Expression GWAS of PGIP1 Identifies STOP1-Dependent and STOP1-Independent Regulation of PGIP1 in Aluminum Stress Signaling in Arabidopsis

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To elucidate the unknown regulatory mechanisms involved in aluminum (Al)-induced expression of POLYGALACTURONASE-INHIBITING PROTEIN 1 (PGIP1), which is one of the downstream genes of SENSITIVE TO PROTON RHIZOTOXICITY 1 (STOP1) regulating Al-tolerance genes, we conducted a genome-wide association analysis of gene expression levels (eGWAS) of PGIP1 in the shoots under Al stress using 83 Arabidopsis thaliana accessions. The eGWAS, conducted through a mixed linear model, revealed 17 suggestive SNPs across the genome having the association with the expression level variation in PGIP1. The GWAS-detected SNPs were directly located inside transcription factors and other genes involved in stress signaling, which were expressed in response to Al. These candidate genes carried different expression level and amino acid polymorphisms. Among them, three genes encoding NAC domain-containing protein 27 (NAC027), TRX superfamily protein, and R-R-type MYB protein were associated with the suppression of PGIP1 expression in their mutants, and accordingly, the system affected Al tolerance. We also found the involvement of Al-induced endogenous nitric oxide (NO) signaling, which induces NAC027 and R-R-type MYB genes to regulate PGIP1 expression. In this study, we provide genetic evidence that STOP1-independent NO signaling pathway and STOP1-dependent regulation in phosphoinositide (PI) signaling pathway are involved in the regulation of PGIP1 expression under Al stress.

Keywords: aluminum stress, Arabidopsis thaliana, GWAS, PGIP1, phosphoinositide signaling, transcription factor, NO signaling, STOP1
INTRODUCTION

In the last few decades, extensive studies in molecular physiological research for aluminum (Al) toxicity in acid soils (pH < 5.5) have found that activation of Al-tolerance genes governs Al resistance in plants (Delhaize and Ryan, 1995; Liu et al., 2014; Kochian et al., 2015). Several transcription factors that activate the transcription of critical Al-resistant genes (e.g., ALUMINUM-ACTIVATED MALATE TRANSPORTER 1 (ALMT1); Tokizawa et al., 2015) have been identified, including SENSITIVE TO PROTON RHIZOTOXICITY 1 (STOP1; Ichii et al., 2007). Al-inducible expression of STOP1-regulated genes plays critical roles in Al tolerance in Arabidopsis (Sawaki et al., 2009) and is conserved in various plant species (Ohyama et al., 2013). It is important to identify the mechanisms regulating gene expression related to Al tolerance, which would be helpful in the field of breeding and the management of crops in acidic soil.

Several mechanisms regulating the expression of STOP1-regulated Al-tolerance genes have been reported. For example, the expression of AtALMT1 under Al stress involves calcium signaling that includes CALCIINEURIN B-LIKE PROTEIN 1 (Ligaba-Osena et al., 2017) and CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 2 (Tokizawa et al., 2015), and phosphatidylinositol signaling that includes PHOSPHATIDYLINOSITOL 4-KINASE (Wu et al., 2019). In addition, WRKY DNA-BINDING PROTEIN 46 suppresses AtALMT1 (Ding et al., 2013). These regulators have been characterized in most Al-stress root responses. However, long-term stress leads to high accumulation of Al in the shoot, which is also directly related to shoot growth inhibition (Larsen et al., 1997, 2005; Sadhukhan et al., 2020). Although most of the Al signaling mechanism in shoot is unknown, for example recently we have found Al-inducible expression of ALUMINUM SENSITIVE 3 (ALS3; encodes a bacterial-type ABC transporter-like protein that is involved in the translocation of Al (Larsen et al., 2005)) is STOP1-dependent and shows a specific response to Al in the shoots of Arabidopsis (Sawaki et al., 2016). By contrast, Al-inducible expression of ALS3 in the shoots is dependent on similar signaling mechanisms in the roots, including phosphatidylinositol signaling, although the genes involved in the pathway differ between the shoots and roots (Wu et al., 2019; Sadhukhan et al., 2020). These observations suggest that understanding the regulatory mechanisms of gene expression in shoots is critically important to explore the complexity of the Al signaling pathway.

POLYGALACTURONASE-INHIBITING PROTEIN 1 (PGIP1) gene expression is regulated by STOP1 and is strongly induced in the shoot along with ALS3 by mineral stress (especially Al) and acidic soil conditions (Sawaki et al., 2016). Although the contribution of PGIP1 to Al tolerance has not been studied yet, it has been speculated that PGIP1 plays a role in stabilizing the pectin in the cell wall under acidic conditions (Sawaki et al., 2009; Kobayashi et al., 2014). Al binds preferentially to unmethylated pectin, catalyzed by pectin methylesterase via nitric oxide (NO) signaling (Sun et al., 2016), which negatively affects cell wall structure and function by increasing rigidity and reducing cell expansion and mechanical extensibility, thus inhibiting plant growth (Tabuchi and Matsumoto, 2001; Sun et al., 2016). In contrast to ALS3, which is specifically expressed in Al, PGIP1 is also responsive to abiotic stress. Specifically, PGIP1 is induced by oligogalacturonides, a known degradation product of the cell wall in plant defense (Ferrari et al., 2003; Davidsson et al., 2017). This suggests that analysis of the response of PGIP1 expression will provide an opportunity to study Al signaling pathways in the shoot that may reveal the cross talk between stress signaling pathways, including Al stress, when compared to previous studies of ALS3 (Sadhukhan et al., 2020).

GWAS on the expression level difference of an Al-response gene is a powerful tool to identify the unknown upstream signaling pathways regulating the gene of interest (Atwell et al., 2010; Wang et al., 2020). GO enrichment and gene co-expression network analyses can add to the power of eGWAS in identifying functional candidate genes (Kobayashi et al., 2016; Sadhukhan et al., 2020; Song et al., 2021). We have conducted a genome-wide association study targeting gene expression level (eGWAS) that identified cis-mutations in the promoters of NOD26-like intrinsic protein 1; 1 (NIP1; 1), which regulates hydrogen peroxide sensitivity; and AtALMT1; multidrug and toxic compound extrusion (MATE), which encodes an Al-responsive citrate transporter, is the determinant of the expression levels of these genes in roots (Sadhukhan et al., 2017; Nakano et al., 2020a, 2020b). In addition to the cis-locus, the eGWAS of AtMATE also revealed trans-loci associated with gene expression (Nakano et al., 2020b). Through eGWAS, we also found the involvement of phosphatidylinositol and calcium signaling in the regulation of ALS3 expression under Al stress in Arabidopsis shoots (Sadhukhan et al., 2020). To identify the factors involved in Al signaling related to PGIP1 expression, we conducted an eGWAS based on the expression level of PGIP1 in Arabidopsis thaliana accessions with a reverse genetics approach. We propose both STOP1-dependent and STOP1-independent Al signalings for the transcriptional regulation of PGIP1 in the shoots of A. thaliana.

