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Original Paper

The *Arabidopsis* ubiquitin ligase *ATL31* is transcriptionally controlled by WRKY33 transcription factor in response to pathogen attack

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Abstract ATL31, an *Arabidopsis* RING-type ubiquitin ligase, plays a critical role in plant carbon/nitrogen (C/N)-nutrient responses during post-germinative growth and in defense responses to pathogen attack. *ATL31* expression under these stress conditions suggested the presence of transcriptional regulators mediated by these stress signals. We recently reported that the expression pattern of *WRKY33*, a transcription factor involved in plant defense responses, is highly correlated with that of *ATL31*. In this study, we investigated the detailed relationship between the *ATL31* gene and WRKY33. Using transient reporter analysis, we found that WRKY33 could significantly activate *ATL31* transcription in plant cells. Transcript analysis of stable transgenic *Arabidopsis* plants overexpressing *WRKY33* confirmed that the expression of *ATL31* in response to the PAMPs flg22 and chitin was enhanced compared with wild-type plants, while expression was repressed in *wrky33* mutants. Further detailed transient reporter analysis revealed that transactivation by WRKY33 is required and mediated through a specific W-box *cis*-acting element in the promoter region of the *ATL31* gene. In contrast, WRKY33 did not regulate *ATL31* expression during the C/N response. Taken together, these results demonstrate that WRKY33 acts as a transcription factor of *ATL31* and positively regulates its expression during activation of plant defense responses.

Key words: Defense response, PAMPs, WRKY transcription factor, ubiquitin ligase.

Plants are affected by abiotic and biotic stresses, with species preservation requiring them to perceive and develop optimal responses to environmental conditions (Atkinson and Urwin 2012). Transcriptional induction of stress-related genes is required for plant adaptation, a process involving the appropriate temporal and spatial binding of transcription factors to DNA sequences present in promoter regions of target genes (Franco-Zorrilla et al. 2014).

WRKY transcription factors are a family of transcriptional regulators, involved in developmental processes and biotic and abiotic stress responses (Bakshi and Oelmüller 2014; Rushton et al. 2010). To date, 72 members of the WRKY family have been identified in *Arabidopsis*. About 60 amino acids are common to the DNA binding domains of all WRKY proteins and contain the sequence WRKYGQK and a zinc-finger structure. The WRKY domain recognizes the *cis*-element W-box with the sequence (T/C)TGAC(C/T). In addition, WRKY transcription factors have been divided into three groups based on the number of WRKY domains and the structure of their zinc fingers (Rushton et al. 2010; Yamasaki et al. 2013).

The transcription factor WRKY33 is an important transcriptional regulator involved in plant defense responses, salt resistance (Jiang and Deyholos 2009) and thermotolerance (Li et al. 2011). Several studies on the role of WRKY33 in plant defense responses have shown that, following the recognition of pathogen-associated molecular patterns (PAMPs) and *Pseudomonas*, MPK4 released from the MKS1-WRKY33 complex stimulates the binding of WRKY33 to the promoter of the *PAD3* gene to positively regulate the synthesis of the antimicrobial protein camalexin (Andreasson et al. 2005; Baccelli et al. 2014; Qiu et al. 2008). In addition, WRKY33 has been shown to positively regulate plant defenses against necrotrophic pathogens through other mitogen-activated protein (MAP) kinase pathways. Thus, WRKY33 can be activated via MPK3/6 to induce camalexin biosynthesis and the *ACS2*/*ACS6* genes for ethylene production (Li et al. 2012; Mao et al. 2011). Moreover, nuclear-encoded sigma factor binding proteins (SIBs) 1 and 2 interact with the C-terminal WRKY domain of WRKY33, which enhances the DNA

Abbreviations: PAMPs, pathogen-associated molecular patterns; W-box, WRKY DNA binding element; MAP kinase, mitogen-activated protein kinase; C/N-nutrient, carbon/nitrogen-nutrient.

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binding activity of WRKY33 to its defense-related target genes against necrotrophic pathogens (Lai et al. 2011). WRKY33 has also been reported to bind to its own promoter, suggesting a feedback regulation for plant resistance to pathogen attack (Mao et al. 2011).

The *Arabidopsis Tóxicos en Levadura* (*ATL*) gene family encodes plant-specific putative RING-type ubiquitin ligases with a transmembrane domain and is composed of 91 and 121 members in *Arabidopsis* and rice, respectively (Aguilar-Hernández et al. 2011; Guzmán 2012; Koiwai et al. 2007). ATL31 and its closest homolog ATL6 have been reported to play important roles in the carbon/nitrogen (C/N)-nutrient response by regulating the stability of 14-3-3 proteins through ubiquitination (Sato et al. 2009, 2011). We also recently found that both *ATL* genes are involved in plant defense responses (Maekawa et al. 2012). For example, *ATL31* gene expression is strongly induced in response to PAMPs and pathogen infection; furthermore, *ATL31* expression was found to highly correlate with the expression of the transcription factors, *WRKY33* and *WRKY53* (Aoyama et al. 2014; Maekawa et al. 2012). Moreover, we demonstrated that *ATL31* gene is transcriptionally up-regulated under C/N stress conditions and in senescent leaves, with the close correlation with the expression of the senescence related-transcription factor *WRKY53* (Aoyama et al. 2014). However, it still remains unclear how *ATL31* is transcriptionally regulated during plant defense responses.

In this study, we demonstrate that WRKY33 positively regulates *ATL31* expression in *Arabidopsis* cells via a specific W-box *cis*-acting element in the *ATL31* promoter. In addition, our analyses of responses to bacterial and fungal PAMPs, flg22 and chitin, as well as to *Pseudomonas* bacteria in plants overexpressing *WRKY33* and those with the *wrky33-1* mutant provided genetic evidence suggesting that WRKY33 plays a positive role in plant disease resistance by promoting *ATL31* expression.

Materials and methods

Plant materials and growth conditions

Wild-type Columbia-0 and all other materials used in this study were grown under conditions described previously (Yasuda et al. 2014). The *wrky33-1* mutant (SALK_006603; Zheng et al. 2006) was obtained from the Arabidopsis Biological Resource Center (ABRC) (Ohio State University, Columbus, OH, USA).

