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Broad-spectrum resistance gene *RPW8.1* balances immunity and growth via feedback regulation of WRKYs

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Summary

Arabidopsis *RESISTANCE TO POWDERY MILDEW 8.1* (*RPW8.1*) is an important tool for engineering broad-spectrum disease resistance against multiple pathogens. Ectopic expression of *RPW8.1* leads to enhanced disease resistance with cell death at leaves and compromised plant growth, implying a regulatory mechanism balancing *RPW8.1*-mediated resistance and growth. Here, we show that *RPW8.1* constitutively enhances the expression of transcription factor *WRKY51* and activates salicylic acid and ethylene signalling pathways; WRKY51 in turn suppresses *RPW8.1* expression, forming a feedback regulation loop. *RPW8.1* and *WRKY51* are both induced by pathogen infection and pathogen−/microbe-associated molecular patterns. In ectopic expression of *RPW8.1* background (*R1Y4*), overexpression of *WRKY51* not only rescues the growth suppression and cell death caused by *RPW8.1*, but also suppresses *RPW8.1*-mediated broad-spectrum disease resistance and pattern-triggered immunity. Mechanistically, WRKY51 directly binds to and represses *RPW8.1* promoter, thus limiting the expression amplitude of *RPW8.1*. Moreover, *WRKY6*, *WRKY28* and *WRKY41* play a role redundant to *WRKY51* in the suppression of *RPW8.1* expression and are constitutively upregulated in *R1Y4* plants with *WRKY51* being knocked out (*wrky51 R1Y4*) plants. Notably, *WRKY51* has no significant effects on disease resistance or plant growth in wild type without *RPW8.1*, indicating a specific role in *RPW8.1*-mediated disease resistance. Altogether, our results reveal a regulatory circuit controlling the accumulation of *RPW8.1* to an appropriate level to precisely balance growth and disease resistance during pathogen invasion.

Introduction

Plant broad-spectrum disease resistance confers resistance against multiple species of pathogens or many races/isolates of the same pathogen. Identification and utilization of genes that confer broad-spectrum resistance is the most effective and environmentally friendly method to reduce the loss of crops, vegetables and fruits caused by diverse pathogens. In recent years, many genes conferring broad-spectrum immunity have been identified and used in breeding programmes. In crops, certain resistance (*R*) genes have been identified to confer broad-spectrum immunity. Most *R* genes encode proteins belonging to the family of nucleotide-binding site-leucine rich repeat (NBS-LRR) immune receptors (NLRs) that recognize pathogen-secreted effectors leading to activation of effector-triggered immunity (ETI) (Cui *et al*., 2015). For example, *Pi50*, *Pigm*, *Pizh*, *Pi2* and *Pi9* are well-known NBS-LRR-encoding genes in rice conferring broad-spectrum resistance against *Magnaporthe oryzae*, the causative agent of rice blast disease (Deng *et al*., 2017; Xie *et al*., 2019). Similarly, *Xa21*, *Xa7*, *Xa23*, *Xa41*(*t*) and *Xa47*(*t*) are *R* genes conferring broad-spectrum resistance against *Xanthomonas oryzae pv. oryzae* (*Xoo*), the causative agent of rice bacterial blight (Chen *et al*., 2021; Chen and Ronald, 2011).

Besides *R* genes, some defence regulators are also involved in the regulation of broad-spectrum immunity. In rice, more than 50 broad-spectrum defence regulators have been characterized. For example, *OsMYB30* improves *bsr-d1*-mediated broad-spectrum blast resistance by activating lignin biosynthesis genes to strengthen cell walls (Li *et al*., 2017). *OsWRKY45* mediates blast resistance conferred by coiled-coil (CC)-NB-LRR protein Pb1 (Inoue *et al*., 2013). Rice cysteine-rich-receptor-like kinases OsCRK6 and OsCRK10 contribute to *OsNPR1* (non-expressor of pathogenesis-related genes 1)-mediated resistance to *Xoo* (Chern *et al*., 2016). In barley, *mlo* is a nonspecific recessive gene conferring broad-spectrum disease resistance to powdery mildew. Because of its excellence in conferring broad-spectrum immunity, the resistant *mlo* allele has been widely used in most high-yield European spring barley for more than four decades (Dreiseitl, 2020).

A series of plant receptor-like kinases (RLKs) and receptor-like proteins (RLPs) act as pattern recognition receptors (PRRs), which recognize pathogen−/microbe-associated molecular patterns (PAMPs/MAMPs) or damage-associated molecular patterns (DAMPs) to induce basal, non-host resistance against multiple pathogens, contributing to broad-spectrum disease resistance. For example, *Pi-d2*, encoding a receptor-like kinase protein, confers gene-for-gene resistance to multiple races of *M. oryzae* (Chen *et al*., 2006). *Bph3*, a locus encoding plasma membrane-localized lectin receptor kinases (OsLecRK1-OsLecRK3), enhances resistance to brown planthopper (BPH) and white-backed planthopper in susceptible rice varieties (Liu *et al*., 2015). *Xa21*, encoding a leucine-rich-repeats serine/

threonine kinase, confers high levels of resistance to most races of *Xoo* (Ercoli *et al*., 2022). In tomato, the *I-3* gene, encoding an S-receptor-like kinase (SRLK), confers resistance against multiple races of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) when incorporated into cultivated tomato from a wild tomato species (Catanzariti *et al*., 2015). In apple, *Vf* genes, encoding receptor-like proteins, represent the best-studied apple scab *R* genes (Malnoy *et al*., 2008); *HcrVf1* and *HcrVf2*, derived from wild species *Malus floribunda* 821, confer scab resistance when expressed in a susceptible cultivar (Belfanti *et al*., 2004; Malnoy *et al*., 2008).

RESISTANCE TO POWDERY MILDEW 8 (*RPW8*) is an *Arabidopsis thaliana* locus identified from wild-type accession Ms-0 containing two naturally polymorphic, dominant *R* genes, *RPW8.1* and *RPW8.2*, which confer resistance to powdery mildew (Orgil *et al*., 2007; Xiao *et al*., 2001). Both *RPW8.1* and *RPW8.2* are atypical *R* genes encoding proteins containing a CC domain homologous to the CC domain of CC-NBS-LRR type R proteins (Xiao *et al*., 2001). A typical RPW8 domain is present in the N-termini of a subclass of NLRs termed *helper NLRs* (*hNLRs*), which are genetically required for the immune signalling of diverse sensor *NLRs* that directly or indirectly recognize their cognate effectors (Jubic *et al*., 2019). For example, *NRG1*, a *hNLR* containing a RPW8 domain, is required for the hypersensitive response (HR) cell death and full oomycete resistance (Castel *et al*., 2019), suggesting an important role for *RPW8* genes and *RPW8* domains in *NLR*-mediated immunity.

Transgenic lines ectopically expressing *RPW8.1* from its native promoter in Col-*gl* background (*R1Ys*) showed greatly enhanced broad-spectrum resistance to powdery mildew fungus, downy mildew oomycete (Ma *et al*., 2014) and *Pseudomonas syringae* bacterium (Li *et al*., 2018) in Arabidopsis. Studies also reveal that *RPW8.1* boosts basal defence responses against multiple pathogens in Arabidopsis, including callose deposition, production of reactive oxygen species (ROS), expression of defence-related genes and hypersensitive response-like cell death with elevated H_2O_2 accumulation (Li *et al*., 2018). Moreover, transgenic rice lines expressing *RPW8.1* display significantly enhanced broad-spectrum resistance against fungal pathogen *M. oryzae* and bacterial pathogen *Xoo* (Li *et al*., 2018). These results demonstrate that *RPW8.1* could be used as an important tool for engineering broad-spectrum disease resistance in plants.

Plant innate immune system contains multiple layers of defence responses to fight against the invasion of diverse pathogens. However, excessive, or prolonged defence responses usually impede growth resulting in yield penalties (Bergelson and Purrington, 1996; Nelson *et al*., 2018). Conversely, inactivation of immune responses easily leads to great yield loss upon pathogen invasion (Shi *et al*., 2013). Therefore, controlling defence responses at a precise level and time is important for plants to maintain normal growth in complicated environments. Plants have developed various regulatory mechanisms to precisely control the growth-immunity trade-off. In recent years, several proteins have been identified as key regulators of growth-immunity trade-offs in plants. In Arabidopsis, BRASSINAZOLE-RESISTANT1 (BZR1) mediates suppression of immune responses when fast growth is required (Lozano-Duran *et al*., 2013). HOMOLOGUE OF BRASSINOSTEROID ENHANCED EXPRESSION 2 (BEE2) INTERACTING WITH IBH1 (HBI1), a bHLH transcription factor, negatively regulates a subset of genes involved in immunity and in turn, PTI signals repress *HBI1* transcription to mediate a trade-off between growth and PTI (Fan *et al*., 2014). Conversely, transcription factor WRKY45 functions to enhance immunity but suppresses growth in rice (Goto *et al*., 2015; Goto *et al*., 2016). Therefore, limiting the expression levels of defence genes to an appropriate level is critical for plants to maintain normal growth. Breeders have selected genes that balance growth and immunity to improve disease resistance without causing yield penalty. In rice, the *Pigm* (*Pyricularia-Gumei*) locus precisely controls a yield-immunity trade-off. PigmS (Pigm Susceptible) interacts with PigmR (Pigm Resistant) to attenuate the immune response and yield penalty conferred by PigmR, thus maintaining yield under blast disease conditions (Deng *et al*., 2017). The Ideal Plant Architecture 1 (IPA1) protein improves both yield and blast disease resistance by maintaining an optimal balance between growth and immunity via switching DNA binding specificity upon phosphorylation or dephosphorylation at a critical residue in the DNA binding domain (Wang *et al*., 2018).

The transgenic *R1Y* lines ectopically expressing *RPW8.1* with its native promoter exhibited a stunted morphology and spontaneous lesions in leaves but enhanced disease resistance (Li *et al*., 2018; Ma *et al*., 2014). In contrast, the wild-type accessions expressing *RPW8.1*, such as Wa-1 and Ms-0, show no cell death (Orgil *et al*., 2007), indicating the existence of a regulatory mechanism to control RPW8.1 to an appropriate level avoiding excessive cell death and growth inhibition in wild-type accessions. In this study, we found that *WRKY51* (*At5g64810*) was constitutively up-regulated in *RPW8.1*-expressing *R1Y* lines but down-regulated in *RPW8.1* mutant lines. We generated transgenic lines to overexpress or mutate *WRKY51* in an *RPW8.1*-expressing line (*R1Y4*) or wild-type accession and examined their disease resistance and defence responses. Our results reveal a regulatory circuit mediated by WRKY51, WRKY6, WRKY28 and WRKY41 that limits *RPW8.1* expression to avoid excessive defence responses and fitness costs.

