Role of the tomato fruit ripening regulator MADS-RIN in resistance to *Botrytis cinerea* infection

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Abstract

Tomato MADS-RIN (RIN) transcription factor has been shown to be a master activator regulating fruit ripening. Recent studies have revealed that in addition to activating many other cell wall genes, it also represses expression of XTH5, XTH8 and MAN4a, which are positively related to excess flesh softening and cell wall degradation, which might indicate it has a potential role in pathogen resistance of ripening fruit. In this study, both wild type (WT) and RIN-knockout (RIN-KO) mutant tomato fruit were infected with Botrytis cinerea, to investigate the function of RIN in defence against pathogen infection during ripening. The results showed that RIN-KO fruit were much more sensitive to B.cinerea infection with larger lesion sizes. Transcriptome data and qRT-PCR assay indicate genes of phenylalanine ammonialyase (PAL) and chitinase (CHI) in RIN-KO fruit were reduced and their corresponding enzyme activities were decreased. Transcripts of genes encoding pathogenesis-related proteins (PRs), including PR1a, PRSTH2 and APETALA2/Ethylene Response Factor (AP2/ERF) including ERF.A1, Pti5, Pti6, ERF.A4 were reduced in RIN-KO fruit comparing to WT fruit. Moreover, in the absence of RIN the expression of genes encoding cell wall modifying enzymes XTH5, XTH8, MAN4a has been reported to be elevated, which is potentially correlated with cell wall properties. When present, RIN represses transcription of XTH5 by activating ERF.F4 a class II (repressor class) ERF gene family member and ERF.F5. These results support the conclusion that RIN enhances ripening-related resistance to grey mould infection by upregulating pathogen-resistance genes and defense enzyme activities as well as reducing accumulation of transcripts encoding some cell wall enzymes.

Key words: MADS-RIN, tomato fruit, Botrytis cinerea, pathogen resistance, cell wall
Introduction

The tomato (Solanum lycopersicum, previously Lycopersicon esculentum) fruit is recognized as a model system for investigating fleshy fruit development, especially for climacteric fruit ripening (Pesaresi et al., 2014; Quinet et al., 2019; Li et al., 2021). As the fruit ripen and senesce, susceptibility to infection by numerous microorganisms increases, directly leading to pre- and postharvest fruit decay and economic loss. A wide variety and high diversity of secondary metabolites and defence responses produced by fruit are involved in the protection against biotic stress (Yang et al., 2018). Grey mould disease, caused by Botrytis cinerea (B. cinerea), is one of the most prevalent diseases that infect tomatoes. It enters mainly through wounds and an infection can cause the entire plant to die, with resulting yield losses in the field and rotting of fruit during storage (Blanco-Ulate et al., 2013; Elad et al., 2007; Zhang et al., 2019). Therefore, improving resistance to B. cinerea is important for tomato production, prolonging postharvest storage and fruit quality maintenance.

The regulatory mechanisms underlying tomato fruit ripening involve coordinated induction or repression of multiple gene transcripts, including transcription factors (TFs) which target structural genes encoding enzymes in pathways affecting color, flavor, aroma, cell wall properties and levels of ripening-related hormones (Giovannoni, 2004, 2007; Giovannoni, 2017; Grierson, 2013; Shinozaki et al., 2018; Li et al., 2021). These ripening changes can affect the resistance to B. cinerea infection in tomato fruit. Several different types of TFs, including members of the GRAS, MYB, bHLH and MADS families, play important roles in modulating fruit resistance to B. cinerea at the transcriptional level. For example, overexpression of a GRAS gene SlFSR in rin mutant fruit significantly shortened fruit postharvest shelf-life by activating transcription of cell wall modifying genes (PL, PE, TBG4, XTH5, PG, CEL2, XYL1, MAN1, EXP) and decreased susceptibility to postharvest pathogens, thus prolonging storage life of fruit (Zhang et al., 2018a). SlFSR silencing in WT tomato fruit led to lower levels of gene transcripts encoding various cell wall modifying proteins and reduced enzymatic activities of cellulose (CEL), β-D-xylosidases (XYLs), tomato β-galactosidases (TDBGs), polygalacturonase (PG), and prolonged the shelf-life of fruit (Zhang et al., 2018a). MADS-RIN-deficient tomato fruit only partially ripen and do not initiate system-2 ethylene synthesis (Li et al., 2020). Overexpression of another MADS TF, SIMMYB75, up-regulated the jasmonic acid (JA)-mediated signaling pathway, activated peroxidase (POD) and superoxide dismutase (SOD) activities, decreased levels of fruit skin wax and downregulated SlFSR transcripts (Liu et al., 2021). Silencing of SIMYC2, encoding a bHLH TF, promoted methyl jasmonate (MeJA)-induced fruit resistance to B. cinerea, possibly by regulating defensive enzyme activities, pathogenesis-related protein (PRs) expression, and altering α-tomatine, phenolic acids and flavonoid compounds (Du et al., 2017; Min et al., 2020). Other TF families also participate in fruit ripening-related pathogen resistance, such as the AP2 TF, SISHN3, which is related to cuticle permeability and up-regulates transcription of pathogenesis-related gene family members AOS, PRI1a (Buxdorf et al., 2014) and NAC-NOR which activates ripening events (Cantu et al., 2009; Li et al., 2021). Moreover, multiple members of the
APETALA2/Ethylene Response Factors (AP2/ERFs) family have been reported to function in tomato fruit resistance to *B. cinerea* (Ouyang *et al.*, 2016; Yu *et al.*, 2018).