MATERIALS AND METHODS

Plant Materials

Seeds of 83 worldwide natural A. thaliana accessions (Atwell et al., 2010; Cao et al., 2011; Horton et al., 2012) used in our previous GWAS (Sadhukhan et al., 2020; Nakano et al., 2020b) and T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, United States), the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, United Kingdom), and the RIKEN BioResource Center (RIKEN BRC, Tsukuba, Japan). Prior to experimental use, the procured seeds were multiplied by a single-seed descent process. Homozygosity was confirmed in T-DNA insertion mutant line using primers from the SALK database, following their protocols.1 The sequences of the primers are given in Supplementary Table S1. The T-DNA insertion mutants used in this study were pgip1 (SALK_001662), STOP1-KO (SALK_114108), at1g64105

1http://signal.salk.edu/tdnaprimer2.html
Plant Growth Conditions and Stress Treatment

Seedlings were grown on nylon mesh floating on modified MGRL solution (Fujiiwara et al., 1992; 2% solution with 200 μM CaCl₂; initial pH 5.6) for 10 days at 22°C using a 12-h photoperiod with 37 μmol m⁻² s⁻¹ photon flux density. The culture solution was renewed every 2 days. After 10 days, the seedlings were transferred to another modified MGRL solution (without P and pH 5.0) containing 25 μM AlCl₃·6H₂O (Sawaki et al., 2016). The shoots were harvested after 24 h of Al treatment and immediately frozen with liquid nitrogen for RNA extraction. The same Al toxic solution containing 50 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO), an NO scavenger, was used to evaluate the effect of NO (D’Alessandro et al., 2013; Sun et al., 2016). The concentration of cPTIO was based on preliminary experiments from which maximum suppressed responses were obtained without affecting the plant root (Supplementary Figures S1, S2).

Soil culture was conducted using commercial acidic soil (PROTOLEAF, Tokyo, Japan; pH 4.2; 1.25 w/v soil/water solution; Agrahari et al., 2020). The acidic soil was neutralized by the addition of CaCO₃ (Koyama et al., 2000; Sawaki et al., 2016; Agrahari et al., 2020; 4.0 g kg⁻¹; pH 5.1; 1.25 w/v soil/water solution) and used as the control soil. Plants (100 seeds) were grown for 2 weeks at 22°C during a 12-h photoperiod with 37 μmol m⁻² s⁻¹. Throughout the experiment, the plants were irrigated daily with deionized water to maintain soil moisture. The soil pH (water) and exchangeable Al were determined using the method described by Koyama et al. (2000).

RNA Extraction and Real-Time Quantitative Reverse Transcription PCR

Total RNA was isolated from the shoots using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s instructions. The RNA quality was analyzed using the A260/A280 ratio on a NanoVue Plus spectrophotometer (Biochrom, Holliston, United States). Total RNA was reverse-transcribed using ReverTra Ace quantitative PCR master mix with genomic DNA remover (Toyobo, Osaka, Japan) following the manufacturer’s instructions. The gene expression levels were quantified using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) according to the manufacturer’s instructions. The gene expression levels were quantified using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) according to the manufacturer’s instructions. For all quantifications, a standard curve was constructed using a cDNA dilution series, and the transcript levels of selected genes were quantified relative to that of the stable internal reference gene, Ubiquitin 1 (UBQ1; AT3GS2590; Kobayashi et al., 2007, 2014). We have checked the invariant expression of UBQ1 for all experimental condition in used lines in this study (Supplementary Figure S3). We included a control with no reverse transcriptase to assess genomic DNA contamination, and the amplification efficiency of all primers was confirmed. The primer sequences used to amplify the selected genes are shown in Supplementary Table S2.

Expression Genome-Wide Association Study of PGIP1

The eGWAS analyses were carried out using TASSEL v3.0 software following a mixed linear model (MLM; Bradbury et al., 2007) using a total of 160,748 genome-wide single nucleotide polymorphism (SNP) information from public databases (Atwell et al., 2010; Cao et al., 2011; Horton et al., 2012) which excluded SNPs of missing data or those with less than 5% minor allele frequency, as described earlier (Nakano et al., 2020b). The heritability (h²) was estimated by the following formula h² = (the additive genetic variance)/(the additive genetic variance + the residual variance). The suggestive SNPs were determined by quantile–quantile (Q–Q) plot analysis (Zhang et al., 2018) using a free statistics software, and the genes closest to the SNPs (Table 1) were identified using the TAIR 10 database.

Bioinformatics of Genes Associated With Significant SNPs

Gene ontology (GO) analysis was performed using an online tool available in the TAIR database. Gene polymorphisms were mined from the 1,001 Genomes database and POLYMORPH database. The genes upregulating PGIP1 expression in T87-cultured cells of Arabidopsis were identified by the Regulatory-network Research (RnR) database. Co-expression network analysis was conducted on the eGWAS-detected and RnR database-listed genes using the ATTED-II database (Obayashi et al., 2018). cis-elements and corresponding transcription factors (TFs) of the promoters were predicted using PlantPAN 3.0 (Chang et al., 2008).

In planta Complementation Assay of STOP1

The STOP1 complementation Arabidopsis transgenic plant was constructed as described by Ohyama et al. (2013). STOP1 genomic DNA containing the promoter (~2,848 from the first ATG) and downstream (~626 from the stop codon) regions was cloned into a binary vector (promoterless pBIG2113SF).

References

Bradbury, J., Zhang, Y., Kroon, D., Casstevens, T., Ramdoss, Y., and Buckler, E. S. (2007) TASSEL: Software Architecture for Genetic Improvement and Selection. Front. Plant. Sci 8, 21.

Cao, N., Zhan, X., and Bradbury, J. (2011) Fitness-related quantitative trait loci (QTL) for growth parameters and flowering time in Arabidopsis thaliana. J. Exp. Bot. 62, 3509–3521.

