A mutant did not change the splicing ratio of TaXa21-H and TaWRKY53 (Figure 7c). These data indicate that Pst_A23 binding to the cis-element likely results in the decreased AS of TaXa21-H and TaWRKY53 and the R1R2A mutation affects its regulatory role on AS of TaXa21-H and TaWRKY53.

Silencing of TaXa21-H and TaWRKY53 compromises plant resistance to Pst

The LRR-receptor-like kinase gene TaXa21 and transcription factor gene TaWRKY53 have been reported to positively regulate adaption to various stresses in cereals (Van Eck et al., 2014; Wang et al., 2019; Wu et al., 2008). To determine whether the decreased functional transcripts caused by Pst_A23 overexpression contribute to the reduced wheat resistance, we silenced TaXa21-H and TaWRKY53 using barley stripe mosaic virus (BSMV) in the wheat cultivar Suwon 11 to confirm the function of these two genes in the resistance of wheat to Pst infection. Fourteen days after inoculation of the avirulent Pst race CYR23, which normally does not grow well in Suwon11, more uredinia appeared on the leaves of TaXa21-H and TaWRKY53-knockdown plants than on the control plants (BSMV:00) (Figure 8a). TaXa21-H and TaWRKY53 transcript levels were reduced by 70%–75% at 24 and 48 hpi in TaXa21-H and TaWRKY53-knockdown plants (Figure 8b). The lengths of fungal hyphae and the infected leaf areas per infection site were greater at 48 hpi in TaXa21-H and TaWRKY53-knockdown plants than in the controls (Figure 8c). Moreover, the expression of the defence-related genes TaPR1 and TaPR2 in the knockdown plants was reduced 3–7-fold at 24 and 48 hpi, respectively, compared to that in the controls (Figure 8d). Taken together, these data show that silencing of TaXa21-H and TaWRKY53 impairs wheat resistance to Pst, indicating their positive regulator role in wheat resistance to Pst infection. Thereby, it is reasonable that the repressed functional transcripts of TaXa21-H and TaWRKY53 by Pst_A23 might contribute to the compromised wheat resistance to stripe rust.

Figure 7 Pst_A23 binds to the cis-element of TaWRKY53 and TaXa21-H to interfere with splicing. (a) Affinity of Pst_A23 for the cis-elements of TaWRKY53 and TaXa21-H genes was confirmed using RNA-EMSA. The dilution gradient of Pst_A23:GST was incubated with RNA, and GST was used as the negative control. (b) Validation of alternative splicing of target genes TaWRKY53 and TaXa21-H in Pst_A23-OE lines. Splice isoforms were detected using RT-PCR with specific primers for gene isoforms. Schematic representation of splicing events are in the left panel. Black and red arrows indicate the primers used to amplify the unspliced RNA and spliced RNA form respectively. The splicing ratio was calculated by determining the level of spliced RNA normalized to the level of unspliced RNA using RT-qPCR. The mean and SEM were calculated from three biological replicates. *P < 0.05; **P < 0.01; ns, not significant using Student’s t-test in comparison with WT. (c) Detection of alternative splicing of TaWRKY53 and TaXa21-H in wheat leaves transiently expressing Pst_A23 and R1R2A mutant. The mean and SEM were calculated from three biological replicates. *P < 0.05; **P < 0.01; ns, not significant using Student’s t-test in comparison with dsRed.
Discussion