Ripe fleshy fruit are more sensitive to mould infection than unripe fruit (Florlani *et al.*, 2019) and the implication is that changes that occur during ripening render fruit more susceptible to pathogen attack. Tomato fruit ripening is regulated by the plant hormone ethylene and various TFs, including NAC-NOR, MADS-RIN (RIN) and SPL-CNR, which affect fruit color, flavor, texture and secondary metabolism. Loss of function of any of these TF delays or disrupts ripening (Gao *et al.*, 2019; Li *et al.*, 2020; Manning *et al.*, 2006; Vrebalov *et al.*, 2002). RIN, together with ethylene, is indispensable for full ripening and RIN activates several ripening-related pathways including directly targeting and activating expression of cell wall genes such as expansin (*Exp1*), PG and pectate lyase (*PL*) (Li *et al.*, 2020; Zhong *et al.*, 2013), all of which have been reported to contribute to change in tomato fruit pericarp firmness and corresponding resistance to *B. cinerea* (Cantu *et al.*, 2008a; Silva *et al.*, 2021; Yang *et al.*, 2017). In addition, RIN also represses aspects of over-ripening, especially excess texture change, in addition to facilitating other ripening processes (Ito *et al.*, 2020; Li *et al.*, 2020). Several studies have indicated that RIN interacts with other TFs such as FRUITFUL homologs FUL1, FUL2 and TOMATO AGAMOUS-LIKE1 (TAGL1) (Fujisawa *et al.*, 2014; Ito *et al.*, 2020; Li *et al.*, 2018; 2019).

Although mutant non-ripening (*nor*) and ripening-inhibitor (*rin*) fruit have similar ripening disrupted phenotype, *rin* fruit are less susceptible to *B. cinerea* than *nor* fruit (Cantu *et al.*, 2009). However, it is unclear how the key ripening regulator MADS-RIN (RIN) affects fruit susceptibility or resistance to *B. cinerea*. The reason for this is that although the *rin* mutation was originally recognized as loss-of-function, it is now known that the *rin* mutation is caused by the genomic DNA fragment deletion and resulting in the fusion of adjacent truncated *RIN* and *MC* genes (*RIN-MC*), which has acquired a repressor activity because the existence of EAR repressing motif in C-terminal of RIN-MC protein (Vrebalov *et al.*, 2002; Ito *et al.*, 2017; Li *et al.*, 2018; 2020). This means that the previous conclusions about RIN function in defence against infection stemming from examination of ripening-related mutants of *rin* (Cantu *et al.*, 2009; Silva *et al.*, 2021) require re-evaluation using different biological materials. Recently, tomato lines manipulated to produce fruit that are deficient in the RIN TF, using CRISPR/Cas9, have become available (Ito *et al.*, 2017; 2020; Li *et al.*, 2020), providing an opportunity to test the role of RIN in resistance to infection.

In this study, we compared *B. cinerea* infection of wild type and *RIN*-knockout (*RIN-KO*) tomatoes and found that RIN-deficient fruit showed reduced transcription of genes involved in various defence responses affecting *phenylalanine ammonialyase* (*PAL*), *chitinase* (*CHI*), *PRs*, AP2/ERFs, altered cell wall gene transcripts and increased sensitivity to infection by *B. cinerea*. In this study, we compared *B. cinerea* infection of wild type and *RIN*-knockout (*RIN-KO*) tomatoes and found that RIN-deficient fruit showed reduced transcription of genes involved in various defence responses affecting *phenylalanine ammonialyase* (*PAL*), *chitinase* (*CHI*), *PRs*, AP2/ERFs, altered cell wall gene transcripts and increased sensitivity to infection by *B. cinerea*. In this study, we compared *B. cinerea* infection of wild type and *RIN*-knockout (*RIN-KO*) tomatoes and found that RIN-deficient fruit showed reduced transcription of genes involved in various defence responses affecting *phenylalanine ammonialyase* (*PAL*), *chitinase* (*CHI*), *PRs*, AP2/ERFs, altered cell wall gene transcripts and increased sensitivity to infection by *B. cinerea*.
Materials and methods

Plant materials and growth conditions

Two homozygous lines of tomato *RIN*-knockout (*RIN*-KO) mutants produced by the CRISPR/Cas9 gene editing system and used in this study were described by Li et al. (2020). The genome DNA editing details are given in Fig. S1. Wild type (WT) tomato (*Ailsa Craig, AC*) and two *RIN-KO* mutants (*RIN-KO-1, RIN-KO-2*) seedlings were grown under conditions of 16 h/8 h light/dark cycle at 26 °C. Fruit were harvested at breaker+5 (B5) stage and selected for uniform shape, color, size without physical injuries, surface-sterilized with 2 % sodium hypochlorite, washed twice and air-dried at room temperature for further use.

Fungal culture and fruit inoculation

*B. cinerea* (ACCC 36028) was from the Agricultural Culture Collection of China, cultured on potato dextrose agar (PDA) plates at 26 °C, 90 % relative humidity (RH). Spore suspensions was obtained and diluted with sterile water to the required final spore concentration. Fruit were wounded by making a uniform hole (2 mm × 4 mm, wide and deep) on the equator. Then, 5 μL of a 1 × 10⁸ conidia/L *B. cinerea* suspension were pipetted into the hole (using sterile water as control). Fruit were stored at 26 °C , 90 % RH in an artificial climate chamber. Three biological replicates with twenty fruit in each replicate were used and five fruit from each replicate were sampled at 0, 24, 48 and 96 h post inoculation (hpi); 1 cm region (both around and below) of pericarp tissues adjacent to and surrounding the infection site was sampled, frozen rapidly in liquid nitrogen and stored at -80 °C for further use.

Measurement of disease symptoms

The lesion size is measured using crossing method and the percentage of inoculation sites with rot symptoms was also used to calculate disease incidence. Lesion size distribution (%) was rated by lesion diameter (LD) at 96 h hpi. 0 mm < small LD ≤ 14 mm; 14 mm < medium LD ≤ 28 mm; large LD >28 mm.
RNA sequencing (RNA-seq) and bioinformatic assay

RNA extraction

RNA was isolated from pericarp of tomato fruit as described (Jaakola, 2001) with small modifications. 1 g frozen sample was extracted with 500 μL extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8.0), 25mM EDTA, 2.0 M NaCl, 0.5g/L spermidine), 500 μL water-saturated phenol and 20 μL β-mercaptoethanol, mixed well and then centrifuged at 12000 rpm, 4 °C for 10 min. The upper phases were transferred to new tubes, an equal volume of chloroform:isoamylol (24:1) was added and repeated the extraction. One quarter volume of 10 M lithium chloride was added to precipitate the RNA overnight at -20 °C, collect it and centrifuged at 12000 rpm for 20 min at 4°C, the supernatant was removed and the precipitate washed twice with 1mL 70% ice-cold ethanol, then dissolved in 100 μL sterilized RNAase-free water. The genomic DNA was removed by DNase I (Takara) and the RNA quality and quantity confirmed by spectrophotometry (NanoDrop 1000, Thermo Scientific, USA).