Generation of transgenic plant

Full-length cDNA fragments of the *WRKY33* gene (At2g38470) were amplified by PCR using the primers described in Supplementary Table S1 and pUni vector (U87915, ABRC) as a template. These fragments were sequenced and introduced into the vector pENTR/D-TOPO (Life Technologies) to generate the plasmid *pENTRWRKY33*. Full-length *WRKY33* was subsequently recombined into the binary vector pGWB5 (Nakagawa et al. 2007), as described by the manufacturer (Life Technologies), placing the *WRKY33* gene under the control of the CaMV 35S promoter (*p35S-WRKY33*). The fusion plasmid was used to transform *Agrobacterium tumefaciens* pGV3101 (pMP90) by electroporation, and then used to transform *Arabidopsis thaliana* ecotype Columbia-0 as described (Yasuda et al. 2014). All PCR products and inserts were verified by DNA sequencing.

Gene expression analysis

RNA was isolated from leaf tissue using Trizol reagent (Invitrogen), treated with RQ1 RNase-Free DNase (Promega), and reverse transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen). PCR amplification, using the primers described in Supplementary Table S1 and normalized cDNA samples, was performed as described (Yasuda et al. 2014). The number of amplification cycles ranged from 19–30, depending on the primer sets. PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. Quantitative real-time PCR was performed using SYBR Premix EX Taq (TAKARA), the primers described in Supplementary Table S1 and a Stratagene Mx3000P instrument (Agilent Technologies), using the protocol described by the manufacturer. Signals were normalized relative to those for 18S rRNA.

Transient C/N stress treatment

Arabidopsis plants were grown on modified MS medium containing 100 mM glucose (Glc) and 30 mM nitrogen (N) (10 mM NH_4NO_3 and 10 mM KNO_3) for 2 weeks after germination and transferred to C/N medium containing 100 mM Glc/30 mM N or 300 mM Glc/0.3 mM N. Plants were harvested at the indicated times after C/N treatment, and transcript levels were quantitated.

Plasmid constructions and transient protoplast transfection

To generate plasmids for transient protoplast analysis, the relevant *ATL31* promoter containing the 5′ ATG upstream regions were amplified by PCR and introduced into the *Hin*dIII–*Bam*HI sites of pBI221 (Jefferson 1987). Overlapping PCR was used to generate the mutant promoter of the *ATL31* gene, in which the sequence TGA CC was changed to CCG GG. For the effector constructs, the plasmid *pENTRWRKY33* was introduced into the vector pUGW2 (Nakagawa et al. 2007), as described by the manufacturer (Life Technologies), with the *WRKY33* gene under the control of the CaMV 35S promoter. Primers used for PCR amplification of promoters and effector are summarized in Supplementary Table S1. The orientation and precise insertion of all constructs were verified by sequencing.

Protoplasts were prepared from *Arabidopsis* T87 suspension cells subcultured for four days (Axelos et al. 1992), as described

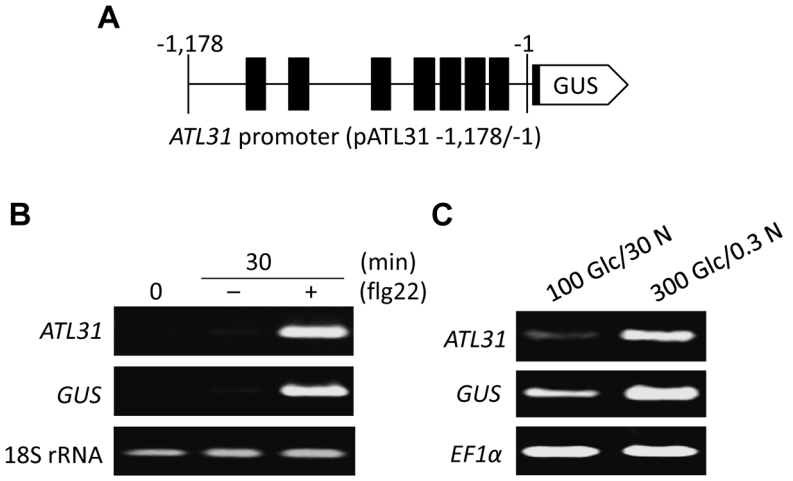

Figure 1. (A) −1,178 bp promoter fragment from *ATL31* is sufficient for high gene expression in response to bacterial PAMPs and C/N-nutrient stress. (A) Construction of a plasmid containing the *ATL31* promoter (-1,178/-1) fused to the *GUS* gene (*pATL31-GUS*). Boxes in black are the putative W-box elements in the *ATL31* promoter. (B) *ATL31* expression of *pATL31-GUS* plants in response to flg22. RT-PCR analysis of endogenous *ATL31* and *GUS* mRNA transcripts in leaves of 2-week-old *pATL31-GUS* plants treated without (−) or with (+) 1 μM flg22 for 30 min. 18S rRNA was used as an internal control. (C) *ATL31* expression of *pATL31-GUS* plants in response to disrupted C/N-nutrient conditions. RT-PCR analysis of endogenous *ATL31* and *GUS* in *pATL31-GUS* plants treated for 24 h in medium containing 100 mM glucose (Glc)/30 mM N (control medium) or 300 mM Glc/0.3 mM N. *EF1α* was used as an internal control.

(Aoyama et al. 2014). Transfected protoplasts were transferred into 3.5 cm petri dishes, incubated under dim light at 22°C for 15 h and lysed. The soluble extracts were split; one half was used for analysis of reporter-GUS activity, while the other half was used for normalization. GUS activity was normalized relative to the amount of transformed reporter-GUS plasmid. Protoplasts transfected with the reporter construct alone was used as controls. Data are shown as the mean±SD of three biological replications. Predicted *cis*-elements in the *ATL31* promoter were searched for using the PLACE database (http://www.dna.affrc.go.jp/PLACE/index.html) (Higo et al. 1999).

Treatment with PAMPs

Crab shell chitin and flg22 (Sigma Genosys) were dissolved in distilled water. Five microliters of crab shell chitin (200 μg ml^{-1} containing 0.05% agar) or flg22 (1 μM containing 0.05% agar) were dropped onto the first to fourth true leaves of 2-week-old *Arabidopsis* plants. Control plants were similarly treated with equivalent amounts of distilled water containing 0.05% agar.