In this study, we have characterized Pst_A23 as a critical effector of the notorious wheat fungal pathogen, *P. striiformis*. We showed that knockdown of Pst_A23 significantly impairs fungal pathogenicity and enhances wheat resistance, providing a potential target for wheat resistance engineering. Our results indicate that Pst_A23 translocates into the host nucleus, where it localizes to nuclear speckles, specific nuclear domains enriched in pre-mRNA splicing factors. This localization pattern together with our transcriptome analysis, which found that overexpression of Pst_A23 results in a total of 600 differential splicing events, provides strong support for functional annotation of Pst_A23 as an effector that reprograms AS process in the host. Additionally, differential splicing events we identified include genes involved in the host defence and immune response, further suggesting that Pst_A23 affects host immunity via perturbations to AS. Furthermore, we provide evidence for direct and selective binding between Pst_A23 and pre-mRNA binding motifs found in specific target genes, such as TaXa21-H and TaWRKY53, which we show contribute to wheat resistance to *Pst* infection. Collectively, our evidence points to Pst_A23 as an important effector that regulates *Pst* infectivity by binding to and regulating AS of a subset of genes involved in defence mechanisms and thus interfering with the ability of the wheat host to launch a robust immune response to the invading pathogen.

In general, effectors of different pathogens and parasites have been demonstrated to reprogram host splicing to subvert host...
immunity or promote infection. Overexpression of the oomycete effector \textit{PsAvr3c} results in AS of 401 genes, in a manner dependent on the serine/arginine-rich RNA-binding proteins SKRPs (Huang \textit{et al.}, 2017). Recently, Huang \textit{et al.} (2020) identified nine \textit{Phytophthora infestans} splicing regulatory effectors (SREs) that are involved in pre-mRNA splicing using the luminescence-based AS reporter system in \textit{N. benthamiana}, among which, SRE3 was found to specifically bind U1-70k to manipulate plant AS machinery. The Cyst nematodes effector 3D08 interacts with an auxiliary spliceosomal protein SMU2 in the plant nucleus, leading to the AS changes in genes functionally linked to feeding site formation (Verma \textit{et al.}, 2018). Until now, all the SREs were found to regulate host splicing via binding to the splicing factors, including the core snRNP or the auxiliary RNA binding proteins (RBPs) (Fu \textit{et al.}, 2007; Huang \textit{et al.}, 2017, 2020; Nicaise \textit{et al.}, 2013). Interestingly, we present evidence that \textit{Pst}_{A23} uses a fundamentally different mechanism whereby it exerts its effects on AS by binding to the specific pre-mRNA motifs (Figure 9). The evidence we assembled to support this claim includes cellular localization studies that showed that \textit{Pst}_{A23} localizes to nuclear speckles, and that this localization depends on the presence of an arginine-rich domain, R2-rich domain. Moreover, our \textit{in vitro} studies showed that \textit{Pst}_{A23} binds directly to specific RNA motifs identified as enriched in the splice sites of genes whose AS was altered upon \textit{Pst}_{A23} overexpression, thus tying \textit{in vitro} observations with \textit{in vivo} studies into a cohesive mechanistic framework.

The outcome of AS is precisely tuned by recognition of the splicing factors and their recognition RNA sequence in the pre-mRNAs (Galganski \textit{et al.}, 2017). Different serine/arginine-rich splicing factors were found to recognize specific RNA motifs to modulate the selection of alternative splice sites or the AS form, such as exon-inclusion and exon skipping (Galganski \textit{et al.}, 2017). Otherwise, the short sequences of cis-elements in the intronic or exonic region were also shown to play a critical role in inducing or suppressing the selection of nearby 5’- or 3’-splicing sites by binding to the trans-acting factors, such as the RBP SR proteins (Day \textit{et al.}, 2012; Thomas \textit{et al.}, 2012). Upon \textit{Pst}_{A23} overexpression, alternative intron retention was prevalent among the aberrant splicing event. One possible explanation is that most intron-containing genes in plants contain a tandem acceptor site (XAGXAG, where X is any nucleotide) (Reddy \textit{et al.}, 2013), and \textit{Pst}_{A23} interacts with RNA motifs that are similar to tandem acceptor sites, resulting in the reduction of intron retention as observed for TaXa21-H. Moreover, \textit{Pst}_{A23} as an RNA-binding protein could bind the cis-element of specific genes, such as \textit{TaWRKY53}, leading to impacted alternative 5’ splice site usage in \textit{TaWRKY53}. Therefore, we speculate that \textit{Pst}_{A23} binding to the RNA motifs affects the regulation of splice site recognition and leads to subtle differences in splicing outcomes. As an RBP, \textit{Pst}_{A23} may compete with other splicing factors in binding the splice site or nearby cis-element thus disturbing the splicing recognition or directly functioning as a splicing factor that recognizes specific RNA sequence to alter the splicing sites or the splicing form. Another possibility is that except for RNA binding, in the spliceosome \textit{Pst}_{A23} likely interacts with other components of the spliceosome, such as the splicing factor or the core small nuclear ribonucleoproteins to interfere with the recognition and splicing of the target RNA by the spliceosome.