Results

RPW8.1 boosts *WRKY51* expression and the activation of salicylic acid and ethylene signalling pathways

In this study, we try to explore how plants control the expression of *RPW8.1* to balance the growth and disease resistance. As *RPW8* locus contains *RPW8.1* and *RPW8.2*, we first explored whether *RPW8.2* affected the expression of *RPW8.1*. We mutated *RPW8.2* (*rpw8.2*) in a *RPW8*-contained accession Wa-1 (Orgil *et al*., 2007) using the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology (Figure S1a–c). Intriguingly, *RPW8.1* mRNA level was decreased in *rpw8.2* (Figure S1d), indicating that *RPW8.2*, at least, did not suppress *RPW8.1* expression and there existed other mechanisms controlling *RPW8.1* expression.

To explore how plants control *RPW8.1*-mediated cell death and growth penalty, we conducted RNA-seq experiments to compare the transcriptomic profiles of a transgenic line expressing *RPW8.1-YFP* from the native *RPW8.1* promoter in Col-*gl* background (*R1Y4*) and the Col-*gl* control that does not contain the *RPW8* locus (Table S1). We found that 1197 genes were constitutively up-regulated in *R1Y4* (Table S2), including 16 *WRKYs* (Table S3). *WRKY51* displayed the highest constitutive elevation among the 16 *WRKYs* 1 week post-appearance of the spontaneous cell death (Figure S2a) and constitutively greatly up-regulated (by 10 to 30-fold) in *R1Y4* and *R1Y5* when the

spontaneous cell death appeared (Figure 1a), indicating that *WRKY51* was constitutively elevated by *RPW8.1*.

We generated *RPW8.1* mutants (*rpw8.1*) using the CRISPR/Cas9 technology in *RPW8*-contained accessions Ms-0 and Wa-1 (Figures S3 and S4). Compared to wild-type plants, *rpw8.1* plants expressed significantly decreased mRNA levels (by over threefold) of *WRKY51* (Figure 1a). Moreover, the other 15 *WRKYs* were also constitutively suppressed in the *rpw8.1* mutant compared to the Ms-0 control except *WRKY58* (Figure S2b). These results indicate that the up-regulation of *WRKY51* requires *RPW8.1*.

We also examined the expression of these WRKYs in *RPW8.2*-expressing line *R2Y3* to detect the effect of *RPW8.2* on *WRKYs*. Different from that in *R1Y4*, seven of the 16 tested *WRKYs* were slightly and constitutively elevated in *R2Y3*, but their relative mRNA levels were less than 2-fold of those in the Col-*gl* control (Figure S2c). Moreover, *WRKY51* in *R2Y3* was significantly suppressed (Figure S2c). These results suggest that the up-regulation of *WRKY51* is independent of *RPW8.2*.

It was reported that *RPW8.1* enhances SA signalling and ethylene signalling (Xiao *et al.*, 2003; Xiao *et al.*, 2005; Zhao *et al.*, 2021). Moreover, Gene Ontology (GO) pathway analysis showed that the up-regulated genes in *R1Y4* are most enriched in SA-related pathways: 'salicylic acid biosynthetic process', 'salicylic acid metabolic process' and 'response to salicylic acid' (Table S4; Figure S5a), confirming the prominent role of SA in *RPW8.1*-mediated immunity. We then tested whether the hormone signals were involved in *RPW8.1*-enhanced *WRKY51* expression by examining the marker genes of the two signalling pathways (*PR1* and *PR2* for SA, *ERF1* and *ERF2* for ethylene) in plants with or without *RPW8.1*. Consistently, the mRNA levels of these marker genes were greatly elevated in *R1Y4* compared to the Col-*gl* control without hormone treatment, whereas suppressed in *rpw8.1* compared to the Ms-0 control (Figure S5b,c). However, these marker genes and *WRKY51* were not largely induced by benzothiadiazole (BTH) and ethrel (ETH) treatment in *R1Y4* (Figure S5d) but greatly induced to similar levels comparable to Ms-0 control in *rpw8.1* 12 or 24 h post-treatment (Figure S5d,e). Based on these data, we deduced that *RPW8.1* functioned upstream of SA and ETH to enhance *WRKY51* expression.

WRKY51 suppresses *RPW8.1* expression

To explore whether *WRKY51* was involved in *RPW8.1*-mediated growth penalty and resistance, we generated transgenic lines

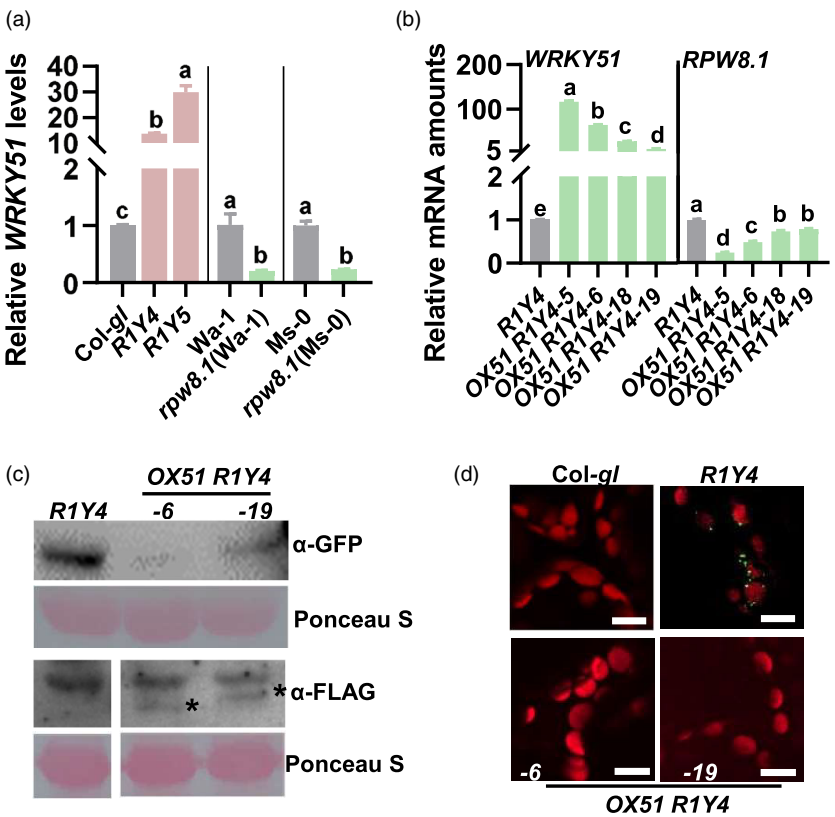


Figure 1 *WRKY51* is up-regulated by *RPW8.1* and feedback-suppresses *RPW8.1* expression. (a) Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) show the relative mRNA levels of *WRKY51* in native promoter-expressed *RPW8.1* transgenic lines (*R1Y4 & R1Y5*) in Col-*gl* background and *rpw8.1* mutants in Ms-0 and Wa-1 background. (b) mRNA levels of *WRKY51* and *RPW8.1* in the transgenic lines *OX51 R1Y4* (35S-expressed *WRKY51* and native promoter-expressed *RPW8.1*) and the *R1Y4* control. (c) Western blots for RPW8.1 protein amount in the presence or absence of *WRKY51* overexpression. Protein was extracted from five-week-old plants for immunoblot analysis using antibodies against green fluorescent protein (GFP) (for RPW8.1) and Flag (for WRKY51) individually. '*' indicates WRKY51-Flag bands. (d) Subcellular localization of RPW8.1-YFP (yellow fluorescent protein) in *OX51 R1Y4* and *R1Y4*. Representative confocal images are acquired from leaves of five-week-old plants. YFP-tagged RPW8.1 protein was pseudo-colored green, and auto-fluorescent chloroplasts were pseudo-colored red. Scale bar = 10 μm. For (a) and (b), data are shown as mean ± SD (n = 3 independent samples). The letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis.

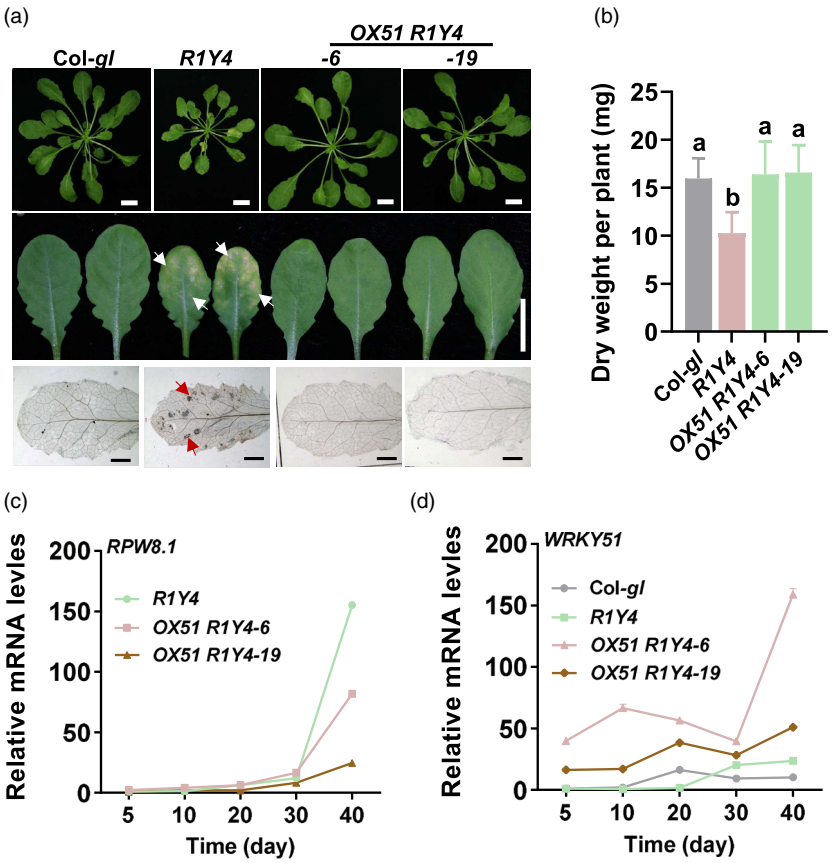


Figure 2 *WRKY51* rescues *RPW8.1*-suppressed plant growth. (a) Representative five-week-old plants and leaves of *R1Y4*, *OX51 R1Y4* and Col-*gl* control. The white arrows indicate necrotic lesions and the red arrows indicate cell death nearby the necrotic lesions. Cell death was stained with trypan blue in the lower panel. Scale bar: 1 cm for upper and middle panels and 1 mm for the lower panel. (b) Dry weights of *R1Y4*, *OX51 R1Y4* and Col-*gl* control. Data are shown as mean ± SD (n = 6 independent samples). The letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis. (c-d) Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) show the relative mRNA levels of *RPW8.1* and *WRKY51* in the indicated lines during the growth period. Data are shown as mean ± SD, n = 3 independent samples.