Treatment of plants with pathogens

Pseudomonas syringae pv. *tomato* DC3000 was grown overnight at 27°C in NYGB liquid medium [0.8% (w/v) nutrient broth, 0.2% (w/v) yeast extract, 0.2% (w/v) K_2HPO_4, 0.05% (w/v) KH_2PO_4, 0.5% (w/v) glucose, 0.025% (w/v) $MgSO_4$]. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgCl_2$ to a final density of 10^5 colony-forming units (cfu) ml^{-1}. Leaves of 3-week-old *Arabidopsis* plants were infected with bacteria and sampled 24 h later.

Results

The promoter region of ATL31 *is a putative target of WRKY33*

We previously reported that *ATL31* expression is enhanced in response to both C/N-nutrient stress and pathogen attack (Aoyama et al. 2014; Maekawa et al. 2012). We also found W-box like elements (T/C)-TGAC(C/T) in the 5′-upstream sequence of the *ATL31* coding region (pATL31 -1,178/-1) (Aoyama et al. 2014) and that WRKY53 may be a direct transcription factor of the *ATL31* gene, responsible for disrupting C/N-nutrient stress in *Arabidopsis* plants (Aoyama et al. 2014). However, the transcription factor mediating the induction of *ATL31* in response to pathogen attack remains unclear. *ATL31* expression was found to highly correlate with expression of the transcription factor *WRKY33*, especially in plant defense responses (Maekawa et al. 2012, Supplementary Figure S1). WRKY33 is activated after perception of PAMPs by phosphorylation cascades, suggesting an essential role of WRKY33 in early plant defense responses (Denoux et al. 2008; Qiu et al. 2008; Wan et al. 2004). Since WRKY33 binds to W-box elements of its own promoter and promotes its transcription (Mao et al. 2011), transcriptionally correlated genes may be putative targets of WRKY33.

We therefore cloned the fragment -1,178/-1 of the *ATL31* promoter (pATL31 -1,178/-1) from the *Arabidopsis* genome and prepared transgenic plants expressing the *β-glucuronidase* (*GUS*) gene under the control of the *ATL31* promoter (*pATL31 -1,178/-1-GUS*) (Figure 1A). Transcription analysis confirmed that the pATL31 -1,178/-1 region was sufficient to promote the expression

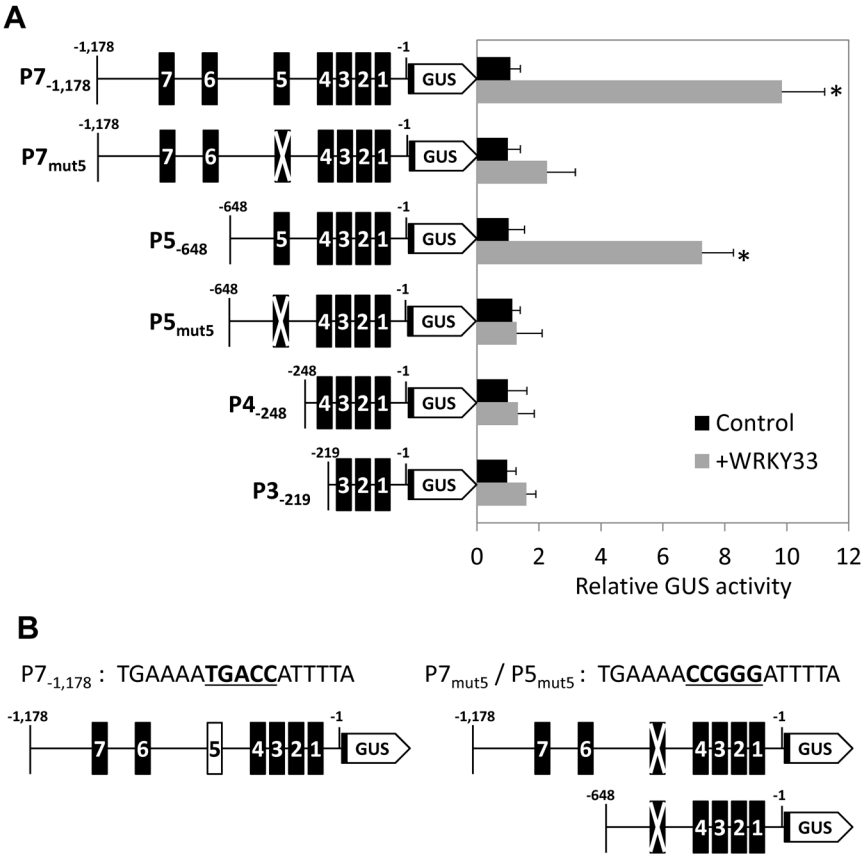

Figure 2. Transcriptional activation of *ATL31* by WRKY33 in *Arabidopsis* protoplast cells. (A) Transient reporter analysis was performed using plasmids containing a 5′ deletion and mutations of the *ATL31* promoter fused to the *GUS* gene. Plasmids containing each *ATL31* promoter and the *WRKY33*-coding region were co-transfected into protoplast cells. Each *ATL31 promoter-GUS* construct is schematically shown on the left. The positions of the W-box elements (T/C)TGAC(C/T) within the promoter are shown in numbered black boxes, and mutations in the W-box elements are indicated by white X's in the left-hand scheme. GUS activity was measured after 15 h incubation and normalized to transfection efficiency. The negative control consisted of cells transfected with *ATL31 promoter-GUS* without *WRKY33* effector and set to 1. Means±SD of relative GUS activity from three independent experiments are shown. Asterisks indicate significant differences compared with the negative control by Student's *t*-test ($p<0.05$). (B) Nucleotide sequences of W-box 5 and its mutation that abolishes binding and function. The putative *cis*-element in W-box 5 is in bold and underlined. "$P7_{-1,178}$" indicates the wild-type sequence while "$P7_{mut5}$/$P5_{mut5}$" indicates the mutated version of W-box 5.

of *GUS* in response to the bacterial flagellin-derived peptide flg22 (Figure 1B). The expression of *GUS* transcript was also increased by C/N-nutrient stress (300 mM Glc/0.3 mM N), consistent with previous results showing that the pATL31 -1,178/-1 region was sufficient for transcriptional promotion by WRKY53 (Aoyama et al. 2014) (Figure 1C). To assess whether WRKY33 directly promotes *ATL31* transcription, we performed transient gene expression assays in protoplast cells. Co-transfection of *pATL31 -1,178/-1-GUS* (*$P7_{-1,178}$-GUS*) with *WRKY33* resulted in a significant increase in GUS activity when compared with co-transfection of the *$P7_{-1,178}$-GUS* construct with an empty effector vector (Figure 2A), suggesting that WRKY33 protein promotes *ATL31* transcription.