Cao, N., Zhang, Y., and Bradbury, J. (2014) Using the RnR database: tools for gene expression and biological function analysis of Arabidopsis. Front. Plant Sci 5, 404.
### TABLE 1 | GWAS identified SNPs with directly linked to protein-coding genes that were associated with PGIP1 expression levels in the shoots of 83 Arabidopsis thaliana accessions under Al stress.

| Chr | Physical position | GWAS p-value | Lower expression group | Higher expression group | Directly associated gene of detected SNP | SNP location in the directly associated gene | Functionally candidate‡‡ | Al-responsive expression†† | Short description |
|-----|------------------|--------------|------------------------|------------------------|----------------------------------------|-----------------------------------------------|--------------------------|--------------------------|-------------------|
| 1   | 3,476,243        | 7.46 × 10⁻⁵  | 71/G 0.34              | 12/A 0.54              | AT1G10540 Intron                      | Intron                                        | 1.04                     |                         | NAT8 (nucleobase-ascorbate transporter 8) |
| 1   | 22,938,272       | 1.88 × 10⁻⁴  | 79/G 0.37              | 4/A 0.39               | AT1G2050 Exon                         | Exon                                          | 1.14                     |                         | Ankyrin repeat family protein |
| 1   | 23,795,163       | 1.39 × 10⁻⁴  | 74/C 0.33              | 9/G 0.68               | AT1G6410 Exon                         | Exon                                          | 1.23*                    |                         | NAC027 (NAC domain containing protein 027) |
| 2   | 8,175,062        | 1.75 × 10⁻⁴  | 17/T 0.16              | 66/C 0.37              | AT2G18880 Exon                        | Exon                                          | 1.01                     |                         | VEL2 (vernalization5/VIN3-like 2) |
| 2   | 9,317,842        | 2.59 × 10⁻⁴  | 9/C 0.29               | 74/A 0.38              | AT2G21850 Exon                        | Exon                                          | 0.97                     |                         | Cysteine-Histidine-rich C1 domain family protein |
| 2   | 9,354,086        | 9.32 × 10⁻⁵  | 10/A 0.23              | 73/C 0.38              | AT2G21950 Exon                        | Exon                                          | 0.98                     |                         | Skip6 (SKP1 interacting partner 6) |
| 3   | 8,902,459        | 2.93 × 10⁻⁴  | 13/T 0.27              | 70/A 0.39              | AT3G24480 Exon                        | Exon                                          | 0.74                     |                         | LRX4 (leucine-rich repeat extension 4) |
| 4   | 4,969,631        | 3.16 × 10⁻⁴  | 35/T 0.27              | 48/A 0.44              | ATSG15300 Exon                        | Exon                                          | 2.40*                    |                         | Pentatricopeptidase repeat (PPR) superfamily protein |
| 5   | 9,229,573        | 1.45 × 10⁻⁴  | 13/C 0.30              | 70/A 0.38              | ATSG26300 Intron                      | Intron                                        | 0.64                     |                         | TRAF-like family protein |
| 5   | 9,241,705        | 1.44 × 10⁻⁴  | 17/G 0.28              | 66/A 0.39              | ATSG26330 Exon                        | Exon                                          | 0.92                     |                         | Cysteine-Histidine-rich protein |
| 5   | 14,889,845       | 2.74 × 10⁻⁴  | 62/G 0.35              | 21/T 0.46              | ATSG37500 Exon                        | Exon                                          | 0.77                     |                         | GOKK (gated outwardly-rectifying K⁺ channel) |
| 5   | 15,560,442       | 1.99 × 10⁻⁴  | 9/A 0.28               | 74/G 0.37              | ATSG38860 Intron                      | Intron                                        | 1.15                     |                         | BLM3 (BES1-interacting Myc-like protein 3) |
| 5   | 15,574,085       | 2.08 × 10⁻⁴  | 77/G 0.35              | 6/A 0.59               | ATSG38900 Exon                        | Exon                                          | 2.61*                    |                         | Thioredoxin superfamily protein |
| 5   | 17,460,312       | 6.27 × 10⁻⁵  | 62/C 0.35              | 21/T 0.48              | ATSG43460 Intron                      | Intron                                        | 0.98                     |                         | HR-like lesion-inducing protein-like protein |
| 5   | 20,724,766       | 5.60 × 10⁻⁵  | 73/T 0.34              | 10/A 0.59              | ATSG50940 Intron                      | Intron                                        | 1.05                     |                         | RNA-binding KH-domain-containing protein |
| 5   | 23,783,404       | 2.34 × 10⁻⁴  | 66/A 0.33              | 17/C 0.51              | ATSG58900 Exon                        | Exon                                          | 1.24*                    |                         | Homeodomain-like transcriptional regulator (R-R-type MYB protein) |
| 5   | 23,790,818       | 1.47 × 10⁻⁴  | 70/A 0.34              | 13/G 0.53              | ATSG58910 Intron                      | Intron                                        | 1.45*                    |                         | LAC16 (laccase 16) |

1 Number of accessions for each SNP allele.
2 Mean value of relative fold change (RFC) in PGIP1 expression for accessions carrying the tolerant or sensitive SNP allele.
3 The fold change between Al treatment and control. Asterisk indicates showed greater than 1.2-fold change (p < 0.05). The expression data in the shoot obtained from our previous microarray data (Sawaki et al., 2016).
4 The circle indicates gene that may be functionally related to regulation of gene expression based on their GO term and publications. The GO term is shown in Supplementary Figure S6.
5 The most focused SNPs with a value of p < 10⁻³.5 in the GWAS for expression levels of PGIP1 in the shoots of 83 A. thaliana accessions under 24-h Al treatment are presented. SNP locations within the directly associated gene are shown as exon or intron or upstream (denoted as a minus sign). Gene symbols and a short description of each gene from the literature and the TAIR/Araport11 database are indicated. Bold type indicates information on a priori candidate genes.
This construct was introduced into Agrobacterium tumefaciens strain GV3101 and transformed into STOP1-KO plants by the floral dip method (Clough and Bent, 1998). A T3 homozygous line was used for the experiments.

DNA-Protein Binding Assay
The binding of STOP1 to double-stranded, synthetic promoter fragments was studied using an amplified luminescent proximity homogeneous assay (AlphaScreen™, PerkinElmer, Waltham, MA, United States) and a 276 EnSpire Multimode Plate Reader (PerkinElmer) as described by Enomoto et al. (2019). Competition assays were performed with non-biotinylated probes (450 nM) according to the manufacturer’s instructions. The competitor probes, consisting of the promoter fragments −193 to −222 bp upstream from the PGIP1 and −2,694 to −2,723 bp upstream from the AT5G38900 start codons, respectively, were designed around the STOP1-binding site according to the Plant Cistrome Database (Sadhu.khan et al., 2019). The mutated probes were designed following the method of Tokizawa et al. (2015). The forward and reverse probe sequences are listed in Supplementary Table S3.