The abnormal splicing isoforms of wheat genes caused by \textit{Pst}_{A23} overexpression, such as TaXa21-H may be degraded by nonsense-mediated decay and other RNA degradation mechanisms, which results in the decreased expression profile. The predicted amino acid sequence of TaXa21-H is 52% similar to its \textit{Oryza sativa} orthologue OsXa21, which is a well-studied receptor kinase that confers broad-spectrum resistance to \textit{Xanthomonas oryzae pv. oryzae} (Song \textit{et al.}, 1995; Yang \textit{et al.}, 2013). In wheat, TaXa21 was recently shown to play a positive role in a specific type of host resistance to \textit{Pst}, known as high-temperature seedling plant resistance (Wang \textit{et al.}, 2019). TaWRKY53 was reported to regulate oxidative responses to biotic and abiotic stress in cereals by regulating the downstream targets ORK10/LRK10 (a Ser/Thr receptor kinase) and POC1, a peroxidase in the apoplast (Van Eck \textit{et al.}, 2014; Wu \textit{et al.}, 2008). Here, we illustrated that transient silencing of TaXa21-H and TaWRKY53 impairs wheat resistance to \textit{Pst}, which may partially account for the increased susceptibility of \textit{Pst}_{A23}-OE lines, indicating that \textit{Pst}_{A23} is involved in the AS of RNAs from wheat genes participating in resistance to pathogens. In fact, AS regulation of the immune receptor RLKs and WRKY transcription factors have been proved to play roles in plant-microbe interaction. Splicing of RNAs encoding two plant immune receptor kinases, SNC4 and CERK1, is regulated by the conserved splicing factors SUA and R5N2, which are required for the plant defence response to \textit{P. syringae} in \textit{Arabidopsis thaliana} (Zhang \textit{et al.}, 2014). The alternative shorter transcripts of \textit{OsWRKY62} and \textit{OsWRKY76} were shown to increase upon pathogen infection and in \textit{OsiWRKY62/76} RNAi-lines, leading to a reduced repressor activity (Liu \textit{et al.}, 2016). Besides TaXa21-H and TaWRKY53, \textit{Pst}_{A23} overexpression reprogrammed more than 10,000 genes in pathways linked to the biosynthesis of secondary metabolites, plant-pathogen interactions, protein processing in the endoplasmic

![Figure 9](image-url) Working model of \textit{Pst}_{A23} during \textit{Pst}-wheat infection. Effector \textit{Pst}_{A23} is secreted from \textit{Pst} and enters the host spliceosome, where protein \textit{Pst}_{A23} binds to RNA motifs. In the host spliceosome, \textit{Pst}_{A23} acts as a splicing regulator to reprogram the metabolism of plant pre-mRNA, ultimately leading to the reduction of plant resistance to \textit{Pst}. In contrast, in the absence of \textit{Pst}_{A23}, the host spliceosome splices plant pre-mRNA normally.
retticulum, and so on. Combined with the increased resistance in \textit{Pst\_A23-RNAI} lines, our findings indicate that \textit{Pst\_A23} is a crucial pathogenic factor that suppresses plant immunity to enable pathogen growth and development in the host, by directly regulating host pre-mRNA splicing to repress positive regulators of host defence and immune response.