Determination of a specific W-box motif essential for ATL31 *transcription by WRKY33*

To further investigate the mechanism regulating *ATL31* expression, we explored the *ATL31* promoter region necessary for its transcriptional activation by WRKY33. There are seven putative W-box elements in the promoter region. The promoter activity of various deletion constructs of the *ATL31* promoter was tested by transient GUS reporter analysis (Figure 2A). A deletion 5′ to position -648 ($P5_{-648}$; containing W-box 1 to 5) resulted in the retention of strong GUS activity, similar to that of $P7_{-1,178}$. Further deletions, to positions -248 ($P4_{-248}$; containing W-box 1 to 4) and -219 ($P3_{-219}$; containing W-box 1 to 3) almost completely abolished the GUS activity (Figure 2A). These results suggest that the fifth element (W-box 5) is an essential *cis*-element for *ATL31* transcriptional activation by WRKY33.

To determine the importance of W-box 5 for WRKY33 recognition, we also evaluated the effect of its mutation, in which the W-box motif TGA CC was changed to CCG GG (Figure 2B). Mutation of W-box 5 in either $P7_{-1,178}$ ($P7_{mut5}$) or $P5_{-648}$ ($P5_{mut5}$) was sufficient to abolish the promoter activity (Figure 2A). These data indicate that W-box 5 in the promoter of the *ATL31* gene is

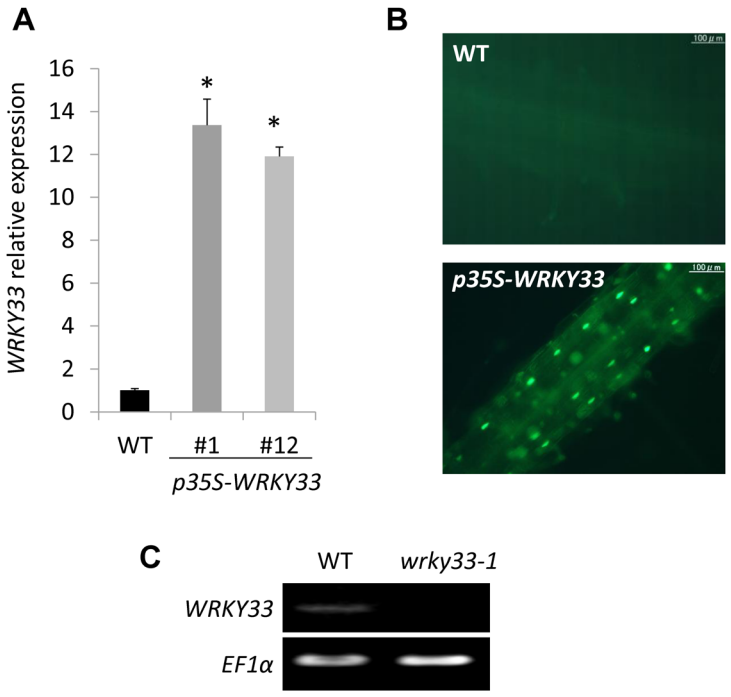

Figure 3. Characteristics of transgenic *Arabidopsis* plants overexpressing the *WRKY33* gene and loss- of function mutants. (A) *WRKY33* transcripts by quantitative RT-PCR. mRNA was purified from WT and two independent lines of *p35S-WRKY33* (#1 and #12) plants. All analyses were performed in triplicate and results normalized to the expression of 18S rRNA. Expression level in WT was set to 1. Means±SD are shown. Asterisks indicate significant differences compared with WT by Student's *t*-test ($p<0.05$). (B) Localization of the WRKY33-GFP fusion protein. GFP fluorescence was observed in the root tissue of wild-type (WT) and *p35S-WRKY33 Arabidopsis* plants. Bar=100 μm. (C) *WRKY33* expression in *wrky33-1* mutant plants. *WRKY33* expression was analyzed by RT-PCR, with *EF1α* expression used as an internal control.

critical for its transcriptional regulation by WRKY33.

PAMP-inducible ATL31 *transcription is reciprocally affected in a* WRKY33 *overexpressor and a mutant wrky33*

To evaluate the physiological involvement of WRKY33 in *ATL31* transcription, we investigated the *ATL31* expression pattern in plants overexpressing *WRKY33* and in those with a *wkry33* loss-of function mutant. Transgenic *Arabidopsis* plants overexpressing *WRKY33* fused with green fluorescent protein (GFP) were generated (*p35S-WRKY33*). Isolation of *p35S-WRKY33* was confirmed by transcript analysis showing higher *WRKY33* expression level than in wild-type (WT) plants (Figure 3A). GFP fluorescence of WRKY33-GFP protein in *Arabidopsis* root tissue showed nuclear localization (Figure 3B), as previously described (Lippok et al. 2007), confirming the successful overexpression of *WRKY33* in *p35S-WRKY33 Arabidopsis* plants. We also evaluated *WRKY33* transcription in the *wrky33-1* mutant using a set of primers that spans the entire open reading frame (Figure 3C), as described (Mao et al. 2011). Since *WRKY33* is up-regulated by flg22 (Qiu et al. 2008) and by chitin, a polysaccharide found in fungal cell walls (Wan et al. 2004), as well as the *ATL31* gene (Maekawa et al. 2012), we evaluated the effect of these PAMPs on the transcription of *ATL31* in *p35S-WRKY33* and *wrky33-1* mutant plants. We found that *ATL31* transcripts in WT were increased 9-fold by flg22 treatment, similar to the up-regulation of the *GST1* gene, a typical flg22-responsive marker gene (Figure 4A and B). *ATL31* expression in *p35S-WRKY33* plants was further increased by flg22 treatment, approximately 30-fold (Figure 4B). In contrast, *ATL31* expression was repressed in the *wrky33-1* mutant, with the levels of expression similar in flg22 and mock-treated plants (Figure 4B), clearly demonstrating that WRKY33 is required for *ATL31* transcription in response to flg22 in *Arabidopsis* plants.