overexpressing FLAG-tagged *WRKY51* using the 35S promoter in the *R1Y4* background (*OX51 R1Y4*). We obtained more than 20 independent lines and examined *WRKY51* mRNA and protein levels in four independent lines. All tested *OX51 R1Y4* lines displayed markedly elevated *WRKY51* mRNA and protein (probed with FLAG antibody) levels in comparison with *R1Y4* control (Figure 1b,c). Surprisingly, *OX51 R1Y4* showed significantly decreased (down to 20%) mRNA and protein (probed with GFP antibody) levels of *RPW8.1* compared with *R1Y4* control (Figure 1b,c). Consistent with this result, the YFP signals from RPW8.1-YFP in *OX51 R1Y4* were greatly reduced compared to that in *R1Y4* (Figure 1d). These results indicate that *WRKY51* feedback suppresses the expression of *RPW8.1*.

WRKY51 rescues *RPW8.1*-suppressed plant growth and cell death

We then looked at the effects of *WRKY51* on *RPW8.1*-suppressed plant growth and biomass. *R1Y4* showed obviously stunted plant stature and cell death-caused necrotic lesions in leaves compared to Col-*gl* control (Figure 2a). Intriguingly, the stunted morphology, necrotic lesions in leaves and decreased biomass caused by *RPW8.1* in *R1Y4* were completely rescued by *WRKY51* overexpression in *OX51 R1Y4*, which showed normal stature and biomass, as well as leaves without cell death like Col-*gl* control (Figure 2a,b). Consistently, although *RPW8.1* RNA levels were gradually increased in both *OX51 R1Y4* and *R1Y4* control during development, they remained relatively suppressed due to *WRKY51* overexpression, showing lower levels in *OX51 R1Y4* than in *R1Y4* control from day 30 when the cell death-necrotic lesions appeared in *R1Y4* (Figure 2c). In contrast, *WRKY51* RNA levels were remarkably elevated in *OX51 R1Y4* lines than in Col-*gl* and *R1Y4* control from day 30 (Figure 2d), but were suppressed in *rpw8.1* from day 20 compared to the Ms-0 control (Figure S6). These results indicate that, during development, RPW8.1 induces the expression of *WRKY51*, which in turn suppresses *RPW8.1* expression to alleviate *RPW8.1*-mediated cell death and growth penalty.

WRKY51 and *RPW8.1* were simultaneously induced by pathogens and PAMPs

WRKY51 was constitutively up-regulated in *RPW8.1* lines (Figure 1a), in which PTI responses and disease resistance were boosted compared with Col-*gl* control (Li *et al.*, 2018). Intriguingly, RNA-seq data showed that *WRKY51* is responsive to PAMPs chitin and flg22 (Table S1). Based on these results, we predicted that pathogens could induce the expression of *WRKY51* and *RPW8.1* simultaneously to limit the expression amplitude of *RPW8.1*. We first examined the expression of *WRKY51* and *RPW8.1* in wild-type Ms-0 following pathogen inoculation or PAMPs treatment. The expression of both genes was induced by pathogens (powdery mildew or *P. syringae*) and PAMPs (flg22 and chitin) (Figure 3a), indicating that pathogen infection could induce *WRKY51* and *RPW8.1* expression simultaneously.

We then explored *WRKY51* expression in *R1Y4* and Col-*gl* control, and *RPW8.1* expression in *OX51 R1Y4* and *R1Y4* control. Again, *WRKY51* and *RPW8.1* RNA levels were induced in all the

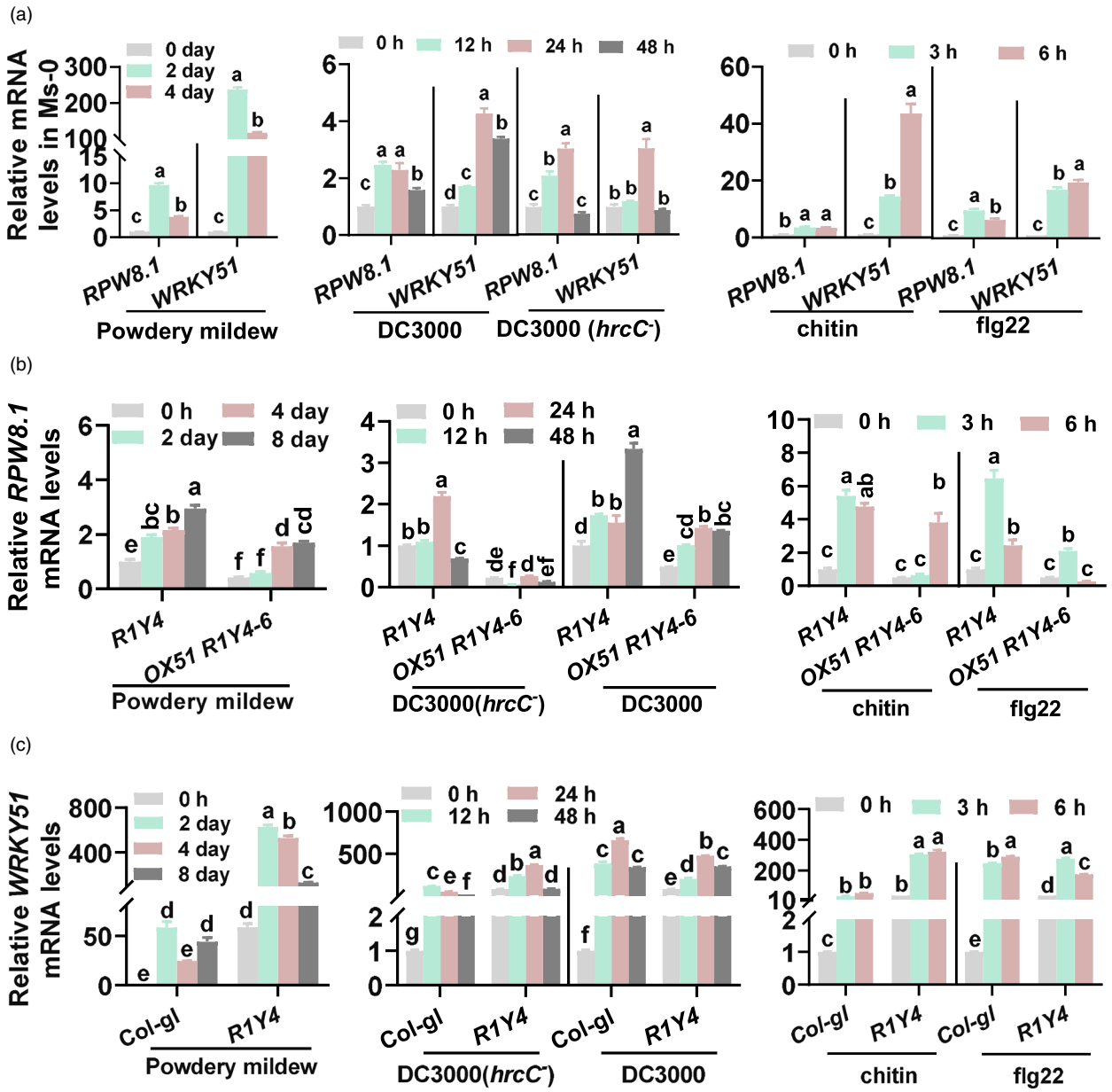


Figure 3 *WRKY51* and *RPW8.1* were simultaneously induced by pathogens and PAMPs. (a) Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) show the relative mRNA levels of *RPW8.1* and *WRKY51* induced by pathogens (powdery mildew and *Pseudomonas syringae*) or PMAPs (flg22 and chitin) in Ms-0 background expressing *RPW8.1*. (b) RT-qPCR show the relative mRNA levels of *RPW8.1* induced by pathogens or PMAPs in *OX51 R1Y4* (containing 35S-*WRKY51* and native promoter-*RPW8.1*) and *R1Y4* (containing native promoter-expressed *RPW8.1*) control. (c) RT-qPCR show the relative mRNA levels of *WRKY51* induced by pathogens or PMAPs in *R1Y4* and Col-*gl* control. In (a), (b) and (c), data are shown as mean ± SD ($n = 3$). The letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis.

examined lines (Figure 3b,c). Two points are worth of noting. First, *RPW8.1* was less induced in *OX51 R1Y4* than in *R1Y4* by pathogens and PAMPs (Figure 3b), implying that *WRKY51* may decrease *RPW8.1* expression during pathogen inoculation. Second, *WRKY51* RNA levels were significantly induced to higher levels in *R1Y4* than in Col-*gl* control by chitin and powdery mildew, but not by flg22 and *P. syringae*, at both time points (Figure 3c), implying that *RPW8.1* has an enhancement effect on *WRKY51* RNA expression and that this effect has a probable preference toward fungi (chitin and powdery mildew) than bacteria (flg22 and *P. syringae*).