RNA-seq and bioinformatic analysis

Total RNA were prepared from both WT and RIN-KO-2 fruit at 0, 24 and 48 hpi for RNA-seq, with three biological replicates for each time point. Pairwise comparison of differentially expressed transcriptome genes (DEGs) of WT (WT_24 hpi compared to WT_0 hpi) and RIN-KO (RIN-KO_24 hpi compared to RIN-KO_0 hpi) fruit were made using a BioManager package. The pair-end sequencing was performed on an Illumina machine by Biomarker (China), the fastq format raw data were pretreated using in-house perl scripts, reads with less than two mismatches were used to construct transcripts using HISAT2 tools, the clean data for this paper have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SUB9451512. The DEGs were selected with the threshold of Fold Change ≥ 1.5 and FDR-corrected P-value < 0.01 in each sample. The transcriptome data was visualized using the pheatmap package.

Gene Ontology (GO) enrichment analysis

GO enrichment analysis was performed using GOseq R packages (Young et al., 2010) on DEGs. Genes were classified into biological process, cellular component and molecular function.
Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

The KOBAS (version 2.0; http://obas.cbi.pku.edu.cn/download.do) was used for KEGG enrichment analysis, then visualized with R Project (R version 3.4.0; https://www.r-project.org/).

Quantitative real-time PCR (qRT-PCR)

Total RNA of 1 μg was used for cDNA synthesis with an RT-PCR reagent Kit (TaKaRa, Japan). qRT-PCR was conducted using FastStart Essential DNA Green Master (Roche, cat. no. 06402712001, Switzerland) with a LightCycler480 (Roche, Switzerland). Relative gene expression levels were measured by the \(2^{-\Delta\Delta Ct}\) method (Livak and Schmittgen, 2001), and samples were normalized using an internal reference gene (Elongation Factor1α, Solyc06g005060) (Chapman et al., 2012). Three biological replicates were performed for each with three technical repeats. The primer pairs are listed in Table S7.

Enzyme activity

Frozen 1 g samples of frozen fruit powder were extracted with 3.6 mL 200 mM borate buffer (pH 8.8), vortexed and centrifuged at 12,000 rpm for 20 min at 4 °C, and 2 mL supernatant samples collected and transferred to new tubes. The PAL (EC 4.3.1.24) activity was measured as described (Yu et al., 2014) with small modifications. The reaction mixture contained 500 μL crude enzyme and 200 μL 50 mM L-phenylalanine (dissolved in 200 mM borate buffer, pH 8.8) in a 1.5 mL tube and was incubated for 1 h without shaking at 37 °C. The reaction was stopped by adding 40 μL 6M HCl. PAL activity was calculated as the release of cinnamic acid using a Microplate Reader (Synergy H1, Bioteck, USA) under 290 nm and expressed as U/(kg•s).

The CHI (EC 3.2.1.14) activity assay kit (BC0825, Beijing Solarbio Science & Technology, China) was used and CHI activity was measured with a Microplate Reader at 540 nm and expressed as U/(kg•s).

Alignments and phylogenetic analysis of AP2/ERFs

Tomato AP2/ERF amino acid sequences were downloaded from the PlantTFDB database v5.0 (http://planttfdb.gao-lab.org/). Alignment of the proteins was made using ClustalX (version 1.8.1) with the neighbor-joining (NJ) method, and visualized using FigTree (version 1.3.1) according to (Zhang et al., 2018b).
Promoter sequence and motif assay

Promoter sequences 2.0 kb in length were obtained from Sol Genomics Network database (https://solgenomics.net/), the CArG-box elements sequences were from Fujisawa et al. (2013). Two putative AP2/ERF binding motifs (GCC-box, ATCTA binding motif) were described in Li et al. (2020).

Statistical analysis

Microsoft EXCEL 2020 and Graphpad Prism 7 were used for statistical analyses.

Dual-luciferase assay

A dual luciferase trans-activation assay was implemented as described (Shi et al., 2017). The coding sequence (CDS) of RIN, TAGL1, ERF.F4 and ERF.F5 without stop codon were cloned into the pGreenII 62SK vector as effector (Hellens et al., 2005). Promoters of cell wall and ERF genes were inserted into pGreenII 0800-LUC double reporter vector constructs fused to the LUC reporter gene, with a REN luciferase driven by 35S promoter as an internal control (Hellens et al., 2005). The constructed effector and reporter plasmids were transfected into A. tumefaciens strain GV3101 separately and coinfected into tobacco (N. benthamiana) leaves (1mL effector mixed with 100 µL reporter). Tobacco plants were grown in a light/dark cycle of 16 h/8 h at 24 °C. The dual luciferase assay kits (Promega, USA) was used to measure LUC/REN activities three days after infiltration. Each assay included three biological replicates.

Results

Knockout of RIN reduces resistance against B.cinerea in ripening tomato fruit

The role of RIN in resistance against B.cinerea was investigated using tomato fruit from WT and two independent RIN-KO mutant lines (RIN-KO-1, RIN-KO-2), produced using the CRISPR/Cas9 gene editing system as reported by Li et al. (2020). Fruit picked at B5 stage were selected for B.cinerea infection. At this stage, both RIN-KO-1 and RIN-KO-2 mutant fruit were deep yellow/orange color, which indicated partial ripening compared to WT fruit which were red (Fig. 1A). 100% disease incidence occured in WT tomato fruit after inoculation with a spore suspension at a concentration of $1 \times 10^8$ conidia/L (Fig. S2). No visible disease symptoms were apparent until 24 hpi, but enlarged lesions were observed at 48 hpi (Fig. 1A). At 96 hpi, lesions in the two RIN-KO lines were an average of 2.6- and 2.8-fold larger compared to WT fruit (Fig. 1B). At 96 hpi, over 30% of the lesions in RIN-KO fruit were classified as large (exceeding 28 mm) but in WT fruit the majority were medium (from 15-28 mm) (Fig. 1C), indicating that RIN-KO fruit were less resistant to B.cinerea than WT fruit.
Analysis of changes in RIN-regulated genes in response to B. cinerea infection