Similar transcriptional alteration of *ATL31* by WRKY33 was observed in response to chitin, a fungal PAMP. The level of expression of the chitin responsive gene *MPK3* was increased 15 min after chitin treatment (Figure 5A). *ATL31* expression in WT plants was up-regulated 2-fold by chitin treatment, but was increased 10-fold in *p35S-WRKY33* plants (Figure 5B). In contrast, chitin treatment did not increase *ATL31* expression in *wrky33-1* mutant plants (Figure 5B). These results suggest that *ATL31* transcription is rapidly promoted in response to PAMPs derived from both fungi and bacteria under WRKY33 control.

WRKY33 regulates ATL31 *expression in response to pathogenic bacteria*

Since we previously found that ATL31 plays a role in

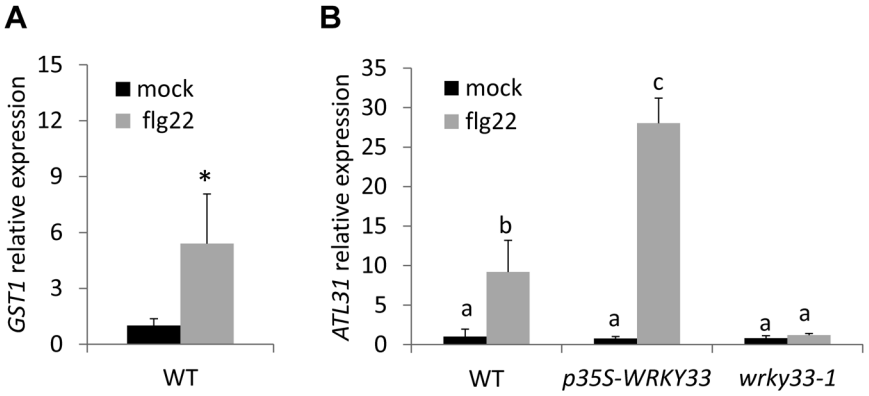

Figure 4. *ATL31* expression in *WRKY33* overexpressor and *wrky33* mutant plants in response to flg22. (A) Expression of *GST1* in WT plants 15 min after treatment with 1 μM flg22. (B) Expression of *ATL31* in WT, *p35S-WRKY33* and *wrky33-1* mutant plants treated with 1 μM flg22 for 15 min. Expression in seedling leaves was analyzed in triplicate by quantitative RT-PCR and normalized to expression of 18S rRNA. Expression level in WT (mock) plants was set to 1. Means±SD are shown. Asterisk indicates significant differences compared with mock treatment by Student's *t*-test ($p<0.05$). Letters indicate significant differences ($p<0.05$) determined by ANOVA, followed by post hoc Tukey test.

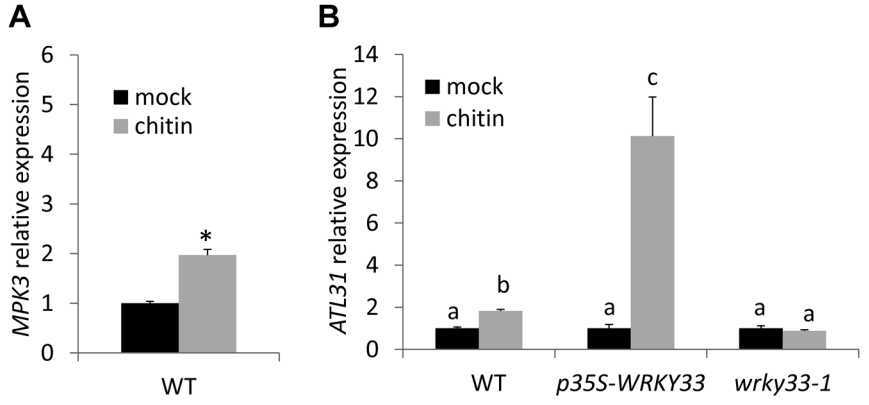

Figure 5. *ATL31* expression in *WRKY33* overexpressor and *wrky33* mutant plants in response to chitin. (A) Expression of *MPK3* in WT plants 15 min after treatment with 200 μg ml^{-1} crab-shell chitin. (B) Expression of *ATL31* in WT, *p35S-WRKY33* and *wrky33-1* mutant plants treated with 200 μg ml^{-1} crab-shell chitin. Expression in seedling leaves was analyzed in triplicate by quantitative RT-PCR and normalized to expression of 18S rRNA. Expression level in WT (mock) plants was set to 1. Means±SD are shown. Asterisk indicates significant differences compared with mock treatment by Student's *t*-test ($p<0.05$). Letters indicate significant differences ($p<0.05$) determined by ANOVA, followed by post hoc Tukey test.

basal immunity against pathogens (Maekawa et al. 2012, 2014), we examined the relationship between ATL31 and WRKY33 in response to the bacterial pathogen *Pst*. DC3000. WT, *p35S-WRKY33* and *wrky33-1* mutant plants were inoculated with *Pst*. DC3000, and transcripts of the *GST1* and *ATL31* genes were measured for up to 24 h by quantitative RT-PCR (qRT-PCR), with the *GST1* gene used as a defense marker in response to *Pst*. DC3000 (Figure 6A). *ATL31* transcripts were increased 8-fold in WT plants challenged with bacterial *Pst*. DC3000. In comparison, *ATL31* transcripts were markedly increased approximately 15-fold in *p35S-WRKY33* plants, but only slightly in *wrky33-1* mutants, after pathogen infection (Figure 6B). These results indicate that *ATL31* is positively regulated by WRKY33 in response to *Pst*. DC3000 infection. Since *ATL31* promotion was completely repressed by flg22 in the *wrky33-1* mutants (Figure 4B), the slight increase in *ATL31* expression observed in response to *Pst*. DC3000 in these mutants suggests the involvement of a flg22-independent pathway that affects *ATL31* expression via an unknown transcription factor derived from *Pst*. DC3000 infection.

WRKY33 is not required for transcriptional activation of ATL31 *in C/N-nutrient responses*

We previously demonstrated that the *WRKY33* gene is transcriptionally activated by C/N-nutrient stress conditions, with an expression pattern similar to *ATL31* in WT plants (Maekawa et al. 2012). We therefore evaluated the effect of C/N-nutrient conditions on the expression of *ATL31* in *wrky33-1* mutant plants.