Previous studies have revealed several rust fungal effectors that are involved in host transcriptome regulation. For example, the Mpl24478 protein of the fungus \textit{Melampsora larici-populina} binds directly to DNA to alter the transcriptome of \textit{N. thalaina}, and the PpEC23 protein of soybean rust \textit{Phakopsora pachyrhizi} interacts with plant transcription factors to regulate the transcriptome (Ahmed \textit{et al.}, 2018; Qi \textit{et al.}, 2016). These effectors regulate gene expression directly at the level of transcription. PgtSR1, an effector from \textit{Puccinia graminis f. sp. tritici} (Pgt), acts as a fungal RNA-silencing suppressor to regulate small RNA metabolism and suppress plant basal defences (Yin \textit{et al.}, 2019). This study is the first to report the role of rust fungus effectors in post-transcriptional regulation of host AS, which throws light on unveiling the mechanisms that govern the altered wheat AS by \textit{Pst} infection as reported (Zhang \textit{et al.}, 2019). However, given that \textit{Pst\_A23} affects several hundreds of AS events, the full extent of its activity and the entire network of mRNA interaction sites remains to be elucidated.

### Experimental procedures

#### Plant cultivation and \textit{Pst} propagation

\textit{N. benthamiana} were grown in the phytotron under a 16 h light/8 h dark cycle at 22 °C. Wheat plants inoculated with \textit{Pst} were incubated at 16°C with 16 h of light and at 14 °C with 8 h of dark. Urediospores of \textit{Pst} race CYR32 and CYR31 were collected from infected plant leaves of the wheat cultivar Suwon 11, and \textit{Pst} CYR23 was collected from the wheat cultivar MingXian169 and stored in a dry at 4 °C until RNA extraction and inoculation were performed (Kang \textit{et al.}, 2002). Germ tubes of \textit{Pst} CYR31 were germinated on the surface of the water for 12–16 h in the dark at 9–11 °C and harvested to store at −20 °C until RNA extraction was performed.

#### Sequence analysis and transcript level detection

The signal peptide of \textit{Pst\_A23} was predicted using SignalP 3.0 (Bendtsen \textit{et al.}, 2004), and the sub-localization was determined using LOCALIZER software (Sper Schneider \textit{et al.}, 2017). Characteristics of the \textit{Pst\_A23} protein were analysed using Expasy (https://www.expasy.org/). To determine \textit{Pst\_A23} transcript levels, total RNA was extracted from the urediospore (U), germ tube (GT), and wheat infected with \textit{Pst} CYR31 at 24, 48, 72, 120 and 168 hpi. For RNA extraction from \textit{N. benthamiana}, \textit{NbaActin} was used to normalize RNA levels. Primers amplifying plant sequences were designed using the qPCR primer database (https://biodb.swu.edu.cn/qprimerdb/). All primers are shown in Table S3.

#### Transient expression assays in \textit{N. benthamiana}

For cell death suppression assay in \textit{N. benthamiana}, the sequence encoding the mature \textit{Pst\_A23}, eGFP, and Bax were subcloned into pGR106 and transformed into \textit{Agrobacterium} GV3101. The transformed \textit{Agrobacterium} strain was washed three times with 10 mM MgCl\textsubscript{2} and diluted to an OD\textsubscript{600} of 0.2. \textit{Agrobacterium} carrying the PVX: \textit{Pst\_A23}, PVX: eGFP were infiltrated into \textit{N. benthamiana} leaves 24 h prior to infiltrating \textit{Agrobacterium} containing Bax using a 1-mL injection syringe. For the PTI assay in \textit{N. benthamiana}, the Flg22 polypeptide was diluted to 10 μM with 10 mM MgCl\textsubscript{2}, and was infiltrated into the same sites on \textit{N. benthamiana} leaves using a 1-mL syringe 48 h after expressing \textit{Pst\_A23} or eGFP.