Al Content in Pectin and Nuclear Magnetic Resonance Analysis
Pectin was extracted from 250 mg powdered Arabidopsis shoot tissue (500 seedlings were grown for 10 days in the hydroponic system mentioned above) in buffer containing 50 mM Tris–HCl (pH 7.2) and 50 mM cyclohexane-trans-1, 2-diamine tetra-acetate (CDTA; Bethke and Glazebrook, 2014). The extraction was continued for 15 min at 95°C with intermittent vortexing, and the sample was then centrifuged at 10,000 × g for 10 min. The supernatant containing pectin was analyzed for Al content using inductively coupled plasma mass spectrometry as described by Watanabe et al. (2015). For nuclear magnetic resonance (NMR) analysis, the supernatant containing pectin was lyophilized and dissolved in D₂O. H-13C-Heteronuclear Single Quantum Coherence (HSQC) NMR was performed at 600.17 MHz on a JEOL ECA 600 NMR spectrometer (JEOL, Tokyo, Japan) equipped with a 5-mm FG/TH tunable probe, using the pulse sequence ‘hsqc_dec_club_pn’. NMR measurements were recorded at 70°C (Siedlecka et al., 2008). Sweep widths of 15 and 170 ppm were used to acquire the 1H and 13C spectra, respectively. For each NMR experiment, 88 scans were collected using a relaxation delay of 1.5 s.

RESULTS

Expression Level Polymorphisms and Amino Acid Polymorphisms Caused by Detected SNP of the Candidate Genes
We examined the 12 candidate genes for expression level polymorphisms (ELPs) and amino acid polymorphisms associated with the PGIP1 expression level. The expression levels of these genes were compared between representative accession groups that carried different detected SNP alleles (Figure 2, Supplementary Figure S7). Five genes, AT1G644105 (NAC027), AT5G38900 (TRX SF) AT5G43460 (HR-like lesion-inducing protein-like protein: HR-like protein), AT5G58900 (Homeodomain-like

Genome-Wide Association Study to Detect Loci Associated With Expression of Arabidopsis thaliana PGIP1 Under Al Stress
We analyzed the expression of PGIP1 in the shoots of wild-type (WT) Arabidopsis, Columbia (Col-0) at different time points after root exposure to 25μM Al. The expression of PGIP1 was induced after 12 h and was markedly induced (five-fold on average) after 24 h of treatment (Figure 1A). It is also a condition of Al accumulation in the shoots (Supplementary Figure S4). Hence, in this study, we chose 24 h as the time point for evaluating PGIP1 gene expression under Al stress to conduct the eGWAS of PGIP1 in the shoots. Next, we analyzed the expression of PGIP1 in 83 Arabidopsis accessions (Supplementary Figure S5; Supplementary Table S4) that had been treated with treated with 25μM Al for 24 h and found that the expression range between log2 RFC −3.3 and log2 RFC 0.1 (RFC: relative fold change; compared to Col-0; Figure 1B; h² = 88.3%). We performed a GWAS following MLM using the PGIP1 expression data, but after Bonferroni correction for multiple testing, we could not detect any significant SNPs at the genome-wide significance level. This could be due to the dependence of the statistical power of GWAS on the population size and allele frequency. Although the Q–Q plot showed only a slight deviated plot, in this study we set a suggestive threshold based on this result (p < 10−5) and selected the top-ranked 17 SNPs as suggestive SNPs associated with PGIP1 expression level variation (Figures 1C,D; Table 1). These potentially associated SNPs were selected for further analysis.

We first characterized 17 protein-coding genes carrying the 17 SNPs directly in their exons, introns, and promoters (Table 1) and then considered a priori candidate genes related to the regulation of PGIP1 expression out of these 17 genes. Out of these, seven genes belonged to the GO term of “regulation of gene expression,” “DNA-binding transcription factor activity,” “intracellular signal transduction,” and “hormone-mediated signaling pathway” and may be functionally associated with PGIP1 expression (Table 1; Supplementary Figure S6). Out of the a priori candidate genes, AT2G21950 (SKP1 interacting partner 6: SKIP6; Farras et al., 2001) and AT3G24480 (leucine-rich repeat extension 4: LRX4; Zhao et al., 2018) are involved in hormonal signaling and cell-wall integrity, respectively. AT5G38900 (Thiorodoxin superfamily protein: TRX SF) is involved in regulation of gene expression via redox signaling (Sevilla et al., 2015). On the other hand, five among the 17 genes were induced more than 1.2-fold by Al in the shoot, according to our earlier transcriptome analysis, carried out under the same experimental conditions (Sawaki et al., 2016; Table 1). Therefore, we finally selected 12 genes with functions related to transcriptional regulation and Al-responsive expression, as a priori candidate genes associated with PGIP1 expression, for subsequent analyses.
transcriptional regulator: R-R MYB), and AT5G58910 (laccase 16: LAC16) exhibited significant differences in the level of expression between accessions carrying different SNP alleles (Figure 2). When comparing the different alleles, the minor allele group with elevated PGIP1 expression showed higher expression levels of each gene than the major allele group with low PGIP1 expression (Table 1; Figure 2). In the case of genes with SNP alleles located directly in exons, we examined the amino acid polymorphisms caused by the detected SNPs using reliable DNA sequences from the 1,001 genome database. The SNPs detected in the exons of five genes caused amino acid polymorphisms, viz. Ser29Cys in NAC027, Ala187Ser in SKIP6, Asn60Lys in LRX4, Phe134Ile in AT5G15300 (pentatricopeptide repeat superfamily protein) and His246Gln in R-R MYB. Among them, NAC027 and R-R MYB showed both ELP and amino acid polymorphism. These polymorphisms might affect the expression level variation of PGIP1 as cis-factors. In this way, eight genes were selected as the first group of possible candidate genes, which were further studied using reverse genetics.