Arabidopsis plants were grown in normal C/N medium containing 100 mM glucose and 30 mM nitrogen for 2 weeks and then transferred to the same C/N medium (control conditions) or modified medium containing 300 mM glucose and 0.3 mM nitrogen (high C/low N). One h after transfer, *ATL31* transcript levels were measured by qRT-PCR. The level of *ATL31* transcripts

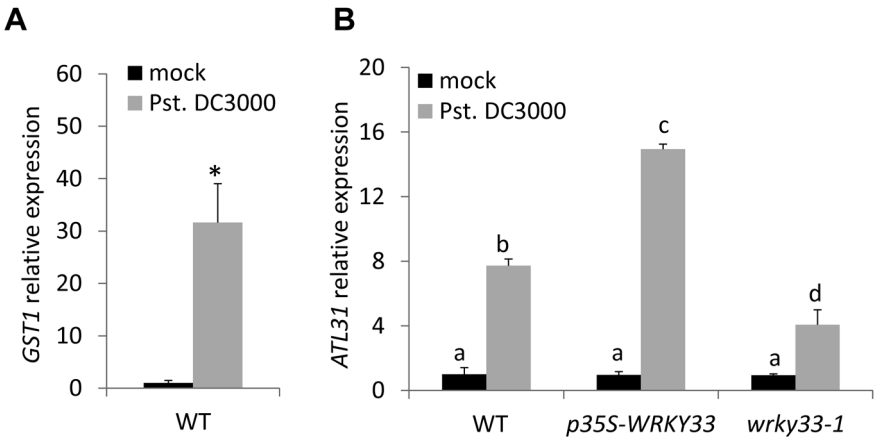

Figure 6. *ATL31* expression in *WRKY33* overexpressor and *wrky33* mutant plants in response to *Pst*. DC3000 infection. (A) Expression of *GST1* in WT plants 24 h after infection with *Pst*. DC3000. (B) Expression of *ATL31* in WT, *p35S-WRKY33* and *wrky33-1* mutant plants inoculated with *Pst*. DC3000. Leaves of 3-week-old plants were inoculated with the bacterial leaf pathogen *Pst*. DC3000. Total RNA was extracted from leaves, analyzed in triplicate by quantitative RT-PCR and normalized to the level of 18S rRNA. Expression level in WT (mock) was set to 1. Means±SD are shown. Asterisk indicates significant differences compared with mock treatment by Student's *t*-test ($p<0.05$). Letters indicate significant differences ($p<0.05$) determined by ANOVA, followed by post hoc Tukey test.

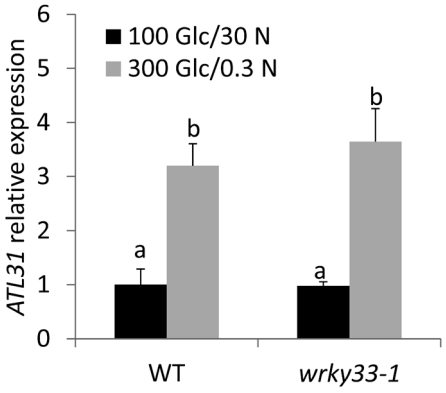

Figure 7. *ATL31* expression in *wrky33* mutant plants in response to C/N-nutrient stress. Expression of *ATL31* in WT and *wrky33-1* mutant plants was analyzed in triplicate by quantitative RT-PCR and normalized to expression of 18S rRNA. mRNA was purified from whole plants 1 h after transfer to C/N medium containing 100 mM Glc/30 mM N (control medium) or 300 mM Glc/0.3 mM N from control medium. Expression level in WT grown in control medium was set to 1. Means±SD are shown. Letters indicate significant differences ($p<0.05$) determined by ANOVA, followed by post hoc Tukey test.

in WT plants were increased approximately 3-fold under high C/low N than under control conditions. Similar findings were observed in the *wrky33-1* mutant (Figure 7), suggesting that WRKY33 does not play a major role in the transcriptional regulation of *ATL31* in plant responses to C/N-nutrient stress.

Discussion

ATL31 *is transcriptionally up-regulated by WRKY33 in plant defense responses*

This study identified WRKY33 as a transcription factor that up-regulates *ATL31* expression in plant defense responses. A specific W-box site was also identified in the *ATL31* promoter for activation by WRKY33. *Arabidopsis* plants overexpressing *WRKY33* showed higher up-regulation of *ATL31* expression than WT plants after PAMP treatment or bacterial infection. In *wrky33-1* mutants, however, this up-regulation was completely repressed in response to PAMPs and partially repressed in response to pathogen infection. Thus, these findings indicate that WRKY33 positively regulates *ATL31* expression during plant defense responses.

Since the activity of WRKY33 is regulated via phosphorylation by MAP kinases during pathogen signaling (Buscaill and Rivas 2014; Ishihama and Yoshioka 2012; Meng and Zhang 2013; Zhang et al. 2002), it suggests that upstream of the transcriptional regulation of *ATL31* by WRKY33 may be regulated by MAP kinase signaling components after recognition of PAMPs and *Pst*. DC3000. Consistent with this hypothesis, the levels of *ATL31* transcription in plants overexpressing *WRKY33* were rapidly increased after treatment with PAMPs but not in their absence. Thus, post-translational regulation of WRKY33 activity is likely required to activate the expression of *ATL31* in response to PAMPs. Taken together, these findings reinforce the hypothesis that *ATL31* is controlled by WRKY33 through MAP kinase cascades involved in pathogen signaling. Further studies are needed to identify the upstream signaling components of WRKY33 in order to understand the detailed mechanism of defense via ATL31 ubiquitin ligase.