WRKY51 suppresses *RPW8.1*-boosted disease resistance and PTI-responses

RPW8.1 expression enhances resistance to fungal pathogen powdery mildew and bacterial pathogen *P. syringae* (Li *et al.*, 2018; Ma *et al.*, 2014). We then assessed whether *WRKY51* could affect *RPW8.1*-mediated resistance. *OX51 R1Y4* supported significantly more bacterial and fungal growth than *R1Y4* control with completely or partially compromised *RPW8.1*-mediated immunity, restoring susceptibility to pathogens to disease levels comparable to wild-type Col-*gl* (Figure 4a-c). These results were

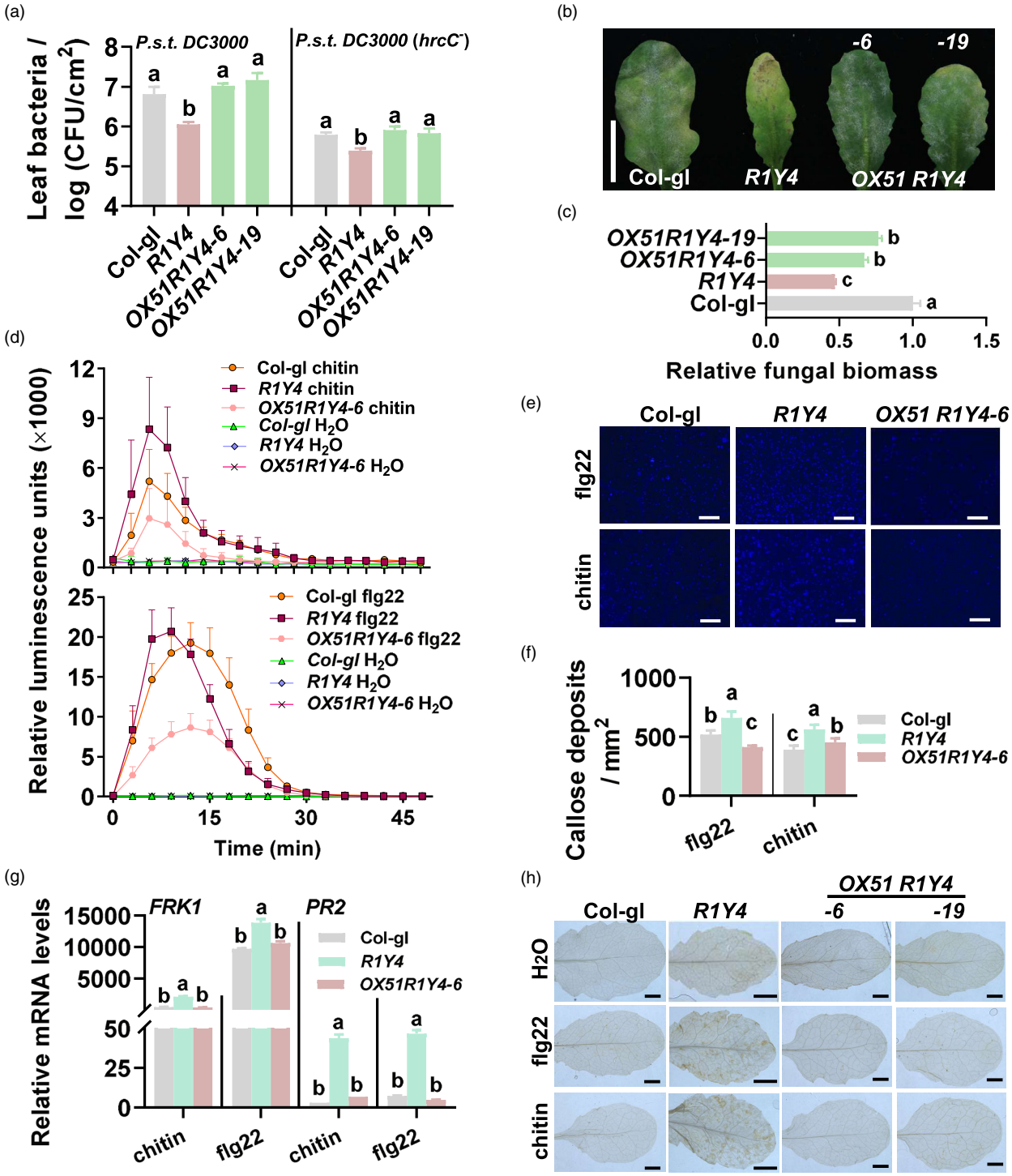


Figure 4 *WRKY51* suppresses *RPW8.1*-mediated resistance and PAMPs-triggered immune responses. (a) Bacterial growth in *OX51 R1Y4* (containing 35S: *WRKY51* and native promoter-expressed *RPW8.1*), *R1Y4* (containing native promoter-expressed *RPW8.1*) and Col-*gl* control 3 days post-inoculation (dpi) with *Pseudomonas syringae* DC3000 or DC3000(*hrcC*⁻). (b) Disease symptoms of indicated lines 10 dpi with powdery mildew. Scale bar = 1 cm. (c) Fungal biomass of powdery mildew from (b). Fungal biomass was examined by quantitative polymerase chain reaction (qPCR) and shown as the ratio of powdery mildew *GDSL-like lipase* DNA against Arabidopsis *actin2* DNA. (d) Reactive oxygen species (ROS) bursts triggered by chitin and flg22 in indicated lines. (e) Callose depositions triggered by chitin and flg22 in *OX51 R1Y4*, *R1Y4* and Col-*gl*. Callose depositions stained by aniline blue appear as bright spots. Scale bars = 0.1 mm. (f) Quantitation of callose depositions in (f). (g) Reverse-transcription qPCR (RT-qPCR) show the relative mRNA levels of *FRK1* and *PR2* in indicated lines (3 h post-treatment (hpt) for *FRK1* and 12 hpt for *PR2*) treated with chitin or flg22. Data were normalized to that of Col-*gl* at 0 hpt. (h) H_2O_2 accumulation in indicated lines 48 hpt with or without chitin or flg22 treatment. 3, 3-diaminobezidin (DAB) was used to stain H_2O_2 (reddish-brown). Scale bar = 1 mm. For a–h, data are shown as mean ± SD (n = 3 independent samples for c and h; n = 4 for a, d and e; n = 6 for g). The letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis.

consistent with the suppressed *RPW8.1* expression in *OX51 R1Y4* lines, and indicate that pathogen-induced *WRKY51* suppresses *RPW8.1*-mediated disease resistance.

We next tested whether *WRKY51* suppressed *RPW8.1*-boosted PTI hallmarks, including reactive oxygen species (ROS) bursts, callose deposition, defence-related gene expression and H_2O_2 accumulation. We found that these PTI hallmarks were affected by *WRKY51* overexpression. First, chitin and flg22 treatments induced higher ROS bursts and more callose depositions in *R1Y4* than in Col-*gl* control. In contrast, chitin and flg22 induced ROS bursts in *OX51 R1Y4* to levels even lower than those in Col-*gl* control (Figure 4d). Second, callose depositions after induction were lower in *OX51 R1Y4* than in *R1Y4* at levels like that in Col-*gl* control (Figure 4e,f). Third, *R1Y4* showed higher RNA levels of two PTI-related marker genes, *FRK1* and *PR2*, than Col-*gl* control upon chitin and flg22 treatments, whereas *OX51 R1Y4* showed RNA levels comparable to those in Col-*gl* control (Figure 4g). Fourth, flg22 and chitin induced less hydrogen peroxide (H_2O_2) accumulation in *OX51 R1Y4* than in *R1Y4* as revealed by 3,3′-diaminobenzidine (DAB) staining (Figure 4h). These results indicate that PAMPs-induced *WRKY51* compromises *RPW8.1*-boosted PTI responses by suppressing PAMPs-induced expression of *RPW8.1*.

WRKY51 directly binds to the *RPW8.1* promoter and represses its activity

As *WRKY51* encodes a transcription factor, we then tested whether WRKY51 protein suppresses *RPW8.1* expression via repressing the activity of the *RPW8.1* promoter by co-expressing RFP-HA-tagged WRKY51 (35S:*WRKY51-HA-RFP*) with a reporter construct containing YFP-tagged RPW8.1 driven by the *RPW8.1* promoter (*R8.1*^{P}:*R8.1-YFP*) containing 1137 bp sequence before the translational start site (ATG) in *Nicotiana benthamiana*. As shown in Figures 5a,b, the RPW8.1-YFP fluorescence intensity and protein level driven by the *RPW8.1* promoter were greatly reduced in the presence of *WRKY51-HA-RFP* compared with HA-YFP, indicating that WRKY51 suppressed *RPW8.1* promoter activity.

Because WRKY proteins are known to bind to the W-box (TTGACC/T) (Rushton *et al.*, 2010), we analysed the *RPW8.1* promoter sequence and found three putative W boxes (TTGACC/T) at −137 to −132 bp, −163 to −158 bp and −274 to −269 bp (Figure 4c). We then tested the *cis*-element on the *RPW8.1* promoter for WRKY51-mediated suppression employing a luciferase reporter assay system in *N. benthamiana*. To this end, we co-expressed *WRKY51* (*35S*:*WRKY51*) with a luciferase reporter driven by wild-type *RPW8.1* promoter (*R8.1*^{P}:*FLUC*) or by a mutated *RPW8.1* promoter (*mR8.1*^{P}:*FLUC*) in which the three W-boxes were mutated. A *YFP* construct (*35S*:*YFP*) was used as a negative control. *Renilla luciferase* (*35S*:*RLUC*) was co-expressed as an internal reference to normalize transfection efficiency. Compared to the YFP control, WRKY51 greatly reduced luciferase expression from the *RPW8.1* promoter at two higher concentrations of the *WRKY51* construct, but did not affect, or even enhance luciferase expression from the mutated promoter (Figure. 5d–f), suggesting that *WRKY51* might bind to the W box and repressed *RPW8.1* promoter activity.