For localization in \textit{N. benthamiana}, \textit{Agrobacterium} cells carrying \textit{Pst\_A23:GFP}, R1A:GFP, R2A:GFP, R1R2A:GFP, and ATSR45: REP were treated with suspension buffer (10 mM MgCl\textsubscript{2}, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), and 10 μM AS, pH 5.6), diluted to an OD\textsubscript{600} of 0.5 and transiently expressed with P19 (silencing suppressor) in leaves from 3- to 4-week-old \textit{N. benthamiana} plants. Forty-eight hours after inoculation, the epidermis was immersed in 4′6-diamidino-2-phenylindole (DAPI) for 3–5 min and then observed on an Olympus BX-51 microscope. Nuclear protein was extracted from \textit{N. benthamiana} leaves 48 h post-inoculation using the nuclear extract method as previously described (Xing \textit{et al.}, 2015).

#### Silencing and overexpression assays in wheat

To localize \textit{Pst\_A23} in wheat protoplasts, we prepared the protoplasts and transformed them with the fusion plasmids \textit{Pst\_A23:GFP} and the EV using PEG-mediated transformation as described by Yoo \textit{et al.} (2007). Green fluorescence was detected with Zeiss LSM880 confocal laser microscope at 24 h post-transformation.

For \textit{Pseudomonas} infection of wheat and \textit{N. benthamiana}, a nonpathogenic strain, \textit{EtHan}, was used to deliver foreign effectors in plants (Upadhyaya \textit{et al.}, 2014). The sequence encoding the mature \textit{Pst\_A23}, R1R2A, and dRed were constructed into a pEDV6 vector and transformed into \textit{P. fluorescens} \textit{EhAn}. The \textit{EhAn} bacteria carrying pEDV6: \textit{Pst\_A23}, pEDV6: R1R2A, and pEDV6: dRed were washed with 10 mM MgCl\textsubscript{2} three times and diluted to OD\textsubscript{600} = 1.0 for wheat infection. \textit{P. syringae} DC3000 was diluted to OD\textsubscript{600} = 0.4 for wheat leaves. \textit{EhAn} carrying constructs were infiltrated into the second leaves of two-leaf stage \textit{Swon11}. For the cell death suppression assay in wheat, 24 h post infiltration of \textit{EhAn}, \textit{P. syringae} DC3000 was injected into the same site to induce cell death and the phenotype was observed ad 5 dpi. For the PTI assays, 24 h post-inoculation of \textit{EhAn} carrying the construct, the infiltrated leaves were collected for callose deposition staining and RT-qPCR analyses for the expression of pathogenesis-related genes.

For examination of the effect of \textit{Pst\_A23} on \textit{Pst} virulence, at 24 hpi, wheat leaves injected with \textit{EhAn} carrying constructs were further infiltrated with \textit{Pst} race CYR23. And the \textit{Pst} inoculated wheat leaves were sampled at 48 h post-\textit{Pst} infection for detection of fungal development.