Physiological significance of WRKY33 and ATL31 in plant defense responses

WRKY33 has been shown to directly regulate enzymes, such as PAD3 and CYP71A13, related to the biosynthesis of the antimicrobial camalexin (Birkenbihl et al. 2012; Lai

et al. 2011; Mao et al. 2011; Qiu et al. 2008). However, little is known about other direct downstream targets of WRKY33 in defense responses. Recently, we reported that ATL31 is involved in plant defense responses against both bacterial and fungal pathogens (Maekawa et al. 2012, 2014). ATL31 is a membrane localized ubiquitin ligase involved in vesicle trafficking with PEN1/SYP121 SNARE protein. *ATL31* overexpressors showed increased callose deposition and papilla formation specific to the cell wall, thus protecting plant cells from fungal infection, leading to increased resistance to nonadapted powdery mildew fungus (Maekawa et al. 2014). These findings suggest a new aspect of WRKY33 function in plant defense responses. On the other hand, WRKY33 was shown to play an essential role in resistance to necrotrophic pathogens, such as *Botrytis cinerea* and *Alternaria brassicicola*, but was not critical for resistance against biotrophic pathogens, including *Pst*. DC3000 (Zheng et al. 2006). We found that, in *wrky33-1* mutants, enhanced *ATL31* expression in response to *Pst*. DC3000 was partially repressed, but was completely cancelled after treatment with flg22, a PAMP derived from bacteria. Thus, as yet additional unknown transcription factor(s) may be associated with *ATL31* expression at different stages of pathogenesis.

Transcriptional regulation of ATL31 *in biotic and abiotic stress response*

We recently reported that *ATL31* is highly up-regulated under high C/low N growth conditions, with similar expression patterns observed for *WRKY33* and *WRKY53* (Aoyama et al. 2014; Maekawa et al. 2012). This study, assessing the function of WRKY33 in C/N-responsive *ATL31* expression, found that WRKY33 does not play a major role in C/N-responsive transcriptional regulation, since *ATL31* transcript levels in *wrky33-1* mutants were not affected by high C/low N stress condition. The *ATL31* promoter region contains several W-box elements that can also be recognized by WRKY53 (Aoyama et al. 2014). While reporter analysis showed that WRKY33 requires a specific W-box element to promote *ATL31* transcription (this study), WRKY53 could promote *ATL31* expression via multiple W-box elements in the promoter (Aoyama et al. 2014). WRKY33 belongs to the group I WRKY subfamily with two WRKY domains and C2H2 zinc finger, while WRKY53 belongs to the group III with one WRKY domain and C2HC zinc finger (Rushton et al. 2010). These findings suggest that different molecular mechanisms regulate *ATL31* expression in response to pathogen attack and C/N-nutrient stress. It should be noted that *WRKY33* is transcriptionally activated in response to high C/low N stress condition (Maekawa et al. 2012). Thus, WRKY33 could be involved in C/N response via regulation of unknown target expressions. In addition to W-box elements, the *ATL31* promoter region also contains other putative *cis*-elements such as MYB transcription factor DNA-binding elements, abscisic acid-responsive elements (ABRE), light and nitrate-dependent GAT A transcription factor DNA-binding elements, implicating that unknown multiple regulatory mechanisms of *ATL31* expression are mediated by variable environmental cues.

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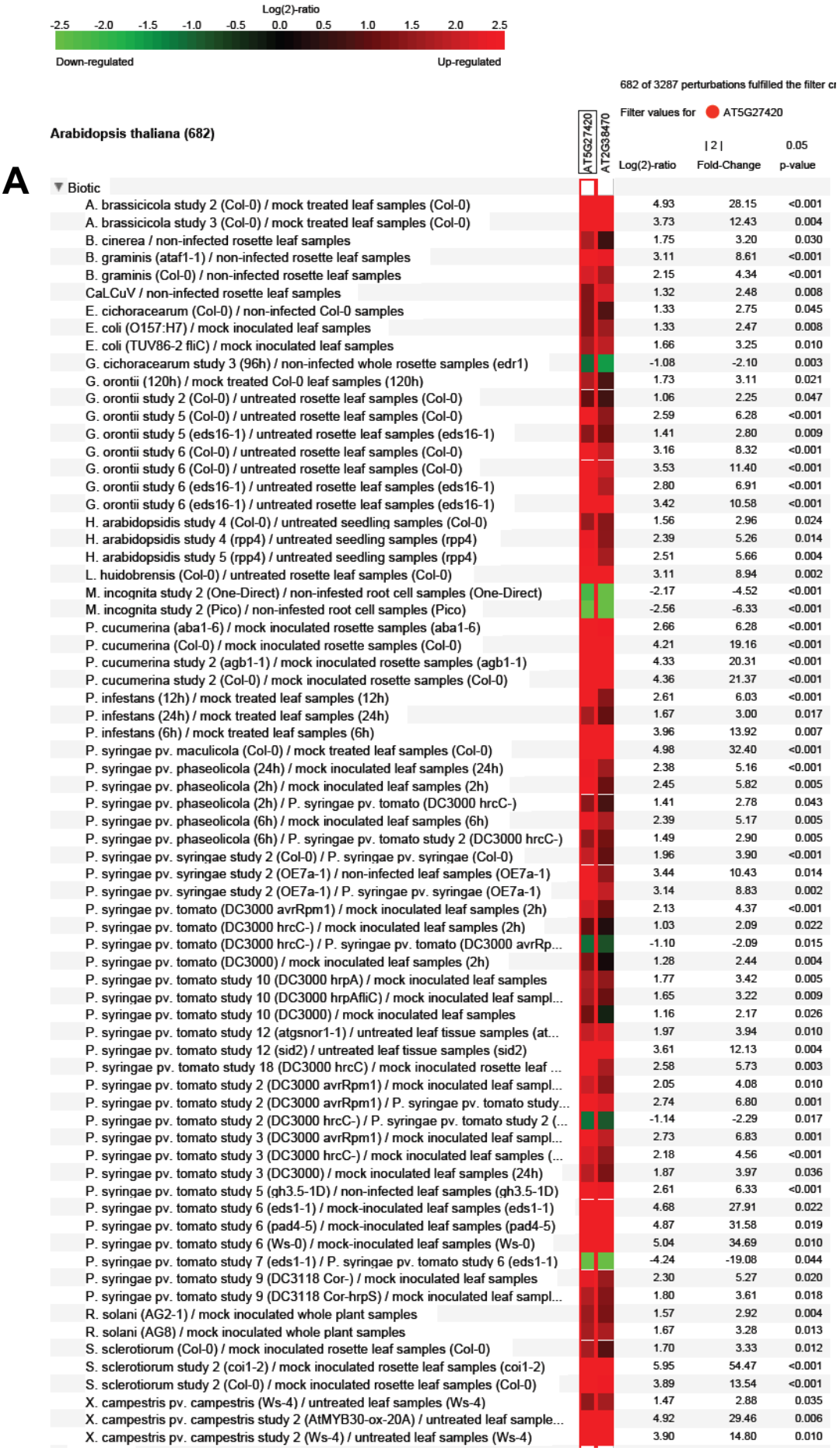