The RIN-KO tomato fruit were much less resistant to B. cinerea than WT, and RNA-seq in WT and RIN-KO fruit was performed to identify genes regulated by RIN, using the threshold of fold change $\geq 1.5$, $q < 0.01$ and FPKM $> 10$, discarding genes expressed at very low level. Significant difference were found in comparison of WT_24 hpi and WT_0 hpi (WT_24 hpi/ WT_0 hpi), and RIN-KO_24 hpi and RIN-KO_0 hpi (RIN-KO_24 hpi/RIN-KO_0 hpi). Analysis of WT_24 hpi/WT_0 hpi identified 567 upregulated and 164 downregulated differentially expressed gens (DEGs) (Fig. 2A and Fig. S3A) and RIN-KO_24 hpi/RIN-KO_0 hpi identified 654 upregulated and 204 downregulated DEGs (Fig. 2A and Fig. S3B). Of these DEG changes, 342 upregulated (Fig. 2A) and 141 downregulated (Fig. 2A) were only found in WT fruit, where RIN is expressed, but not in RIN-KO fruit, which suggests that their enhanced expression in response to B. cinerea infection is dependent on the presence of RIN.

The DEGs affected by B. cinerea in WT (342 upregulated plus 141 downregulated) fruit were classified by Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment assay and were found to be related to 91 (RIN-KO) and 79 pathways (WT) respectively. The top 20 genes are displayed in bubble diagrams in Fig. 2B. In WT fruit, there was a larger number of response genes enriched in the metabolic pathway (Rich Factor (RF) = 0.037), biosynthesis of secondary metabolites (RF = 0.046), and in phenylpropanoid biosynthesis (RF = 0.074) than in RIN-KO fruit (Table S1, Fig. 2B), which suggests that RIN in WT tomato fruit upregulates expression of genes involved in several biochemical pathways associated with plant response and resistance to B. cinerea infection.

Gene Ontology (GO) assay of DEGs revealed potential functions of RIN in cellular component, biological process and molecular function. DEGs specifically occuring in WT (342 upregulated plus 141 downregulated) and RIN-KO (429 upregulated plus 181 downregulated) were categorized by GO enrichment analysis and the top 10 GO terms are shown in Fig. 2C (details in Table S2). Of all the GO enrichment categories, oxidation-reduction processes in biological function ranked the highest in both WT and RIN-KO fruit. Most DEGs in RIN-KO were enriched in cellular component processes, included the cell periphery (100 DEGs), plasma membrane (75 DEGs) and the extracellular region (37 DEGs), as given in Table S2.
RIN mediates effects on PAL and CHI in response to *B. cinerea* infection

The expression pattern of DEGs (FPKM > 10) identified as being enriched in the metabolic pathway, biosynthesis of secondary metabolites and phenylpropanoid biosynthesis categories by KEGG pathway enrichment analysis are shown in Fig. S4, with further details for a total of 82 genes in Table S3. Most (88%) were significantly upregulated in WT fruit in response to infection, but their transcripts were reduced or inhibited in *RIN-KO* fruit after infection, which indicated this response was dependent on RIN (Fig. S4). Most (85%) transcripts of these 82 DEGs reached a peak of expression at 48 hpi, which was consistent with the spread of disease area (Fig. 1A and Fig. S4). Several of these DEGs were classified as encoding enzymes involved in fruit defense, including 2 *PAL* genes (*PAL1, PAL2*) and 4 *CHI* genes (*CHI1, CHI2, CHI3, CHI4*). The increase in expression reached a peak at 48 hpi, but this change did not occur in *RIN-KO* fruit (Fig. 3A, Table S3), indicating their dependence on RIN. Consistent with the gene expression patterns, enzyme activities of PAL and CHI were significantly higher in WT than in *RIN-KO* fruit and they also reached a peak at 48 hpi (Fig. 3B). This result suggests that expression of *PALs* and *CHIs*, and corresponding enzyme activities are increased in response to *B. cinerea* infection which is dependent on RIN.

RIN up-regulates expression of pathogen resistance-related AP2/ERF genes

As mentioned above, expression of 342 genes was up-regulated specifically in WT fruit (Fig. 2A) in response to *B. cinerea* infection. These were selected for further analysis, in order to know more about RIN function. Among these DEGs, 5 PRs and 4 ERFs were significantly upregulated in WT fruit compared to *RIN-KO* fruit (details in Table S4). Expression of 2 PRs, recognized as pathogen response marker genes, and 4 ERFs, reported as being involved in pathogen response, were also measured by qRT-PCR assay using *RIN-KO-1* fruit samples, to verify validity of RNA-seq gene expression data obtained with *RIN-KO-2* fruit samples and compared to responses in WT fruit. Transcripts for the pathogen response marker genes *PR1a* (Sun et al., 2019) and *PR-STH2* (Du et al., 2017) increased markedly after infection and were significantly higher in WT than in *RIN-KO* fruit (Fig. 4A). In addition, the expression of four pathogen resistance *ERFs* (*ERF.A1, Pti5* (also named *ERF.G2*), *Pti6* (also named *ERF.C6*), *ERF.A4*) also increased in WT fruit in response to infection, but showed no change in their expression in infected *RIN-KO* fruit, and transcripts of all these genes reached a peak at 48 hpi (Fig. 4A). In order to know more about the response of *ERFs* to *B. cinerea* infection, a total of 164 AP2/ERF members and their full length protein sequences were obtained to build a phylogenetic tree to understand potential functions (Fig. S5). Among these AP2/ERF genes, 38 had relative higher level transcripts (selected by FPKM > 10) and 19 of these gene transcripts were significantly upregulated in WT fruit during infection comparing to *RIN-KO* fruit, with 84% (16/19)
of them reaching a peak at 48 hpi (Fig. 4B). This result suggests that RIN positively regulates the expression of downstream PRs and ERFs that are involved in fruit response to infection.