Reverse Genetic Characterization of the Candidate Genes

To examine the effect of the candidate genes on PGIP1 expression, its expression level was quantified in the T-DNA insertion knockout (KO) or knockdown mutants (KD; Supplementary Figure S8) of the eight candidate genes. PGIP1 expression was significantly lower in the three T-DNA insertion mutants of nac027, trx sf and r-r myb than in the WT under Al treatment (Figure 3A).
In particular, the reduction of \( \text{PGIP1} \) expression in the \( r-r \ myb \) was as high as approximately 40%. In contrast, other mutants (\( \text{at2g21950}, \text{at3g24480}, \text{at5g15300}, \text{at5g43460}, \text{at5g58910} \)) showed no significant difference of \( \text{PGIP1} \) expression compared to the WT under Al treatment (Figure 3A). From this analysis, we found that \( \text{NAC027}, \text{TRX SF} \) and \( R-R \ MYB \) are involved in the regulation of \( \text{PGIP1} \) expression. We also analyzed the patterns of expression of \( \text{NAC027}, \text{TRX SF} \), and \( R-R \ MYB \) under Al stress in the shoots of WT \( \text{A. thaliana} \) after 24 h of treatment. We observed a significant induction of these genes relative to the control (Figure 3B). The Al-induced responses of \( \text{NAC027} \) were weak, but their gene expression showed Al responses similar to \( \text{PGIP1} \) expression. These results suggest that \( \text{NAC027}, \text{TRX SF}, \) and \( R-R \ MYB \) are related to the regulation of Al-induced \( \text{PGIP1} \) expression. In addition, Al-responsive \( \text{PGIP1} \) and its regulatory system were involved in Al tolerance. In acidic soils containing higher exchangeable Al, \( \text{PGIP1} \), like the Al-hypersensitive \( \text{stop1} \), was much more inhibited in growth than the WT (Supplementary Figure S9). On the other hand, the growth was recovered in the neutralized soil (Supplementary Figure S9). Similarly, the growth of \( \text{trx sf} \) and \( r-r \ myb \) was inhibited than the WT in acidic soil, although the growth of \( \text{nac027} \) was not severely inhibited (Supplementary Figure S9). This may be consistent with the lower degree of repression of \( \text{PGIP1} \) in \( \text{nac027} \) and the weaker induction of Al on the expression level of \( \text{NAC027} \) compared to the other two genes (Figure 3).

### Relationship Between \( \text{STOP1} \) and Genes Regulating \( \text{PGIP1} \) Identified by eGWAS

We investigated the relationship between \( \text{STOP1} \) regulation and the eGWAS-detected genes because it has been reported that \( \text{STOP1} \) regulates \( \text{PGIP1} \) expression (Sawaki et al., 2009). There was no difference in expression levels of \( \text{STOP1} \) in the \( \text{nac027}, \text{trx sf}, \) and \( r-r \ myb \) (Supplementary Figure S10A). In contrast, we found a significant reduction in the gene expression level of \( \text{TRX SF} \) in the \( \text{STOP1-KO} \), whereas the other two genes showed similar expression levels compared to WT (Figure 4A). In addition, in the \( \text{STOP1-complemented} \).
line, the expression of TRX SF was fully recovered similar to Col-0 (Figure 4B). These results suggest that TRX SF is regulated by the STOP1 transcription factor, while none of the three genes affect the expression level of STOP1.

In our previous eGWAS of ALS3, we found the involvement of phosphoinositide (PI)-dependent phospholipase C9 (PLC9) signaling upstream of the STOP1 regulation system, which regulates the expression of ALS3 and PGIP1 in the shoots of A. thaliana (Sadhukhan et al., 2020). Therefore, we examined the expression of TRX SF, NAC027, and R-R MYB in the plc9. We found that the expression of TRX SF was significantly suppressed in plc9 (Figure 4A), similar to the downregulation of PGIP1 (Supplementary Figure S10B). These findings suggest that TRX SF is regulated by a PI signaling-mediated STOP1-dependent pathway. In contrast, we found that the expression of ALS3 remained unchanged in the trx sf, nac027, and r-r myb mutants (Supplementary Figure S10A). These results suggest that TRX SF differentially regulates transcription of PGIP1 and ALS3, which are co-regulated by STOP1 in Arabidopsis shoots.

**In vitro Binding Analysis of STOP1 to TRX SF and PGIP1 Promoter Regions**

Next, we performed a promoter binding analysis to reveal whether STOP1 directly regulates TRX SF and PGIP1 in the STOP1-dependent pathway. The STOP1-binding positions were

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**FIGURE 3 | PGIP1 expression in the mutants of genes identified by eGWAS.** (A) Relative expression levels of PGIP1 in the shoots of WT (WT, Col-0) and T-DNA insertion mutants of eight final candidate genes having expression level polymorphisms (Figure 2) and/or amino acid polymorphisms (see main text). Approximately 100 seedlings of the WT and independent homozygous T-DNA insertion mutants were grown for 10 days and treated with 0 (-Al) or 25 μM AlCl3 (+Al) for 24 h. PGIP1 expression was measured by qRT-PCR. Expression levels are expressed as relative fold changes compared to WT (-Al); significant reductions in relative fold change of PGIP1 from the Al-treated WT sample are indicated by asterisks (Student’s t-test, p < 0.05). (B) Fold changes (+Al/−Al) of AT1G64105 (NAC027), AT5G38900 (TRX SF), and AT5G58900 (R-R MYB) at 24 h of Al treatment, measured by qRT-PCR, are shown; significant fold increases from the control (−Al) samples are indicated by asterisks, *(Student’s t-test, p < 0.05). Average data of three biological replicates are presented with standard errors. UBQ1 was used as an internal control. Primers used for qRT-PCR are listed in Supplementary Table S2.
predicted by searching enriched sequences (octamer units) in the stress-inducible promoters.13 They were identical to the binding sites provided by the Plant Cistrome Database. Therefore, we searched for putative STOP1-binding sites in the promoters of PGIP1 and TRX SF using the Plant Cistrome Database. Based on DNA affinity purification sequencing (DAP-seq), the database identified the “GGNVS” consensus sequence in the PGIP1 and TRX SF promoters for binding STOP1-like proteins (Figure 5A), as previously identified in rice by Tsutsui et al. (2011). The binding capacity of STOP1 to the sequences available from the Plant Cistrome Database was validated by an in vitro competitive binding assay using the AlphaScreen™ system (Figure 5B). A 30-bp synthetic double-stranded DNA probe, designed around the binding site in the PGIP1 (−193 to −222bp) and TRX SF (−2,694 to −2,723) promoters, could compete for STOP1 protein binding with a known STOP1-binding site in the AtALMT1 promoter (Tokizawa et al., 2015). On the other hand, replacing the “GGNVS” consensus sequences within the PGIP1 and TRX SF probe with A/T stretches abolished STOP1 binding (Figure 5B). These results indicate that STOP1 binds directly to the PGIP1 and TRX SF promoters.