We next conducted DNA affinity purification (DAP)-qPCR and electrophoretic mobility shift assay (EMSA) to validate the binding of WRKY51 to the *RPW8.1* promoter. We selected three fragments with or without putative W-boxes: R8-1 (−206 ~ −49 bp with W1 and W2, two W-boxes), R8-2 (−350 ~ −187 bp with W3, one W-box) and R8-CDS (22 ~ 169 bp without a W-box) (Figure 5c). R8-CDS is localized in exon one and used as a negative control. We conducted DAP-qPCR using a GST-tagged WRKY51 protein expressed in *E. coli*. The DAP-qPCR results showed that R8-1 was significantly highly enriched in the DNA pulled down by GST-WRKY51 compared to the GST control, whereas R8-2 and R8-CDS were not enriched (Figure 6a), indicating that WRKY51 preferentially binds to the W-box in fragment R8-1. To confirm the binding of WRKY51 to the W boxes, we amplified a 58-bp fragment containing W1, a 36-bp fragment containing W2 located near ATG site and a 30-bp fragment containing W3 located far away from ATG site. We also amplified mutated W1 and W2, in which the TTGAC core sequences were mutated to TAAAA. These wild-type probes were tagged with biotin at their 5′-termini (W-biotin). Untagged wild-type and mutant W1 and W2 fragments were used as wild-type (W) and mutant (Wm) competitors, respectively, to compete for binding to WRKY51. Consistent with the DAP-qPCR results, EMSA results showed that GST-WRKY51 bound to W1-biotin moderately and W2-biotin strongly causing a shift-up, respectively. Moreover, the shift-up band was greatly reduced by the addition of unlabeled wild-type competitors, but not by mutated competitors (Figure 6b,c). In contrast, GST-WRKY51 did not cause a shift-up when incubated with W3-biotin (Figure 6d). These results indicate that WRKY51 directly binds to W1 and W2 boxes, but not to W3, in the *RPW8.1* promoter.

WRKY6, *WRKY28* and *WRKY41* play a role redundant to *WRKY51* in the suppression of *RPW8.1*

To further study the role of *WRKY51* on *RPW8.1*-controlled disease resistance and growth, we created homozygous *w51 R1Y4* lines by crossing a T-DNA insertional mutant of *WRKY51* (*w51*, SALK_022198C) with *R1Y4* (Figure S7a,b). Compared to *R1Y4*, *w51 R1Y4* expressed significantly decreased *WRKY51* levels and elevated *RPW8.1* mRNA levels (Figure S7c). However, the protein levels and YFP intensity of *RPW8.1* were almost unchanged in *w51 R1Y4* plants (Figure S7d). Moreover, *w51 R1Y4* displayed a similar phenotype and biomass as *R1Y4* with necrotic lesions and cell death on leaves (Figure 7e,f). Subsequently, *w51 R1Y4* plants displayed the same resistance levels against *P. syringae* and powdery mildew and supported the same pathogen growth levels as *R1Y4* (Figure S8a–c). Moreover, chitin- and flg22-induced callose deposition and H_2O_2 accumulation in *w51 R1Y4* were indistinguishable from those in *R1Y4* control (Figure S8d–f). These results imply the existence of other genes that are redundant to *WRKY51* in regulating *RPW8.1* expression.

We then tested the expression of *RPW8.1*-boosted *WRKYs* in *w51 R1Y4*. Intriguingly, *WRKY6*, *WRKY28* and *WRKY41* mRNA levels were constitutively elevated in *w51 R1Y4* compared to those in *R1Y4* control (Figure 7a), implying a compensatory role of these *WRKYs* for loss of *WRKY51*. To test the role of these three *WRKYs* in the regulation of *RPW8.1*, we co-expressed these *WRKYs* (*35S*:*WRKYs*) individually with *R8.1*^{P}:*FLUC*. Compared to the YFP control, *WRKY6, WRKY28* and *WRKY41* each greatly reduced luciferase expression from the *RPW8.1* promoter, whereas *WRKY54*, a control *WRKY* gene not up-regulated in *w51 R1Y4*, failed to significantly suppress luciferase expression from the *RPW8.1* promoter (Figure. 7b,c). These results suggest that *WRKY6, WRKY28* and *WRKY41* play a role redundant to *WRKY51* in repressing the activity of *RPW8.1* promoter.

Regulation of growth and disease resistance by *WRKY51* depends on *RPW8.1*

To test whether *WRKY51* affects non-*RPW8.1*-regulated growth and disease resistance, we overexpressed *WRKY51* in wild-type

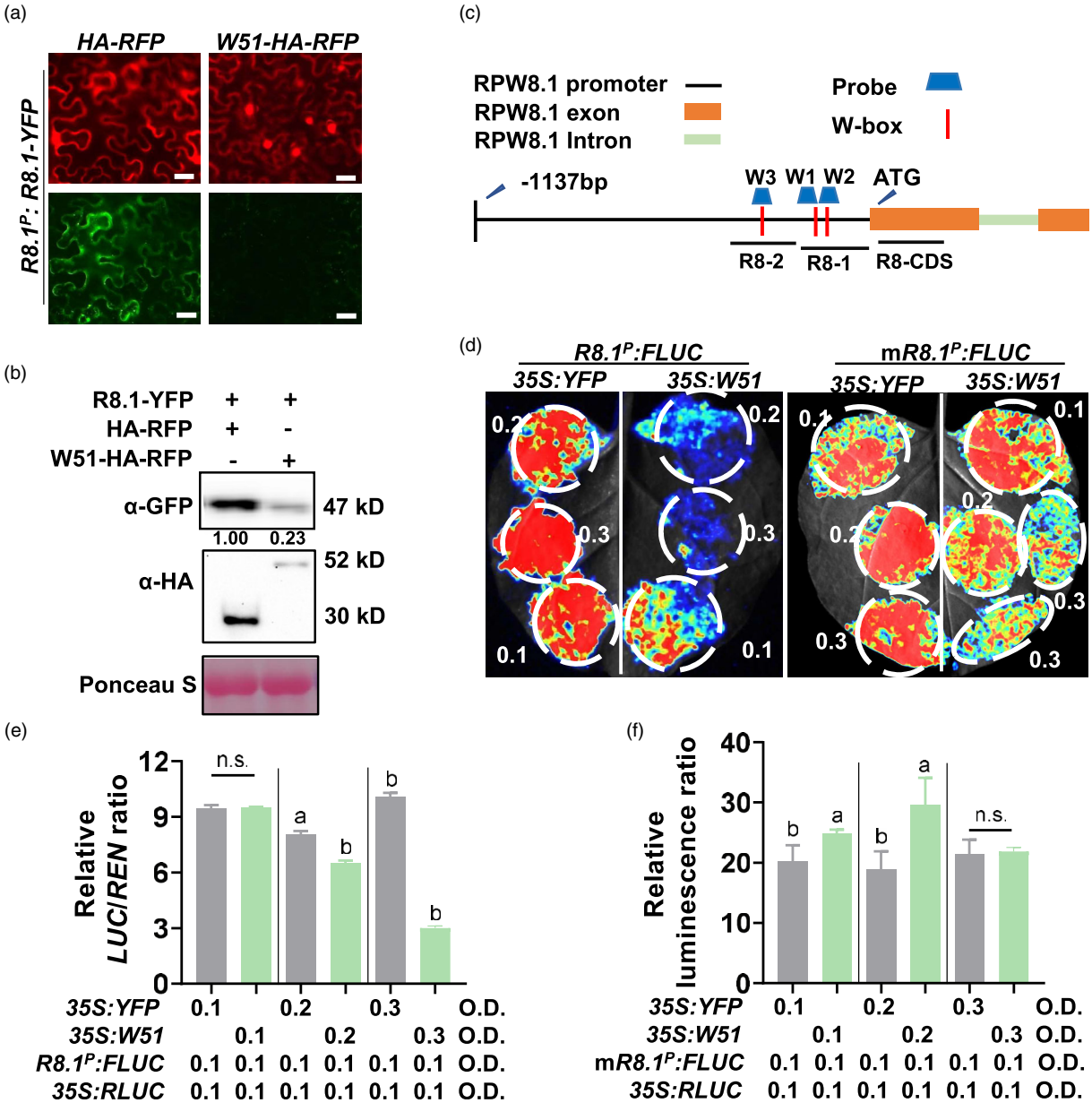


Figure 5 WRKY51 represses the activity of *RPW8.1* promoter. (a-b) Fluorescence intensity (a) and protein amounts (b) of *YFP*-fused *RPW8.1* (*R8.1-YFP*), *RFP*-fused *WRKY51-HA* (*W51-HA-RFP*) and *HA-RFP*. The *RPW8.1* promoter-expressed *R8.1-YFP* construct was transiently co-expressed with construct *35S:HA-RFP* or *35S:WRKY51-HA-RFP* in *Nicotiana benthamiana* leaves via *Agrobacterium*-mediated infiltration. Scale bar = 20 μm. (c) Distribution of fragments and WRKY protein-binding W-boxes in *RPW8.1* promoter. Orange rectangles indicate exons; green rectangle indicates intron; black line indicates promoter region. Short red lines indicate W-boxes. R8-1, R8-2 and R8-CDS indicate the fragments used for DAP-qPCR experiments. Blue trapezoids indicate the probes (W1, W2 and W3) containing W-boxes used in EMSA. ATG: translational start site. (d) Images of firefly luciferase (FLUC) signals expressed from wild-type *RPW8.1* promoter (*R8.1*^{P}) or *RPW8.1* promoter carrying mutated W-boxes (*mR8.1*^{P}). The *R8.1*^{P}*:FLUC* and *mR8.1*^{P}*:FLUC* reporter construct were individually co-expressed with construct *35S:YFP* or *35S:WRKY51* (*35S:W51*) in *N. benthamiana* leaves via *Agrobacterium*-mediated infiltration. LUC light signals were imaged 40 h post-infiltration with *Agrobacterium* at indicated concentrations. *Renilla luciferase* (*RLUC*) was co-expressed as an internal reference to normalize the transfection efficiency. (e-f) Quantitation of firefly luminescence from (d) with different combinations of *Agrobacterium* concentrations. Luminescence signals were detected using a Promega GloMax 96 Luminometer. Data are shown as mean ± SD (n = 3 independent samples). The letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis. 'n.s.' indicate no significant differences.