**RIN inhibits expression of cell wall gene XTH5**

Several studies have revealed that an aberrant texture phenotype occurs in RIN-KO tomato fruit, which might be explained by altered transcripts of several cell wall related genes and may be related to the potential repressor activity of RIN in WT fruit (Ito et al., 2020; Li et al., 2020). In the present study, the expression pattern of 14 cell wall genes was measured at 0, 24 and 48 hpi (Fig. 5A). Note the striking effect of the presence or absence of RIN on activation of expression of some genes and the repression of others. Consistent with previous reports, several genes encoding cell wall modifying enzymes exhibited significantly higher expression (FPKM > 20) in RIN-KO compared to WT fruit, including XTH5, XTH8 and MAN4a (Fig. 5A). The RNA-seq results were verified by qRT-PCR analysis (Fig. 5B), confirming that in the absence of RIN, the transcripts of XTH5, XTH8 and MAN4a were approximately 3.0-fold (48 hpi), 10.0-fold (24 hpi) and 3.0-fold (0 hpi) higher respectively.

As there are two, three and one potential RIN binding sites (the CArG box) present in the promoters of XTH5, XTH8 and MAN4a respectively (Ito et al., 2008) (Fig. S6), the trans-activation activity of the RIN protein on the XTH5, XTH8 and MAN4a promoters was checked using the dual luciferase system. The RIN protein inhibited (~0.7 fold) the LUC reporter gene driven by XTH5 promoter but did not have any obvious trans-activation or inhibitory effect on either the XTH8 or the MAN4a promoters (Fig. 5C). This indicated that RIN repressed the expression of XTH5 but not XTH8 or MAN4a.

We also asked whether some other repressors might cooperatively participate in the process of modulating cell wall gene expression. Gene transcripts of two members of the class II ERF family containing an EAR motif (repressor domain), ERF.F4 and ERF.F5, were significantly higher in the presence of RIN in WT fruit, compared to RIN-deficient fruit (Fig. 4B), and the results were verified by qPT-PCR assay using RIN-KO-1 fruit sample (Fig. 6A). Previously, ERF.F4 has been identified as a direct target of TAGL1 protein (Zhong et al. 2013; Lü et al., 2018) and ERF.F5 as a direct target of RIN protein, based on the results of whole genome chromatin immunoprecipitation (ChIP) assay (Fujisawa et al. 2013; Zhong et al. 2013; Lü et al., 2018).

Promoter analysis showed that from one to three potential RIN or TAGL1 binding sites (CArG box) are present in the promoters of ERF.F4 and ERF.F5 respectively (Fig. S6). The trans-activation activity of the TAGL1 protein mixed with its protein partner RIN on expression from the promoter of the ERF.F4 gene was analyzed using the transient dual luciferase reporter system. The TAGL1 protein significantly activated the LUC reporter gene when driven by the ERF.F4 promoter (~1.5 fold), whereas RIN itself had no significant trans-activation activity. RIN and TAGL1 together had similar trans-activation effects (~1.5 fold) to TAGL1 alone (Fig. 6B), indicating that TAGL1 directly
targets and positively regulates ERF.F4 expression. It also showed that RIN protein slightly activated the LUC gene driven by ERF.F5 promoter (~1.2 fold) (Fig. 6B). Moreover, when the trans-activation activity of ERF.F4 and ERF.F5 proteins on the XTH5 was checked using the dual-luciferase assay system, ERF.F4 and ERF.F5, was found to significantly inhibit the LUC reporter gene driven by XTH5 promoter (~0.5 fold) respectively (Fig. 6B). Furthermore, three putative AP2/ERF binding motifs (ATCTA) are present in the XTH5 promoter (Fig S6), indicating that ERF.F4 and/or ERF.F5 might directly inhibit the expression of XTH5. This evidence suggests that RIN, together with TAGL1, might repress the expression of XTH5 through activating class II ERFs ERF.F4 and/or ERF.F5.

Discussion
RIN alters PALs and CHIs to improve fruit pathogen resistance

Recently, some key players such as PALs from the phenylpropanoid pathway have been reported to be involved in broad spectrum disease resistance (BSR). A noteworthy example is the PAL genes, which are involved in cell wall mediated immunity and BSR (Ning and Wang, 2018), where suppression or knockdown of PALs in Triticum aestivum (Bhuiyan et al., 2009), Arabidopsis thaliana (Cass et al., 2015; Huang et al., 2010), Brachypodium (Cass et al., 2015), Glycine max (Shine et al., 2016) and Capsicum annum (Kim and Hwang, 2014) enhanced pathogen susceptibility (Yadav et al., 2020). The PAL genes have also been shown to be regulated by TFs involved in pathogen defense, and the PAL genes in Panicum virgatum L., Camellia sinensis, Salvia miltiorrhiza and Leucaena leucocephala were reported to be regulated by PvMYB4 (Shen et al., 2012), CsMYB4a (Li et al., 2017), LlMYB1 (Omer et al., 2013), respectively. In addition, it is also believed that salicylic acid (SA) biosynthesis involves PAL-catalyzed steps, and SA dependent signaling regulates the activation of plant defense to microbial pathogen attack (Delaney et al., 1994; Lu, 2014; Robert-Seilaniantz et al., 2011). For example, silencing of PALs in soybean resulted in pathogen-induced SA biosynthesis turnover as well as an impairment in pathogen resistance (Shine et al., 2016). PAL in maize contributed to resistance against sugarcane mosaic virus, which is highly linked to positive regulation of the SA pathway (Yuan et al., 2019), and SA levels in soybean after Phytophthora sojae infection have been shown to be regulated by GmPAL 2.1 (Zhang et al., 2017). In the present study, with RIN-deficient fruit, transcripts of both PAL1 and PAL2 genes and PAL enzyme activity were clearly reduced compared to levels in WT fruit (Fig. 3), supporting the idea that lack of RIN reduces PAL biosynthesis and activity, which could contribute to greater susceptibility to B.cinerea infection.