Al-Inducible NO Signaling Effects
Expression of Genes Regulating PGIP1

NO generation is positively correlated with cell wall pectin demethylation and alteration of cell wall metabolism under Al stress (Zhou et al., 2012; Sun et al., 2016). To establish whether Al accumulation induces NO signaling, we examined NO-inducible marker gene (AT2G06050, AT3G45140, and AT5G42650; Huang et al., 2004) expression in shoots after exposing the roots to Al or Al plus cPTIO (NO scavenger; Shi et al., 2015) for 24h. We found that the expression of the NO marker genes was substantially induced in the Al-treated samples, whereas their expression was suppressed in the samples treated with Al plus cPTIO (Figure 6A). This suggests that Al induces NO signaling in the shoots. Next, we examined whether PGIP1 and ALS3, which are regulated by STOP1, function downstream of NO signaling by quantifying their expression levels in the shoots after exposing the roots to Al or Al plus cPTIO for 24h. The transcript levels of PGIP1 were suppressed significantly in the Al plus cPTIO samples, whereas ALS3 expression was unchanged (Figure 6B). STOP1 expression was neither Al-induced nor affected by cPTIO.

We also assessed whether the eGWAS-identified genes that regulate PGIP1 expression function downstream of NO signaling by quantifying their expression levels in the shoots after exposing the roots to Al or Al plus cPTIO for 24h. We found that only NAC027 expression and R-R MYB expression were significantly suppressed in the Al plus cPTIO samples, while TRX SF remained unchanged (Figure 6B). These results clearly indicate that Al-inducible PGIP1 expression is regulated by the NO signaling pathway through NAC027 and R-R MYB, and this is not regulated by STOP1 (Figure 4A).

Transcriptional Regulation of R-R MYB and NAC027 Included in NO Signaling

The expression of NAC027 and R-R MYB was measured in each KO line. A significant reduction in the expression of NAC027 was observed in the r-r myb compared with the WT
expression (Figure 7A). In contrast, the expression of R-R MYB in the nac027 showed no difference compared with wild type (Figure 7B). In addition, the PlantPAN3.0 database (provides TF binding sites in genome-wide promoters based on DAP-seq analysis of various transcription factors) identified that R-R MYB directly binds to the promoter of NAC027 (Figure 7C).

Interestingly, we found regulators of PGIP1 using the RnR database that is different from the eGWAS-identified factors. The RnR database indicated that overexpression of AT1G51070 [BASIC HELIX-LOOP-HEXIL 115 (BHLH115)], AT2G38090 (duplicated homeodomain-like superfamily protein), and AT2G04780 [FASCICLIN-LIKE ARABINOGALACTAN 7 (FLA7)] upregulated PGIP1 expression, which was at the approximately 99th percentile of expression regulation and about 1.3–2.0-fold expression compared with the control. In fact, we confirmed the downregulation of PGIP1 expression in the KO or KD mutants of these three genes under Al stress (Figure 7D). Among them, AT2G38090 is a member of the R-R-type MYB family (Yan et al., 2006) and is a close homolog of R-R MYB that we found in the eGWAS. This TF also binds to...
the NAC027 promoter as assessed by a PlantPAN3.0 database search, similar to R-R MYB (Figure 7C). Co-expression gene network analysis using the six genes (TRX SF, NAC027, R-R MYB, BHLH115, AT2G38090, and FLA7), whose PGIP1 expression levels were decreased in these KO or KD lines, revealed that R-R MYB was co-expressed with AT2G38090 (R-R type MYB) and FLA7 (Supplementary Figure S11). These results suggest that these genes are involved in the direct/indirect regulation of PGIP1 expression.

DISCUSSION

The transcription of Al-tolerance genes is regulated by a complex mechanism (Delhaize et al., 2012) and involves STOP1 and cross talk with other mechanisms related to stress responses (Daspure et al., 2017). In this study, TRX SF, R-R MYB, and NAC027 involved in the regulatory mechanisms of Al-inducible PGIP1 expression were identified through eGWAS of PGIP1 expression levels under Al stress (Figure 3; Table 1). The STOP1–TRX SF pathway regulate PGIP1 expression through the PI signaling pathway via PLC9, while R-R MYB and NAC027 regulate PGIP1 expression through a STOP1-independent NO-signaling pathway (Figures 4, 6, 8). In contrast, the regulation of ALS3 expression via STOP1 in the shoots was independent of these pathways, including TRX SF, R-R MYB, and NAC027 (Figures 6, 8, Supplementary Figure S10). Taken together, the eGWAS of PGIP1 identified a portion of the complex Al signaling pathways in Arabidopsis shoots.

In the current study, we found plausible causative genes involved in the regulation of PGIP1 expression through the candidate gene-based GWAS (Table 1). Many of these genes showed genomic polymorphisms, but further reverse genetic studies clearly revealed that three genes, TRX SF, R-R MYB, and NAC027, were involved in the regulation of PGIP1 expression. The polymorphism responsible for the variation could not be determined in this study. However, using reliable DNA sequences from the 1,001 Genomes Project, we searched for polymorphisms at the three genes in several high- and low-expression accessions. In this process, we observed a haplotype containing polymorphisms in the intron sequences of TRX, associated with its expression levels. The only amino acid polymorphism caused by the detected SNPs was observed in R-R MYB. Another haplotype including a promoter deletion related to ELP was observed in NAC027 (Supplementary Figure S12). Promoter polymorphisms have been found to significantly impact gene expression level variation (Sadhuakhun et al., 2017; Meloa et al., 2019; Nakano et al., 2020b; Wang et al., 2021). Functional analysis of the promoter sequence polymorphisms of NAC027 will shed light on their potential role in the differential regulation of gene expression.

A key regulatory transcription factor, STOP1, regulates the expression of various genes involved in Al tolerance along with PGIP1 (Sawaki et al., 2009; Wu et al., 2019), in which some genes are directly regulated. Our previous studies found that STOP1 directly regulates transcription of several downstream genes by binding to their promoters under not only Al but also other stress conditions: STOP1 binds to the promoter of Al-inducible AtALMT1 (Tokizawa et al., 2015) and AtMATE (Nakano et al., 2020b), low-oxygen-inducible HsfA2 (Enomoto et al., 2019), and NaCl-inducible CPK23 (Sadhuakhun et al., 2019). In the present study, promoter analyses (i.e., Cistrome database and in vitro
promoter assays; Figure 5) identified a functional STOP1-binding site in the promoters of PGIP1 and TRX SF. This indicates that STOP1 directly activates the Al-inducible expression of PGIP1 and TRX SF. The promoter regions of each gene commonly possessed the minimum consensus sequence of ART1/STOP1 [GGN(T/g/a/C)V(C/A/g)S(C/G); Tsutsui et al., 2011; Tokizawa et al., 2015], although the surrounding sequences of the consensus were different.