Col-*gl* accession (*OX51*) and generated *WRKY51* knockout mutant (*w51*) in Col-*gl* background by crossing a *wrky51* mutant (*w51*, SALK_022198C) with Col-*gl* (Figure 8a). Both *OX51* and *w51* lines displayed a normal morphology and dry weight like wild-type Col-*gl* (Figure 8b,c). Upon inoculation with virulent and non-virulent *P. syringae* and powdery mildew individually, both *OX51* and *w51* displayed a similar disease symptom and supported a similar pathogen biomass as Col-*gl* control

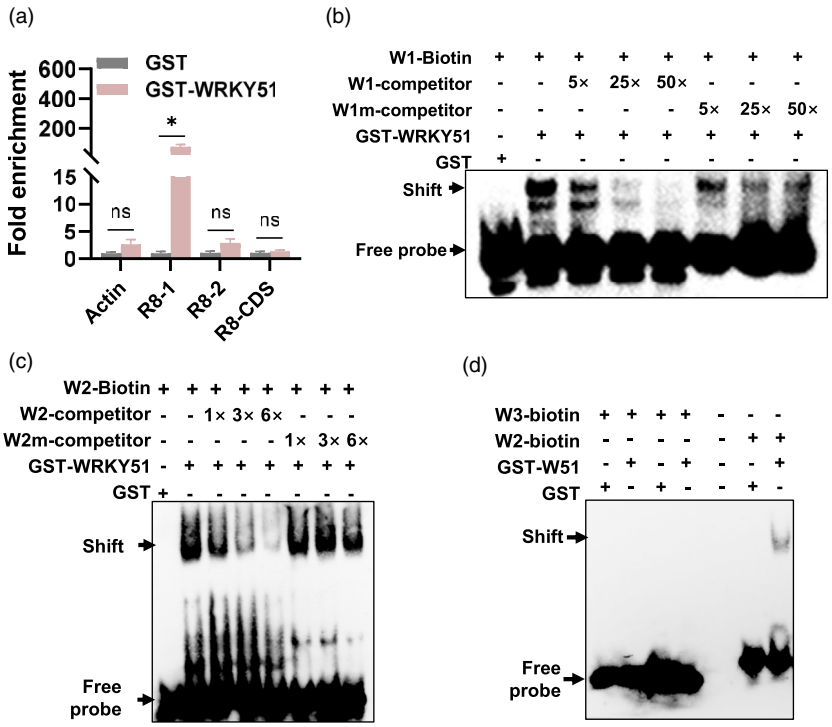


Figure 6 WRKY51 directly binds to the *RPW8.1* promoter. (a) Amounts of R8-1, R8-2 and R8-CDS DNA pulled down by GST-WRKY51 protein or GST control in DNA affinity purification-quantitative polymerase chain reaction (DAP-qPCR). PCR product amounts were normalized to *actin2, R8-1, R8-2, R8-CDS* DNA pulled down by GST, respectively. *Actin2* and *R8-CDS* serve as negative controls. Data are shown as mean ± SD ($n = 3$ independent samples). The star above a bar indicates significant differences at $P < 0.05$ determined by a one-way ANOVA followed by *post hoc* Tukey HSD analysis. (b–d) Binding of WRKY51 protein to probes W1, W2 and W3 in EMSA. W-biotin: W probes labelled with biotin. Unlabeled W1 and W2 were used as competitors in fold excess to probes W1-biotin or W2-biotin as indicated. Wm-competitors: competitors with mutated W-boxes. (–) indicates absence and (+) indicates presence of the reagent.

(Figure 8d–f). Consistently, PAMPs chitin and flg22 induced similar levels of callose deposition and H_2O_2 accumulation in *OX51* and *w51* as in Col-*gl* control (Figure 8g–i). These results indicate that *WRKY51*-mediated regulation of growth and disease resistance depends on *RPW8.1*.

Discussion

Here, we report a regulatory circuit controlling the expression of *RPW8.1* with or without pathogen invasion. *RPW8.1* constitutively enhances *WRKY51* expression, which feedback-suppresses *RPW8.1* expression to an appropriate level avoiding excessive defence responses and cell death. During pathogen invasion, pathogens simultaneously trigger the expression of *RPW8.1* and *WRKY51*. On the one hand, the increased *RPW8.1* level boosts defence responses leading to broad-spectrum disease resistance; on the other hand, the accumulated WRKY51 directly binds to the *RPW8.1* promoter and suppresses *RPW8.1* expression to avoid excessive cell death and growth inhibition. Moreover, *WRKY6, WRKY28* and *WRKY41*, which are also constitutively up-regulated by *RPW8.1*, play a redundant role to *WRKY51* to suppress *RPW8.1* expression. Therefore, plants have evolved a feedback-regulatory circuit to avoid excessive *RPW8.1*-mediated defence responses via enhancing the expression of *WRKYs* (Figure 8j). As a result, plants balance the trade-off between growth and immunity to avoid growth penalties caused by excessive defence responses. These findings provide new insight into the tradeoffs of RPW8.1-mediated immunity with growth and resources for the application of *RPW8.1* to engineer broad disease resistance without growth penalty.

RPW8.1 enhances expression of *WRKYs* associated with the activation of SA and ethylene signalling

We demonstrate that *WRKY51* is induced by PAMPs and pathogens, as well as by *RPW8.1* (Figures 1 and 3). It is intriguing that *RPW8.1* up-regulates a gene that directly represses its transcription in turn. *WRKY51* was boosted in *RPW8.1*-expressing lines with or without pathogen invasion. However, how *RPW8.1* up-regulates *WRKY51* expression remains elusive. RPW8.1 is localized near chloroplasts, whereas WRKY51 is localized in the nucleus. *RPW8.1* encodes an atypical R protein, whose biochemical function remains unknown. RPW8.1 binds to and stabilizes 1-aminocyclopropane-1-carboxylate oxidase 4 (ACO4), an ACC oxidase converting ACC into ethylene; ectopic expression of *RPW8.1* leads to higher ethylene production and activation of ethylene signalling (Zhao *et al.*, 2021). Moreover, *RPW8.1* recruits salicylic acid to activate powdery mildew resistance and HR (Xiao *et al.*, 2005). In this study, we found that *WRKY51* is induced by BTH and ETH in Col-*gl*, *R1Y4* and *rpw8.1* plants, implying that *RPW8.1* enhances the expression of *WRKY51* via downstream SA and ethylene-mediated pathways.

WRKY51 specifically regulates *RPW8.1*-mediated disease resistance

In this study, we identified WRKY51 as a key regulator of *RPW8.1* that controls the amplification of defence responses to an appropriate level to avoid excessive cell death. Intriguingly, WRKY51 specifically participates in *RPW8.1*-mediated disease resistance to balance the trade-off between resistance and growth. Overexpression and knockout of *WRKY51* in non-*RPW8.1* accessions neither affect disease resistance nor penalize plant growth (Figure 8). It is possible that the effects of *WRKY51* are limited to *RPW8.1*-mediated immunity and growth inhibition because WRKY51 directly binds to the *RPW8.1* promoter and controls *RPW8.1* expression.

It was reported that WRKY50 and WRKY51 proteins repress JA signalling, and *WRKY50* and *WRKY51* knockout mutations restore both JA-inducible *PDF1.2* expression and basal resistance to *Botrytis cinerea* (Gao *et al.*, 2011). WRKY51 is a component of a novel JAV1-JAZ8-WRKY51 (JJW) complex that suppresses JA