Transcripts of CHI1-4 genes and CHI enzyme activity were significantly lower in RIN-KO fruit compared to WT fruit (Fig. 3). This might also contribute to the decreased resistance found in RIN-KO fruit, supporting the hypothesis that RIN enhanced resistance to B.cinerea through promoting PAL and CHI biosynthesis and enzyme activities.
Pathogen-defense PR and AP2/ERF genes are activated by RIN

Plants also respond to pathogens through pathogen-associated molecular patterns-triggered immunity (PAMP-PTI) or effector triggered immunity (ETI) mediated by a combination of secretion of antifungal compounds such as chitinases or chitinase-like proteins (Blanco-Ulate et al., 2016; Irieda et al., 2019; Sánchez-Vallet et al., 2015). Plant chitinase acts not only in glycolytic processing of chitin-containing organisms and their chitinase products, chitooligosaccharides, triggering defense responses, such as Ca\(^{2+}\) signaling, ROS accumulation, MAPK cascades activation, induction of defense gene transcription, and callose deposition (Desaki et al., 2018; Faulkner et al., 2013; Fukamizo T, 2019; Spoel and Dong, 2012).

Host PRs in plant cell walls are induced by B. cinerea attack and function in several ways, for example by hydrolyzing cell walls of fungi (CHI, glucanase, mannanase etc), inhibiting fungal enzymes (PG-inhibitor proteins etc), producing toxicity (thaumatin-like proteins, thionins, phytoalexins etc) and other defence signals (PR-1, PDF1.2 etc) (De Wit and Flach, 1979; Blanco-Ulate et al., 2016). In this study, expression of PRs was induced both by ripening and B. cinerea inoculation (Fig. 4A). Expression of 11 PR genes, but not PRIa (Solyc01g106620) or PRSTH2 (Solyc09g091000), was specifically upregulated in WT fruit after inoculation but not in RIN-KO fruit (Table S5), indicating that RIN participates in activating the expression of genes involved in resistance to B. cinerea infection.

The AP2/ERF genes play critical roles not only in fruit ripening but also in plant resistance against biotic stress. For example, overexpression of ERF1 in tomato fruit enhanced resistance against Rhizopus nigricans (Pan et al., 2013). The tomato ERF2 gene participated in MeJA-mediated disease resistance in tomato fruit through promoting activities of defense enzymes and pathogenesis-related protein (Yu et al., 2018). In this study, the increase in ERF transcripts that occurred in WT fruit after infection was inhibited in RIN-KO fruit. Further, among the 16 AP2/ERF genes which were significantly upregulated and reached a peak at 48 hpi in WT fruit (Fig. 4B), four have been reported previously as being involved in tomato resistance to B. cinerea infection, including ERF.A1, ERF.A4, ERF.C5 (also named Pti5) and ERF.G2 (also named Pti6) (Gu et al., 2002; Ouyang et al., 2016).

Moreover, other ERFs have been shown to function in pathogen resistance, such as ERF.C1 (also named TERF1/JERF2), which is believed to be involved in both plant ethylene-jasmonate (ET-JA) interactions and chitosan (CHT)-induced systemic acquired resistance (SAR) response (Czekus et al., 2020; Lorenzo et al., 2003). Expression of genes included ERF.B3 (also named LeERF4) and ERF.D2 could also be induced by Trichoderma harzianum (strain T22) and aphid Macrosiphum euphorbiae infestation (Coppola et al., 2019) and the ERF.D2 (also named ACE43) gene is required for non-host resistance to Xanthomonas oryzae pv. Oryzae in N. benthamiana (Li et al., 2012). However, the
significance of the correlation between these ERFs and RIN in tomato pathogen related resistance, and the mechanisms involved, are unclear.

Absence of RIN up- and down-regulates transcripts of genes encoding cell wall modifying enzymes that may affect the cell wall barriers to B. cinerea

The plant cell wall not only offers a physical barrier to pathogen access but is also involved in early pathogen recognition and plant defense responses (Cantu et al., 2008b). Usually, ripening fruit soften rapidly, involving cell wall degradation with increased susceptibility to pathogen infection, increasing the possibility of skin damage, which can produce wounds for pathogen entry (Blanco-Ulate, 2016). Compared to WT fruit, the internal structure of RIN-KO fruit visibly loses integrity more quickly, leading to greater sensitive to B. cinerea infection (Ito et al., 2020; Li et al., 2020). From WT and RIN-KO GO analysis, following infection with B.cinerea, 71 genes out of a total of 159 common DEGs in the cellular component category, were downregulated in WT compared with RIN-KO fruit.

29 of these 71 genes had relatively high transcript levels (selected as FPKM >10) and their gene expression patterns are visualized in a heatmap based on RNA-seq data (Fig. S7, Table S6). There was a remarkable difference in expression of 14 cell wall modifying genes in WT and RIN-deficient tomato fruit. In the absence of RIN, CEL2, EXP1, PME1.9, XYL1 and Msid1 were delayed (see also Li et al., 2020). In contrast, XTH5, XTH8 and MAN4a transcripts were enhanced compared to WT (Fig. 5A). Recent studies on the fruit of the spontaneous tomato mutants (cnr, nor, rin) showed that differential expression of MAN4a, TBG4, PL, PG2a, PME1 and PME2 is closely associated with fruit firmness (Adaskaveg et al., 2021). Furthermore, overexpressing SlFSR in the rin mutant (which expresses the RIN-MC repressor) upregulated expression of genes such as PG, TBG4, CEL2, XYL1, PL, PE, MAN1, EXP1 and XTH5, and shortened fruit shelf-life significantly (Zhang et al., 2018a).