Similarly, we found that STOP1 may directly regulate TRX SF expression (Figures 4, 5), while TRX SF affected the PGIP1 expression but not STOP1 expression (Figure 3, Supplementary Figure S10), whose regulation contributed to Al tolerance (Supplementary Figure S9). One possible function of TRX SF is to control regulatory proteins by oxidative protein modification, which is a common mechanism of thioredoxin superfamily proteins (Lemaire and Miginiac-Maslow, 2004; Schmidtmann et al., 2014; Mata-Perez and Spoel, 2019). The TRX family regulates gene expression through redox activation of receptors and transcription factors under salicylic acid (SA) and brassinosteroid signaling (Ding et al., 2018; Tian et al., 2018). We previously revealed that TRX1 contributed to Al tolerance using GWAS for Al tolerance in Arabidopsis (Nakano et al., 2020a). These results suggest that TRX-mediated redox signaling is involved in gene regulation related to Al tolerance. However, ALS3 was not involved in the signaling (Supplementary Figure S10), despite the fact that ALS3 expression in shoots is regulated by STOP1 under Al stress, similar to that of PGIP1 (Sawaki et al., 2016).

We found that both NAC027 and R-R MYB (transcription factors respond to various biotic and abiotic stresses, Ascencio-Ibanez et al., 2008; Soitamo et al., 2008; Fang et al., 2018) responded to Al stress and were involved in STOP1-independent regulation of PGIP1, where they function together
in the NO signaling pathway via R-R MYB binding to the NAC027 promoter (Figures 3, 4, 6, 7). However, the contribution of NAC027 to regulation and Al tolerance does not seem to be largely comparable to that of R-R MYB (Figure 3, Supplementary Figure S9), suggesting that R-R MYB, upstream of NAC027, also regulates other genes. NO signaling has been reported to be a second messenger of Al-inducible expression of several Al-tolerance genes (He et al., 2012). Additionally, PECTIN METHYLESTERASE 3, which is induced and activated by Al-dependent NO signaling (Sun et al., 2016; Ye et al., 2018), was detected as a co-expressed gene of R-R MYB and the close homolog of MYB regulating PGIP1 (Figure 7D, Supplementary Figure S11). These results suggest that the co-expressed module involved in NO signaling is related to the Al stress response. In contrast, STOP1 regulated TRX SF along with ALS3 (Figure 6), which was independent of NO signaling not included in the co-expression network.

Furthermore, several genes involved in plant cell wall biogenesis, UDP-GLUCOSE DEHYDROGENASE 4, COTTON GOLGI-RELATED 3, and FLA7, were included in the co-expression network. The cell wall plays important roles not only in the regulation of plant growth and development, but also in the perception and expression of Al toxicity (Tabuchi and Matsumoto, 2001; Eticha et al., 2005; Yang et al., 2011; Kobayashi et al., 2013; Zhu et al., 2014; Kochian et al., 2015; Sun et al., 2016). The PGIP1 was shown to be included in Al tolerance (Supplementary Figure S9), but the details of its role in Al tolerance have not been studied yet; one possibility is that it can protect the binding of Al to negatively charged ligands [e.g., polygalacturonic acid (PGA)] induced by demethylation of cell wall pectin, which is enhanced by Al (Supplementary Figures S4, S13; the degree of pectin methylesterification in Al-treated seedlings decreased to 55% of that without Al treatment). It has been reported that PGIP1 can bind to the PGA region and support the formation of a normal pectin network under biotic stress conditions (Spadoni et al., 2006). A similar alleviation was observed under proton-toxic conditions in the stop1, which showed very low expression of PGIP1 (Kobayashi et al., 2014). Under Al stress conditions, PGIP1 binding to the PGA region might reduce the formation of abnormal pectin networks, which might be caused by unusual Al binding to the PGA region. Further characterization of these events would be useful for identifying the role of PGIP1 in Al tolerance.

CONCLUSION

Through a candidate gene-based GWAS of PGIP1 expression, we successfully identified complex signaling of PGIP1 in response to Al stress in A. thaliana. Furthermore, we propose a model to illustrate that PGIP1 expression is regulated by a STOP1-dependent Al-induced phosphoinositide (PI) signaling through AT5G38900 (TRX superfamily protein) and STOP1-independent Al-induced endogenous NO signaling through AT1G64105 (NAC027 transcription factor) and AT5G58900 (R-R type MYB transcription factor; Figure 8). In addition, our study demonstrates the utility of an eGWAS in understanding the genetic regulation of Al signaling by exploiting the natural variation in the expression levels of key Al-responsive genes. Although a limited number of accessions were used in the current study, a future eGWAS using denser SNP information and a larger accession set, available in recent years (Togninalli et al., 2018), will open up new avenues for better understanding of Al stress signaling in plants.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

RA, YK, and HK conceived and designed the research. RA, TE, EY, TW, HI, AS, and SI performed the experiments. RA, TE, and YN analyzed the data. HK, SP, YY, and YK supervised the study. RA, YK, and HK wrote the manuscript. HK, YK, YY, and MK contributed to new reagents or analytical tools. All authors have approved the manuscript.
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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.774687/full#supplementary-material

REFERENCES
Agrahari, R. K., Kobayashi, Y., Borgohain, P., Panda, S. K., and Koyama, H. (2020). Aluminum-specific upregulation of GmALS3 in the shoots of soybeans: a potential biomarker for managing soybean production in acidic soil regions. Agronomy 10:1228. doi: 10.3390/agronomy10091228

Ascencio-Ibáñez, J. T., Sozzani, R., Lee, T. J., Chu, T. M., Wolfinger, R. D., Cella, R., et al. (2008). Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol. 148, 436–454. doi: 10.1104/ pp.108.121038

Atwell, S., Huang, Y. S., Vilhjálmsson, B. I., Willems, G., Horton, M., Li, Y., et al. (2010). Genome-wide association study of 107 phenotypes in Arabidopsis thaliana inbred lines. Nature 465, 627–631. doi: 10.1038/nature08800