For the \textit{VIGS} assay, the recombinant vectors (BSMV: TaXa21-H and BSMV: TaWRKY53) and the vector containing the virus genome (α, β, and γ) were transcribed to RNA. Transcripts of each vector (α, β, and recombinant vector) were mixed at a 1:1:1 ratio, and 30 μL of transcription mix was added to 160 μL FES buffer for inoculation (Holzberg \textit{et al.}, 2002; Panwar \textit{et al.}, 2013). This mixture was inoculated onto the second leaves of wheat cultivar \textit{Swnow}11 using friction inoculation. Plants inoculated with the virus were grown at 25–27 °C for 10 days. The bleaching phenotype of wheat inoculated with BSMV: PDS served as a positive control for virus infection. Then, the corresponding fourth leaves were inoculated with \textit{Pst} race CYR23. Samples for histological observation and RT-qPCR were harvested at 24 and 48 hpi. Thirty to twenty leaves with \textit{Pst} inoculation were collected to identify disease phenotypes.
For genetic transformation of wheat, sequences specific to \textit{Pst\_A23} were inserted into pANIC\_B8E to silence \textit{Pst\_A23}, and full-length \textit{Pst\_A23} (without signal peptide) with a C-terminal Flag tag was inserted into pANIC\_B8E for overexpression (Mann et al., 2012). Approximately 200–300 isolated wheat embryos (wheat cultivar Fielder) were cultured and infected with \textit{Agrobacterium}. Regeneration and selection were conducted in the corresponding medium with 3 mg/L bialaphos. For the positive selection of transgenic plants, genomic DNA of the transgenic lines was isolated using the CTAB method (Healey et al., 2014) and used for PCR amplification with the specific primers for \textit{Pst\_A23} silencing fragment and full-length \textit{Pst\_A23} (without signal peptide). Meanwhile, the wheat glume was stained with GUS solution as previously described for the selection of positive transgenic plants (Mann et al., 2012). To detect the protein abundance in transgenic wheat plants overexpressing \textit{Pst\_A23}, the nuclear protein of the second leaves of \textit{Pst\_A23}-overexpressing transgenic plants were extracted (Xing et al., 2015), and detected with an anti-Flag antibody by Western blotting. For disease phenotype evaluation of transgenic plants, the second leaves were inoculated with \textit{Pst} CYR32 or CYR31 and samples were harvested at 24 and 48 hpi for observation of hyphal development and host response.

**Histological observation**

For callus staining, the infected \textit{N. benthamiana} and wheat leaves were destained in absolute ethyl alcohol:acetic acid (1:1 v/v) and immersed in chloral hydrate until the leaf segment was transparent. Then the leaf samples were stained with 0.05% aniline blue in 0.067 M K$_2$HPO$_4$ (pH 9.6) and observed under light microscopy. The callous deposits in fields of 1 mm$^2$ were counted using ImageJ software.

For observation of hyphal development, the decolorized and transparent wheat leaf samples at 24 and 48 hpi were treated in 1 mL 1 M KOH at 121 °C for 5 min. Samples were carefully washed three times with 50 mM Tris–HCl (pH 7.4) for 15 min and then stained with 20 µg/mL wheat germ agglutinin Alexa-488 (WGA) (Invitrogen, Carlsbad, CA) for 15 min (Ayliffe et al., 2011). The fungal infection structure was observed under fluorescent microscopy (excitation wavelength 450–480 nm, emission wavelength 515 nm).

For cell death staining with trypan blue, \textit{Pst} inoculated leaves were collected and boiled for 8 min in 0.4% solution of trypan blue and cleared in a solution of 2.5 mg/mL chloral hydrate (Keogh et al., 1980). Stained cell death was observed under a light microscope.

The necrosis mesophyll area, the fungus hyphal length and the infection area were calculated from at least 30 infection sites that substomatal vesicles had formed underneath stomata by CellSens Entry software. Student’s t-test were applied for statistical analysis.

**RNA-seq analysis and AS isoform identification**

The second leaves of \textit{T$_2$} transgenic plants overexpressing \textit{Pst\_A23} and wild-type plants were harvested for total RNA extraction and RNA-seq libraries constructing. These libraries were sequenced using Illumina HiSeq in paired-end mode with a read length of 150 bp (Novogene Bioinformatics Technology Co., Ltd, Beijing, China). The full-length cDNA library of insert size was constructed from the RNA of the WT plants and sequenced by the PacBio Sequel system, and was used as a reference transcriptome. The sequence data were processed using the AMRTlink 5.0 software and corrected using LoRDEC (Salmela and Rivals, 2014). The polished consensus data were aligned to the reference genome (IWGSC RefSeq v1.0, ftp://ftp.ensemblgenomes.org/pub/plants/release-40/fasta/triticum_aestivum/dna) using GMAP (Wu and Watanabe, 2005). RNA-seq data were mapped to the reference genome using Hisat2 (v2.1.0). The gene transcript level was estimated by the FPKMs (fragments Per Kilobase of transcript sequence per Millions base) by Cuffdiff (V2.1.1) and DEGs were identified using the DESeq R package (1.18.0) with the adjusted P-value <0.05 as the threshold for significantly differential expression.