Our finding that the expression of XTH5, XTH8 and MAN4a was lower in ripe WT fruit and higher in susceptible RIN-KO fruit after B.cinerea infection (Fig. 5A-5B), supports the suggestion that cell wall disassembly in ripe fruit might be important for infection susceptibility. Although RIN represses transcription from the XTH5 promoter, it had no obvious regulatory effect on XTH8 and MAN4a expression by dual-luciferase assay (Fig. 5C). In Arabidopsis, the homolog of XTH5, AtXTH30, has been documented to have a potential function in the plant cell cycle in triggering plant immune response (Ascencio-Ibanez et al., 2008). In recent research, the Arabidopsis cwm cell wall mutants have been shown to have altered resistance to at least one pathogen compared to WT plants,
indicating specific functions of plant cell wall composition in modulating the plant immune response (Molina et al., 2021). On the other hand, in potato there is a change in xylan content and xyloglucosyl transferase (XTH-Xet5) in response to cell wall organization during plant-virus interaction (Otulak-Koziel et al., 2018). Previous studies recognized that transcripts of LeERF3/LeERF3b (alternatively named ERF.F5), a repressor class ERF gene, accumulate before and decline sharply after the onset of ripening and do not respond to abiotic stresses and wounding (Chen et al., 2008). Also it has been reported that ERF9 (alternatively named ERF.F4) transcripts significantly increase at later stages of tomato development and are negatively correlated with flavonoid biosynthesis (Ye et al., 2015). In this study, both ERF.F4 and ERF.F5 genes responded to pathogen attack in ripening tomato fruit. The possibility that they are directly upregulated by TAGL1 and RIN, respectively, and this inhibits the expression of XTH5 (Fig. 5-6) requires further investigation. The altered properties of the extracellular matrix caused by the absence of RIN might disrupt the deposition and the retention of defence-related proteins as well as antimicrobial compounds, which could accelerate further disassembly of the host cell wall by B.cinerea (Blanco-Ulate et al., 2014). Further experiments are required to clarify how change to specific cell wall components affect fruit disease resistance.

We propose a working model (Fig. 7) where during ripening RIN activates pathogen response genes and also modulates cell wall related genes, which affects the response to B.cinerea. These genes included (I) PALs and CHIs, (II) pathogen response related PR and AP2/ERF genes, and (III) genes encoding or regulating cell wall enzymes such as XTH5, XTH8, MAN4a, MAN1 and TBG4, and two class II ERF family members activated by RIN and involved in XTH5 inhibition. The resulting changes in a cell wall structure and organization may alter the response and susceptibility to pathogen infection.

Conclusions
The tomato fruit TF RIN is a master activator which, in conjunction with ethylene (Li et al., 2020), regulates fruit ripening, but represses expression of some genes involved in cell wall organization and also strengthens resistance to infection of ripening fruit to infection by the B.cinerea. RIN upregulates transcripts of PALs, CHIs and increases corresponding enzyme activities and also activates the accumulation of transcripts of pathogen-resistance PRs and AP2/ERFs. In RIN-deficient fruit, expression of cell wall modifying genes XTH5, XTH8, MAN4a is increased, whereas in WT RIN inhibits XTH5 transcription through interacting with TAGL1, thus activating expression of repressor genes of ERF.F4 and ERF.F5. Further work will be necessary to clarify this unexpected function of RIN and its interaction with other TFs and target genes in response to various biotic stresses.
Accession numbers

Tomato genes sequence from this article can be found in the Sol Genomics Network (https://solgenomics.net/) under the following accession numbers: RIN (Solyc05g012020), TAGLI (Solyc07g055920). Additional accession numbers are present in Supplement Data.

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Credit authorship contribution statement

HZ and RJ performed the experiments, wrote the article, analysed the data, selected, designed and generated the figures; HZ and SL designed the experiments; YS, CS and ZL contributed to the design of some experiments and to the analysis and discussion and presentation of the data; KC participated in the design of the study and provided support for the project and the tomato program; CZ offered the equipment support and contributed to data analysis. IF and DG contributed suggestions and discussion and edited the final document.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Figure legends

Figure 1 Knockout of RIN reduces tomato fruit resistance to *B. cinerea* infection. A. Phenotype of both wild type (WT) and RIN-knockout (RIN-KO) tomato fruit infected with *B. cinerea* at 0, 24, 48, 96 h post inoculation (hpi). Fruit of two RIN-KO lines and WT at the breaker+5 (B5) ripening stage were infected with *B. cinerea* and observed at 24, 48, 72 and 96 hpi. Bar, 1cm. B. Lesion areas in tomato fruit infected with *B. cinerea*. The error bars indicate the standard deviations (SD) value of three biological replicates. C. Fruit lesion size distribution at the 96 hpi time point. The lesion sizes (lesion diameter) were categorized as small (from 0-14 mm), medium (from 15-28 mm), and large (exceeding 28 mm).

Figure 2 Bioinformatics analysis of differentially expressed transcriptome genes (DEGs) in response to *B. cinerea* in both wild type (WT) and RIN-knockout (RIN-KO) tomato fruit. A. Venn diagrams show the different sets of up-regulated and down-regulated DEGs between WT and RIN-KO fruit. B. KEGG enrichment assay of the up- and down-regulated DEGs in WT and RIN-KO tomato fruit in response to *B. cinerea* at 24 hpi compared to 0 hpi. Rich factor (RF) means the number of genes from selected DEGs in a pathway. C. GO functional enrichment analysis of the up- and down-regulated genes in WT and RIN-KO fruit infected with *B. cinerea* at 24 hpi compared to 0 hpi.

Figure 3 Gene expression patterns and changes in phenylalanine ammonialyase (PAL) and chitinase (CHI) enzyme activities. A. Fragment per kilobase per million mapped reads (FPKM) value of PAL and CHI genes in wild type (WT) and RIN-knockout (RIN-KO) fruit. B. Enzyme activities of PAL and CHI in WT and RIN-KO fruit at 0, 24, 48 and 96 hpi. The error bars indicate the standard deviations (SD) value of three biological replicates.

Figure 4 Accumulation of transcripts of pathogen resistance (PR) and AP2/ERF genes in wild type (WT) and RIN-knockout (RIN-KO) tomatoes. A. qRT-PCR assay of pathogen response-related PRs and ERFs; the error bars indicate the standard deviations (SD) value of three biological replicates. B. A heatmap of differentially expressed ERFs which responded positively to *B. cinerea* infection in WT tomato fruit. The FPKM values were transformed to log_{10} value, and displayed with color ranging from blue (low) to red (high).

Figure 5 Cell wall genes expression patterns in wild type (WT) and RIN-knockout (RIN-KO) tomatoes and inhibition/activation of XTH5, XTH8 and MAN4a promoters by RIN. A. Heatmap of expression of 14 genes involved in fruit cell wall metabolism in WT and RIN-KO fruit. B. qRT-PCR assay of XTH5, XTH8 and MAN4a expression in RIN-KO and WT fruit. C. Trans-activation/repression activities of tomato RIN on the XTH5, XTH8 and MAN4a promoters. The error bars indicate the standard deviations (SD) value of three biological replicates. Lowercase letters
indicate significant differences as determined by Student’s t-test (P < 0.01). The FPKM values were transformed to log_{10} value, and displayed with color ranging from blue (low) to red (high).