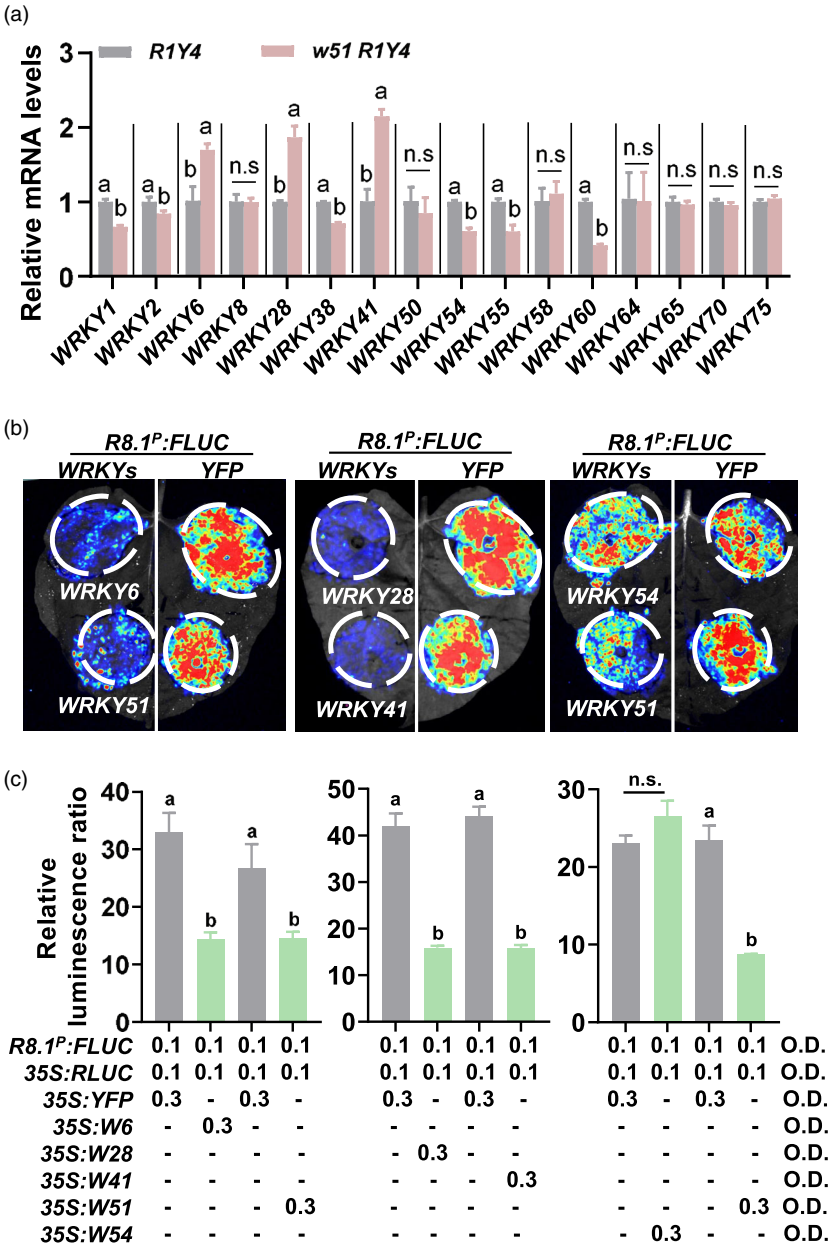
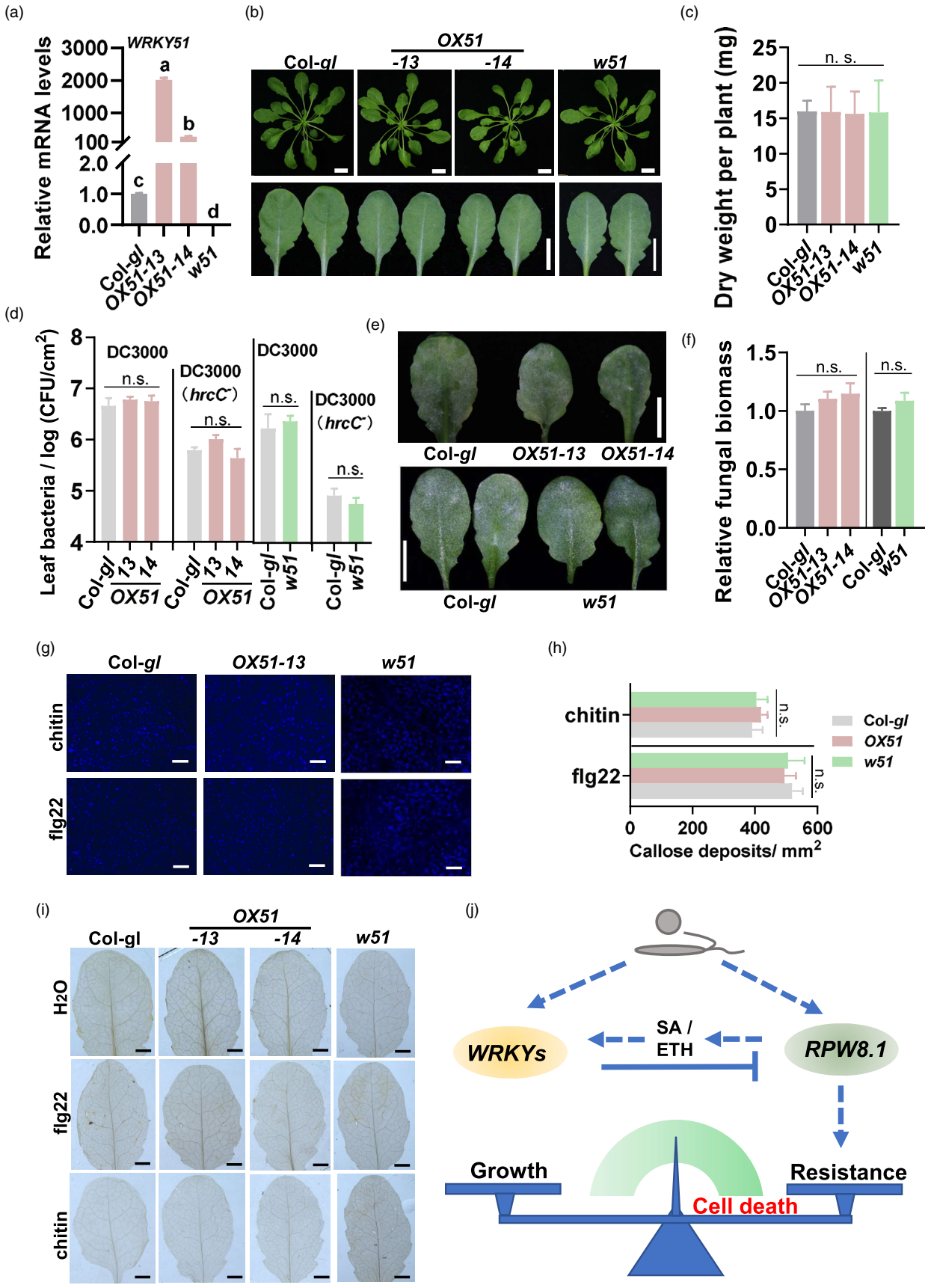


Figure 7 WRKY6, WRKY28 and WRKY41 repress the activity of *RPW8.1* promoter. (a) Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) show the relative mRNA levels of 16 *WRKYs* in *w51 R1Y4* (containing *wrky51* and native promoter-expressed *RPW8.1*) line and *R1Y4* (containing native promoter-expressed *RPW8.1*) control. The letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis. (b) Images of firefly luciferase (FLUC) signals expressed from the *RPW8.1* promoter (*R8.1*^{P}). The *R8.1*^{P}*:FLUC* reporter construct was transiently co-expressed with construct *35S:YFP* or *35S:WRKYs* in *Nicotiana benthamiana* leaves via *Agrobacterium*-mediated infiltration. LUC light signals were imaged 40 h post-infiltration with different combinations of *Agrobacterium*. *Renilla luciferase* (*RLUC*) was co-expressed as an internal reference to normalize the transfection efficiency. (c) Quantitation of firefly luminescence from (b) with different combinations of *Agrobacterium* concentrations. Luminescence signals were detected using a Promega GloMax 96 Luminometer. For a and c, data are shown as mean ± SD ($n = 3$ independent samples). The letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis. 'n.s.' indicate no significant differences.

Figure 8 Regulation of growth and disease resistance by *WRKY51* depends on *RPW8.1*. (a) Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) show the relative mRNA levels of *WRKY51* in *OX51*, *w51* and Col-*gl* control. (b) Morphology of five-week-old plants and leaves of indicated lines. Scale bar = 1 cm. (c) Dry weights of five-week-old seedlings in (b). (d) Bacterial growth in indicated lines 3 days post-inoculation (dpi) with *Pseudomonas syringae tomato* DC3000 and DC3000(*hrcC*$^-$). (e) Disease symptoms of indicated lines seven dpi with powdery mildew. Scale bars = 1 cm. (f) Fungal biomass of powdery mildew in (e). Fungal biomass was examined by quantitative PCR and shown as the ratio of powdery mildew *GDSL-like lipase* DNA against Arabidopsis *actin2* DNA. (g) PAMPs-triggered callose depositions in leaves of indicated lines 18 h post-inoculation (hpi) as stained by aniline blue. Scale bars = 0.1 mm. (h) Quantitation of callose depositions induced by chitin or flg22 in (g). (i) DAB-stained H_2O_2 in leaves of indicated lines treated with or without chitin or flg22 for 48 h. Scale bars = 1 mm. For a, c, d, f and h, data are shown as mean ± SD ($n = 3$ independent samples for a, c and f; $n = 4$ for d; $n = 6$ for h). Letters above bars indicate significant differences at $P < 0.01$ determined by one-way ANOVA followed by *post hoc* Tukey HSD analysis. 'n.s.' indicates no significant difference. (j) A model for the regulatory circuit of *WRKYs* and *RPW8.1* that controls immune response amplitude. Pathogens trigger expression of *WRKYs* and *RPW8.1* simultaneously. On the one hand, elevated levels of *WRKYs* suppress pathogen-induced expression of *RPW8.1* leading to reduced cell death and decreased immune responses. On the other hand, *RPW8.1* also boosts expression of *WRKYs* via SA and ethylene signalling pathway to further limit cell death and immune responses. Thus, plants have evolved a feedback-regulatory circuit where *RPW8.1* enhances expression of *WRKYs*, which further limits *RPW8.1* expression to ensure attenuation of cell death and the balance of growth and immunity.



biosynthesis in the absence of an insect attack; once plants are attacked by insects, the JJW complex is disintegrated, resulting in the activation of JA biosynthesis to enhance plant defence responses against insects (Yan *et al*., 2018). As WRKY51 is up-regulated in *R1Y4* plants, it may be worthwhile to test whether the expression of JA biosynthesis and signalling-related genes were suppressed in *R1Y4*. Nevertheless, Gene Ontology results show that the *RPW8.1*-upregulated genes are enriched in JA biosynthetic process and JA-mediated signalling pathway (Table S4), implying that the regulation of *RPW8.1*-mediated disease resistance and plant growth by WRKY51 is probably independent of JJW complex-mediated signalling.

WRKY51 collaborates with other factors to control activity of the *RPW8.1* promoter

Knockout of *WRKY51* alone has no obvious effects on *RPW8.1*, as *w51 R1Y4* and *R1Y4* plants display similar phenotypes, RPW8.1 levels, disease resistance levels and defence responses (Figures S7 and S8). Thus, *WRKY51* knockout has no clear effects on *RPW8.1*-mediated disease resistance, suggesting the existence of other redundant genes playing a similar role such as *WRKY51* in modulating *RPW8.1*. Consistently, the expression levels of *WRKY6*, *WRKY28* and *WRKY41* were constitutively elevated in the *R1Y4* line (compared to *Col-gl*), and especially in the *w51 R1Y4* line (Figure 7a). Promoter activity analysis reveals that these WRKYs, like WRKY51, can repress *RPW8.1* expression. Moreover, other TFs also have been identified as trans-suppressors of *RPW8.1*. Transient expression of ethylene response factors *ORA59*, *ERF6* and *ERF016* significantly suppress the promoter activity of *RPW8.1*; ORA59 directly binds to the T/A-rich motif in the *RPW8.1* promoter (Zhao *et al*., 2021). Mutations of these TF genes in *R1Y4* result in more severe cell death and ion leakage, as well as more H_2O_2 accumulation upon pathogen infection, supporting their suppressor roles in *RPW8.1*-mediated immune responses (Zhao *et al*., 2021). In summary, WRKY51 collaborates with other factors to control activity of the *RPW8.1* promoter.

Materials and methods

Plant materials and growth conditions

Arabidopsis accessions Col-*gl* (containing a glabrous mutation in Col-0 background), Ms-0, Wa-1 and a transgenic line *R1Y4* expressing *RPW8.1-YFP* in Col-*gl* background (Ma *et al*., 2014) were used. Col-*gl* and *R1Y4* were used to generate *WRKY51* overexpressing lines (abbreviated as *OX51 R1Y4* and *OX51*) and *WRKY51* mutant lines (abbreviated as *w51 R1Y4* and *w51*). Arabidopsis plants were planted in a growth room with 23 °C temperature, 70% relative humidity and a 10−/14-h day/night light setup.

Plant biomass assay

Five-week-old plants were collected and dried in a 42 °C oven overnight. The weight of plants was analysed with three replications (three plants in each replication). Data were analysed by the one-way ANOVA followed by post hoc Tukey HSD analysis with significant differences at $P < 0.01$.