Based on the TPM value (transcripts per million) in each transcriptome, different AS events were analysed using SUPPA software (Alamancos et al., 2015) and the different splicing events were detected using a Mann–Whitney U test under the condition P < 0.05. The splicing ratio was calculated by determining the level of spliced RNA normalized to the level of unspliced RNA using RT-qPCR. Splice form-specific primers (Table S3) were designed to cross the exon–exon junction, and primers detecting unspliced products were designed to span the intron–exon junction. For semi-quantitative analysis of splicing events, cDNA templates from samples were diluted to the same concentration. The reference gene \textit{TaEF} was amplified as a control, and representative splicing genes were detected by primers designed to span the exon–intron–exon junction. Intensities of PCR products were quantified using ImageJ software, and the intensities of \textit{TaEF} PCR products were used as a reference (set to 1).

**RNA electrophoretic mobility shift assay (RNA-EMSA)**

The \textit{Pst\_A23} gene was cloned into pGEX4T-1 and expressed in the \textit{Escherichia coli} BL21 strain. Recombinant proteins were induced by 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) and purified by glutathione GST Sepharose 4b (GE Healthcare). The RNA sequence was synthesized in vitro with biotin at the 5’ end (GENERAL BIOSYSTEMS). The RNA electrophoretic mobility shift assay was performed according to the Thermo Scientific Light ShiftTM Chemiluminescent RNA EMSA Kit (Thermo Fisher Scientific, Waltham, MA). Briefly, the labelled RNA and the recombinant proteins (0–0.4 µg) were incubated for 30 min at room temperature using a dilution gradient of protein in a 20 µl reaction system. The mixture was run on a 6% native polyacrylamide gel under 80 V for 1.5 h in 0.5x TBE buffer, and then RNA was transferred onto a nylon membrane (GE Healthcare). After crosslinking transferred RNA to the membrane, the labelled RNA on the membrane was detected using a chemiluminescent nucleic acid module.

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Conflict of interests
The authors declare no competing interests.

Author contributions
X.W. and C.T. designed the research. Q.X., C.T., J.Z., and M.Y. performed most of the experiments. D.X.W. helped in experiments. J.W. helped in generating transgenic materials. C.T., Q.X., and X.W. analyzed the data, and C.T. wrote the manuscript with the help of Q.X., Z.K., and J.X.W.

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Supporting information

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

Figure S1 Expression pattern of Pst_A23 as determined by RT-qPCR.

Figure S2 Functional evaluation of the signal peptide of Pst_A23.

Figure S3 Western blot detection of protein expression in N. benthamiana.

Figure S4 suppression of Bax/bacteria-induced cell death by Pst_A23 in plants.

Figure S5 Suppression of plant PTI by Pst_A23 in N. benthamiana.

Figure S6 Enhanced wheat stripe rust resistance in Pst_A23-RNAi transgenic plants.

Figure S7 Data analysis of the transcriptome in Pst_A23-overexpressing transgenic wheat.

Figure S8 Predicted RNA motifs and detection of Pst_A23 binding ability to PolyU, PolyC, and ssDNA.

Table S1 Differentially expressed genes (DEGs) in transgenic plants overexpressing Pst_A23 compared to that in the wild-type wheat.

Table S2 Differentially alternatively spliced genes (DASGs) in Pst_A23-overexpressing transgenic lines compared to that in the wild-type plants.

Table S3 Primers and RNA probes used in this study.