**Figure 6 Repression of XTH5 transcription by class II ERFs which are activated by RIN.** A. qRT-PCR assay of ERF.F4, ERF.F5 transcripts in wild type (WT) and RIN-knockout (RIN-KO) tomato fruit. B. Trans-activation/repression activities of tomato RIN and TAGL1 on the ERF.F4 and ERF.F5 promoters and ERF.F4 and ERF.F5 on the XTH5 promoter. The error bars indicate the standard deviations (SD) value of three biological replicates. Lowercase letters indicate significant differences as determined by Student’s t-test (P < 0.01).

**Figure 7 Model outlining the role of RIN transcription factor in ripening-associated resistance to infection by B. cinerea.** (I) RIN induces expression of phenylalanine ammonialyase (PAL) and chitinase (CHI) and related enzyme activities. (II) RIN is necessary for activation of the expression of pathogenesis-related proteins (PRs) and AP2/ERFs involved in response to B. cinerea. (III) In wild type (WT) fruit, RIN represses the expression of the cell wall related gene XTH5 by activating ERF family repressors ERF.F4 and ERF.F5. Arrows with solid lines indicate activation, black dotted line indicate repression. The mechanism responsible for inhibition of XTH8 and MAN4a in the presence of RIN (see Fig 5B) is not yet clear.
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Figure 1 Knockout of *RIN* reduces tomato fruit resistance to *B. cinerea* infection. A. Phenotype of both wild type (WT) and *RIN*-knockout (RIN-KO) tomato fruit infected with *B. cinerea* at 0, 24, 48, 96 h post inoculation (hpi). Fruit of two RIN-KO lines and WT at the breaker+5 (B5) ripening stage were infected with *B. cinerea* and observed at 24, 48, 72 and 96 hpi. Bar, 1 cm. B. Lesion areas in tomato fruit infected with *B. cinerea*. The error bars indicate the standard deviations (SD) value of three biological replicates. C. Fruit lesion size distribution at the 96 hpi time point. The lesion sizes (lesion diameter) were categorized as small (from 0-14 mm), medium (from 15-28 mm), and large (exceeding 28 mm).
Figure 2 Bioinformatics analysis of differentially expressed transcriptome genes (DEGs) in response to B. cinerea in both wild type (WT) and RIN-knockout (RIN-KO) tomato fruit. A. Venn diagrams show the different sets of up-regulated and down-regulated DEGs between WT and RIN-KO fruit. B. KEGG enrichment assay of the up- and down-regulated DEGs in WT and RIN-KO tomato fruit in response to B. cinerea at 24 hpi compared to 0 hpi. Rich factor (RF) means the number of genes from selected DEGs in a pathway. C. GO functional enrichment analysis of the up- and down-regulated genes in WT and RIN-KO fruit infected with B. cinerea at 24 hpi compared to 0 hpi.
Figure 3 Gene expression patterns and changes in phenylalanine ammonia-lyase (PAL) and chitinase (CHI) enzyme activities. A. Fragment per kilobase per million mapped reads (FPKM) value of PAL and CHI genes in wild type (WT) and RIN-knockout (RIN-KO) fruit. B. Enzyme activities of PAL and CHI in WT and RIN-KO fruit at 0, 24, 48 and 96 h post inoculation (hpi). The error bars indicate the standard deviations (SD) value of three biological replicates.
Figure 4 Accumulation of transcripts of pathogen resistance (PR) and AP2/ERF genes in wild type (WT) and RIN-knockout (RIN-KO) tomatoes.

A. qRT-PCR assay of pathogen response related PRs and ERFs, the error bars indicate the standard deviations (SD) value of three biological replicates. B. A heatmap of differential expressed ERFs which positively responded to B. cinerea infection in WT tomato fruit. The FPKM values were transformed to log2(24+h), and displayed with color ranging from blue (low) to red (high).
Figure 5 Cell wall genes expression patterns in wild type (WT) and RIN-knockout (RIN-KO) tomatoes and inhibition/activation of XTH5, XTH8 and MAN4a promoters by RIN. A. Heatmap of expression of 14 cell wall genes expression involved in fruit cell wall metabolism in WT and RIN-KO fruit. B. qRT-PCR assay of XTH5, XTH8 and MAN4a expression in RIN-KO and WT fruit. C. Trans-activation/repression activities of tomato RIN on the XTH5, XTH8 and MAN4a promoter. The error bars indicate the standard deviations (SD) value of three biological replicates. Lowercase letters indicate significant differences as determined by Student’s t-test (P < 0.01). The FPKM values were transformed to log10 values, and displayed with color ranging from blue (low) to red (high).
Figure 6 Repression of XTH5 transcription by class II ERFs which are activated by RIN. A. qRT-PCR assay of ERF.F4, ERF.F5 transcripts in wild type (WT) and RIN-knockout (RIN-KO) tomato fruit. B. Trans-activation/repression activities of tomato RIN and TAGL1 on the ERF.F4 and ERF.F5 promoters and ERF.F4 and ERF.F5 on the XTH5 promoter. The error bars indicate the standard deviations (SD) value of three biological replicates. Lowercase letters indicate significant differences as determined by Student’s t-test (P < 0.01).
Figure 7 Model outlining the role of RIN transcription factor in ripening-associated resistance to infection by B. cinerea. (I) RIN induces expression of phenylalanine ammonialyase (PAL) and chitinase (CHI) and related enzyme activities. (II) RIN is necessary for activation of the expression of pathogenesis-related proteins (PRs) and AP2/ERFs involved in response to B. cinerea. (III) In wild type (WT) fruit, RIN represses the expression of the cell wall related gene XTH5 by activating ERF family repressors ERF:4 and ERF:5. Arrows with solid lines indicate activation, black dotted line indicate repression. The mechanism responsible for inhibition of XTH8 and MAN4A in the presence of RIN (see Fig. 5B) is not yet clear.