Generation of transgenic plants

To generate transgenic lines overexpressing *WRKY51*, the sequence of *WRKY51* was amplified from Col-*gl* cDNA and cloned into binary vector 35S-pCAMBIA1300. The constructs were introduced into Col-*gl* or *R1Y4* via *Agrobacterium*-mediated floral dipping (Clough and Bent, 1998) to generate transgenic plants *OX51* and *OX51 R1Y4*, respectively. To generate mutants *wrky51*, a *wrky51* mutant (SALK_022198C) was crossed with *R1Y4* and Col-*gl*, respectively. Homozygous *w51* in Col-*gl* background and *R1Y4* background (*w51R1Y4*) were obtained by screening F2 individuals with specific primers *w51*-F and T-DNA primer LB (Table S5). We construct *rpw8.1* mutants using the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology in Ms-0. The two *RPW8.1* guide RNA (gRNA) sequences listed in Table S2 were screened by the Cas-OFFinder system (http://www.rgenome.net/cas-offinder/) to avoid potential off-target sites with the screen parameters to allow less than three base-pair mismatches and one DNA/RNA bulge. For the assembly of two gRNAs, the two target sites were incorporated into PCR primers, respectively. The PCR fragment was amplified from pCBC-DT1T2 for dicot targets with four shorter primers, among which two forward or two reverse primers were partially overlapping. The purified PCR fragment, together with the pHEE401E vector, was used to set up restriction-ligation reactions using BsaI and T4 Ligase (New England Biolabs). The reaction was incubated in a thermocycler for 5 h at 37 °C, 5 min at 50 °C and 10 min at 80 °C. The vector was transformed into Agrobacterium strain GV3101 for Arabidopsis transformation.

Pathogen infection

Five-week-old Arabidopsis plants were syringe-infiltrated with the virulent bacterial strain *P. syringae* DC3000 at the concentration of OD_{600} = 0.0005, or the mutant strain DC3000(*hrcC*$^-$) at OD_{600} = 0.002. Bacterial biomass was determined as previously described 3 days post-inoculation (dpi) (Li *et al*., 2010).

The *Golovinomyces cichoracearum* UCSC1 powdery mildew strain was maintained on *pad4 sid2* plants for the generation of fresh inocula. Photos of mildew disease phenotypes were captured at 10 dpi. Relative fungal biomass of powdery mildew was determined by the relative ratio of DNA levels of the powdery mildew *GDSL-like lipase* gene against the Arabidopsis *actin2* gene via quantitative polymerase chain reaction (qPCR) (Wessling and Panstruga, 2012).

Gene expression assay

Five-week-old Arabidopsis plants were pretreated with PAMPs chitin and flg22 or inoculated with pathogens. PAMPs-treated leaves were collected at 0, 3 and 6 hpi. *P. syringae*-inoculated leaves were collected at 0, 12, 24, 48 hpi. Powdery mildew-inoculated leaves were collected at 0, 2, 4, 8 dpi. Total RNAs were extracted using TRIzol reagent (Invitrogen, Shanghai, China) for gene expression assay by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs were used as templates for reverse transcription using the NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (gDNA Purge) (Novoprotein, Suzhou, China). RT-qPCRs were conducted using SYBR mix (Bioground Biotech, Chongqing, China) and gene-specific primers (Table S2). Arabidopsis *actin2* (*ACT2*) gene is used as the internal reference to normalize expression levels.

Reactive oxygen species (ROS) burst

Leaves of four-week-old plants were sliced into strips and about 10 mm^2 strips were incubated in 200 μL water in a 96-well plate for 12 h. Then ROS bursts were triggered by adding 1 μM flg22 or 20 μg/ mL chitin in 200 μL buffer containing 20 mM luminol, 10 μg/ mL horseradish peroxidase (Sigma-Aldrich Shanghai

Trading Co Ltd, Shanghai, China) and determined as relative luminescence units using a GloMax96 Microplate Luminometer (Promega Biotech Co., Ltd, Beijing, China) for about 45 min.

Western blot assays

Total protein samples were extracted with protein extraction buffer (0.25 M Tris–HCL (pH 6.8), 4% sodium dodecyl sulfate, 0.1% bromophenol blue, 40% glycerol); polyclonal antibodies against GFP (BBI Life Science, Shanghai, China), HA (Roche, Shanghai, China), Flag (Sigma-Aldrich Shanghai Trading Co Ltd, Shanghai, China) were individually used with the Clarity Western ECL Substrate System (Bio-Rad, Chengdu, China) to detect protein levels.

Callose Staining

Leaves of four-week-old plants were infiltrated with 1 μM flg22 or 20 μg/ mL chitin and incubated for 12 h. Then the leaves were discolored and stained with 0.01% aniline blue for 30 min (Hauck *et al*., 2003). Callose depositions were captured with a fluorescence microscope under a UV channel (340 to 380 nm) (Zeiss imager A2.0, Germany) and calculated by using the Image J software (Zhang *et al*., 2007).

H_2O_2 accumulation

Leaves of four-week-old plants were collected and submerged in 0.5 mg/mL 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich Shanghai Trading Co Ltd, Shanghai, China) solution for staining overnight. Then the leaves were decolored with 95% alcohol at 65 °C and pictured under a stereomicroscope (OLYMPUS SZX16, Japan).

Dual-luciferase reporter system

The 1137-bp promoter sequence before the transcription start site of *RPW8.1* was amplified with specific primers (Table S2) and cloned into binary vector pCAMBIA1300 to express firefly *luciferase* (*FLUC*), creating construct *R8.1^P:LUC*, via homologous recombination (Zhao *et al*., 2021). The plasmid was introduced into *Agrobacterium* strain GV3101. An *Aagrobacterium* strain expressing *Renilla luciferase* (*RLUC*) driven by the 35S promoter was used as an internal reference to normalize the transfection efficiency. The *Agrobacterium* strains were syringe-infiltrated into *N. benthamiana* leaves with an *Agrobacterium* strain containing *35S:WRKY51* for transient expression assay. We observed the results at 40 hpi. Leaves were sprayed with 0.15 mg/ ml D-luciferin potassium salt (BioVision, USA) containing 0.01% silwet L-77 for imaging. Intensity of firefly luminescence was detected by a low-light cooled charge-coupled device imaging apparatus (Bio-rad, Chengdu, China). The relative intensity of firefly luminescence was normalized to the Renilla luminescence intensity using a dual-luciferase reporter Kit (Beyotime, China) and measured in a GloMax96 Microplate Luminometer following the manufacturer's instruction.

Electrophoretic mobility shift assay (EMSA)

Biotin-labelled probes were prepared by a company (Sangon Biotech, Shanghai, China). For the binding assay, purified GST-WRKY51 protein was incubated with 1.5 nM specific probes in binding buffer (10 mM Tris, 0.2 mM EDTA, 20 mM KCl, 1% glycerol, 0.02% TritonX-100). For the competition assay, cold competitors (unlabelled probes) were added to the reaction at a concentration of 5×, 25×, or 50× of the labelled probe. The samples were incubated at 25 °C for 20 min. Then the reaction mixtures were separated on a 6% native polyacrylamide gel and transferred to a nylon membrane (Amersham Hybond™-N$^+$, GE, America). Later steps followed the instructions of the Chemiluminescent EMSA Kit (Beyotime, China). Luminescence intensity was detected using a low-light cooled charge-coupled device imaging apparatus. The probe sequences are listed in Table S2.

DNA affinity purification (DAP)-qPCR

The experiment was conducted as described before (Li *et al*., 2017) with some modifications. Generally, we sonicated the *R1Y4* genomic DNA (5 μg) into 200 to 500-bp fragments and added 10 μL sonicated DNA sample to 500 μL F-buffer (50 mM Tris–HCl pH = 8.0, 1 mM EDTA, 100 mM KCl, 5% glycerol, 0.1% Triton, 100 mM DTT) containing purified GST-WRKY51 or GST protein with 50 μL pre-washed Pierce™ Glutathione Agarose resins (ThermoFisher, Shanghai, China). The DNA-protein-agarose mixture was incubated at 4 °C overnight. We collected the agarose resins by spinning at 700 g for 5 min at 4 °C and washed five times with 1× PBS + Triton X-100 (0.005%) and two times with 1× PBS. Beads were then resuspended in 25 μL EB (10 mM Tris–HCl, pH = 8.5) and the samples were heated for 10 min at 98 °C. Samples were spun and the supernatants were kept for further qPCR analysis. The equivalent of 1 μL of immunoprecipitated DNA was then amplified by real-time PCR reactions using gene-specific primers. PCR products were normalized to *actin2*.

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Author contributions

Y. L. and W-M. Wang conceived the experimental design, and together with X-M. Y., J-H. Z., X-Y. X., J-F. S., H. S., Z-W. H., L. X., Y-J. L. and Y. Zhu carried out the experiments; G-B. L., S-X. Z., J-L. L., S-H. B., H. W., Z-X. Z., J-W. Z., J. F. and Y-Y. Huang analysed the data; C. L. and M. Pu carried out the planting; Y. L. and W-M. Wang wrote the paper.

Conflict of interest

No conflict of interest in this study.

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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 Mutation of *RPW8.2* leads to decreased expression of *RPW8.1*.
Figure S2. *RPW8.1* constitutively boosts the expression of *WRKYs*.
Figure S3. *rpw8.1* mutant in Ms-0 background.
Figure S4. *rpw8.1* mutant in Wa-1 background.
Figure S5. *RPW8.1* activates SA and ethylene signalling pathways, which enhances the expression of *WRKY51*.
Figure S6. *WRKY51* is suppressed in *rpw8.1* mutant.
Figure S7. *WRKY51* knockout has no major effects on *RPW8.1*-mediated growth inhibition.
Figure S8. *WRKY51* knockout has no major effects on *RPW8.1*-mediated disease resistance and PTI responses.

Table S1 mRNA reads in *R1Y4* and the Col-*gl* with or without PAMPs treatment.
Table S2. Genes up-regulated in *R1Y4* compared to Col-*gl* control.
Table S3. *WRKYs* up-regulated in R1Y4 compared to Col-gl control.
Table S4. GO analysis of the up-regulated genes in *R1Y4*.
Table S5. Primers used in this study.
Appendix S1 Supporting Information.