Supplementary Figure S1
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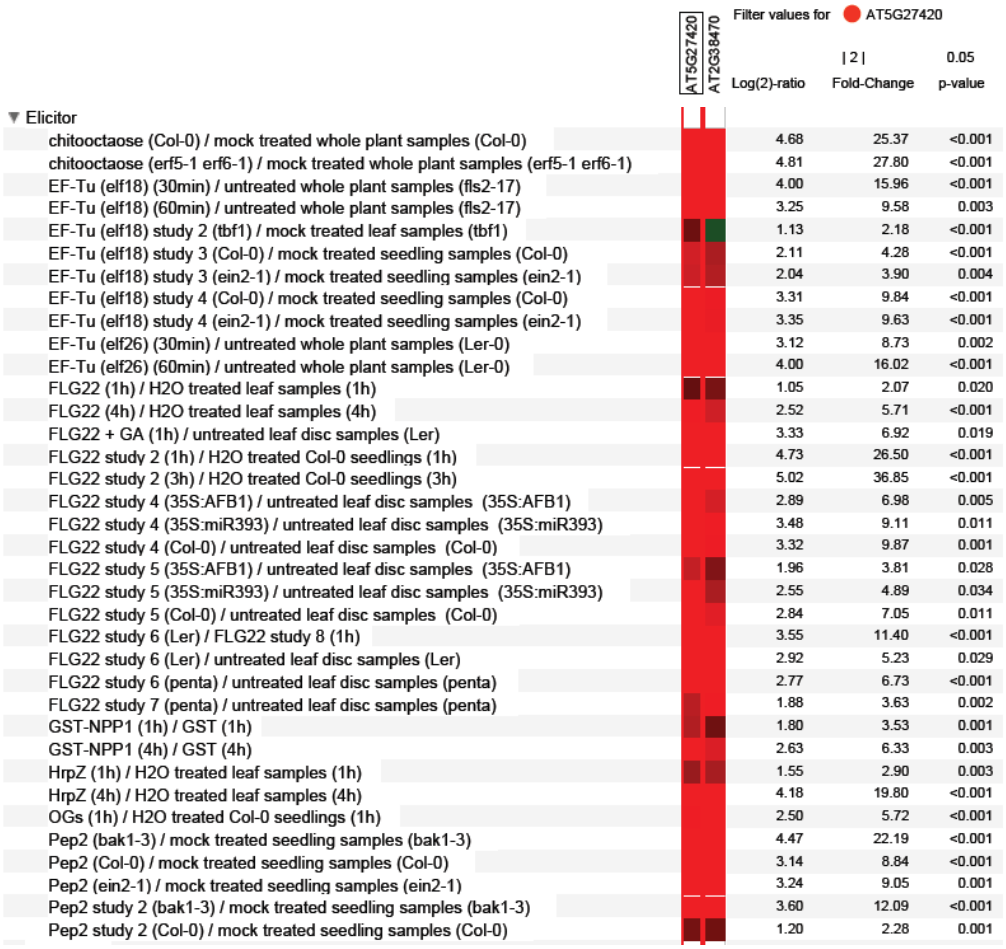
B



Supplementary Figure S1. Gene expression profiles of *ATL31* and *WRKY33* in response to biotic stress and PAMP treatment from a microarray database. Levels of expression of *ATL31* (AT5G27420) and *WRKY33* (AT2G38470) in response to pathogen attack (A) and PAMP treatment (B) are shown in the heat map generated using the public microarray Genevestigator (http://www.genevestigator.ethz.ch). Expression levels and *p*-values were calculated for the *ATL31* gene.

Supplementary Figure S1
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Supplementary Table S1. List of primers

Primer	Sequences (5' → 3')	Used for
-1178 pATL31-F -1178 pATL31-R	AAAAAAGCTTAAAGTCCTTAGTTTG TATGGATCCTGGAGTCCCAAAAAGTTAG	Plasmid construction
-248 pATL31-F -248 pATL31-R	TTCAAGCTTTTCTTCGAAGTCATCGAAC TATGGATCCTGGAGTCCCAAAAAGTTAG	Plasmid construction
-219 pATL31-F -219 pATL31-R	ATTAAGCTTTGTATGACTTTTCACTTCC TATGGATCCTGGAGTCCCAAAAAGTTAG	Plasmid construction
WRKY33 5-entry WRKY33 3-entry	CACCATGGCTGCTTCTTTTCT GGGCATAAACGAATCGAAAAAT	Plasmid construction / RT-PCR
ATL31-F ATL31-R	TACCGGTGGTGGAACTTCCA TATTGACTTCAACTCGGCG	RT-PCR
EF1α-F EF1α-R	GCTGTCCTTATCATTGACTCCACC TCATACCAGTCTCAACACGTCC	RT-PCR
18S rRNA-F 18S rRNA-R	CGGCTACCACATCCAAGGAA GCTGGAATTACCGCGGCT	RT-PCR / qRT-PCR
WRKY33-F WRKY33-R	TTACGCCACAAACAGAGCAC CCAAAAGGCCCGGTATTAGT	qRT-PCR
GST1-F GST1-R	CTCAGACAAAGGAAACAACCTT AAGAACCTTCTGAGCAGAAGG	qRT-PCR
MPK3-F MPK3-R	GAGTTCGAACAACAGCCTCTG CCGTATGTTGGATTGAGTGCT	qRT-PCR
ATL31 exp5-1 ATL31 exp3-1	ACCGGTGGGCTTTTCTTAG AACTGACGATGTTCCTTCACC	qRT-PCR
EF1α-RTF EF1α-RTR	GACATGAGGCAGACTGTTGCA CCGGTTGGGTCCTTCTTGT	Reporter assay
GUS-F GUS-R	GGCTATACGCCATTTGAAGC TTTTTGTCACGCGCTATCAG	Reporter assay / RT-PCR