Bethke, G., and Glazebrook, J. (2014). Cyclohexane diamine tetracetic acid (CDTA) extraction of plant cell wall pectin. Bio Protoc. 4:e1357. doi: 10.10769/ BioProtoc.1357

Bradbury, P. J., Zhang, Z., Kroon, D. E., Castsevvs, T. M., Ramdoss, Y., and Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics 23, 2633–2635. doi: 10.1093/bioinformatics/btm308

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. doi: 10.1373/clinchem.2008.112797

Cao, J., Schneeberger, K., Osowski, S., Günther, T., Bender, S., Fitz, J., et al. (2011). Whole-genome sequencing of multiple Arabidopsis thaliana populations. Nat. Genet. 43, 956–963. doi: 10.1038/ng.911

Chang, W. C., Lee, T. Y., Huang, H. D., and Pan, R. L. (2008). PlantPAN: plant promoter analysis navigator for identifying combinatorial cis-regulatory elements with distance constrain in plant gene group. BMC Genomics 9:561. doi: 10.1186/1471-2164-9-561

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for agrobacterium -mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743. doi: 10.1046/j.1365-313x.1998.00343.x

D'Alessandro, S., Posocco, B.,Costa, A., Zahariou, G., Schiavo, F. L., Carbonera, D., D' Alessandro, S., Posocco, B., Costa, A., Zahariou, G., Schiavo, F. L., Carbonera, D., et al. (2005). Cell-wall pectin and its degree of methylation in the maize root-apex: significance for genotypic differences in aluminum resistance. Plant Cell Envir. 28, 1410–1420. doi: 10.1111/j.1365-3040.2005.01357.x

Fang, Q., Wang, Q., Mao, H., Xu, J., Wang, Y., Hu, H., et al. (2018). AtDIV2, an R-B-type MYB transcription factor of Arabidopsis, negatively regulates salt stress by modulating ABA signaling. Plant Cell Rep. 37, 1499–1511. doi: 10.1007/s00299-018-2321-6

Farrás, R., Ferrando, A., Jäsk, J., Kleinow, T., Librizzi, L., Tubisco, A., et al. (2001). SKP1–SnRK protein kinase interactions mediate proteasomal binding of a plant SCF ubiquitin ligase. EMBO J. 20, 2742–2756. doi: 10.1093/emboj/20.11.2742

Ferrari, S., Vairo, D., Ausubel, F. M., Cervone, F., and De Lorenzo, G. (2003). Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infections. Plant Cell 15, 93–106. doi: 10.1105/tpc.031565

Fujiwara, T., Hirai, M. Y., Chino, M., Komeda, Y., and Naito, S. (1992). Effects of sulfur nutrition on expression of the soybean seed storage protein genes in transgenic petunia. Plant Physiol. 99, 263–268. doi: 10.1104/pp.99.1.263

He, H., Zhan, J., He, L., and Gu, M. (2012). Nitric oxide signaling in aluminum stress in plants. Protoplasma 249, 483–492. doi: 10.1007/s00709-011-0310-5

Horton, M. W., Hancock, A. M., Huang, Y. S., Toomajian, C., Atwell, S., Auton, A., et al. (2012). Genome-wide patterns of genetic variation in worldwide Arabidopsis thaliana accessions from the RegMap panel. Nat. Genet. 44, 212–216. doi: 10.1038/ng.1042

Huang, X., Stettmer, K., Michel, C., Hutzler, P., Mueller, M. J., and Durner, J. (2004). Nitric oxide is induced by wounding and influences Jasmonic acid signaling in Arabidopsis thaliana. Planta 218, 938–946. doi: 10.1007/s00024-003-1178-1

Iuchi, S., Koyama, H., Iuchi, A., Kobayashi, Y., Kitabayashi, S., Kobayashi, Y., et al. (2007). Zinc finger protein STOP1 is critical for low-oxygen tolerance in Arabidopsis. J. Exp. Bot. 58, 3297–3311. doi: 10.1093/jxb/erz124

Itchon, D., Stass, A., and Horst, W. J. (2005). Cell-wall pectin and its degree of methylation in the maize root-apex: significance for genotypic differences in aluminum resistance. Plant Physiol. 140, 128–137. doi: 10.1104/pp.107.102337

Kobayashi, Y., Ito, M., Koyama, H., and Nishiwaki, K. (2004). Nitric oxide-induced malate release and its role for rhizotoxic stress tolerance in Arabidopsis. Plant Physiol. 135, 843–852. doi: 10.1104/pp.107.102335

Kobayashi, Y., Kobayashi, Y., Watanabe, T., Shaff, J. E., Ohta, H., Kochian, L. V., et al. (2013). Molecular and physiological analysis of A3+ and H+ rhizotoxicities at moderately acidic conditions. Plant Physiol. 163, 180–192. doi: 10.1104/ pp.113.222893
Sakurai, N., Ara, T., Enomoto, M., Motoegi, T., Morishita, Y., Kurabayashi, A., et al. (2014). Tools and databases of the komics web portal for preprocessing, mining, and dissemination of genomics data. *Biomed. Res. Int.* 2014:948182. doi: 10.1155/2014/948182

Sawaki, Y., Iuchi, S., Kobayashi, Y., Kobayashi, Y., Ikka, T., Sakurai, N., et al. (2009). STOP1 regulates multiple genes that protect Arabidopsis from proton and aluminium toxicities. *Plant Physiol.* 150, 281–294. doi: 10.1104/pp.108.134700

Sawaki, K., Sawaki, Y., Zhao, C. R., Kobayashi, Y., and Koyama, H. (2016). Specific transcriptionic response in the shoots of Arabidopsis thaliana after exposure to Al rhizotoxicity: - potential gene expression biomarkers for evaluating Al toxicity in soils. *Plant Soil* 409, 131–142. doi: 10.1007/s11104-016-2960-8

Schmidtmann, I., König, A.-C., Orwat, A., Leister, D., Hartl, M., and Finkemeier, I. (2014). Redox regulation of Arabidopsis mitochondrial citrate synthase. *Mol. Plant* 7, 156–169. doi: 10.1093/mp/sst144

Sevilla, F., Camejo, D., Ortiz-Espin, A., Calderón, A., Lázaro, J. J., and Jiménez, A. (2015). The thioredoxin/peroxiredoxin/sulfiredoxin system: current overview on its redox function in plants and regulation by reactive oxygen and nitrogen species. *J. Exp. Bot.* 66, 2945–2955. doi: 10.1093/jxb/erv146

Shi, H., Chen, Y., Tan, D.-X., Reiter, R. J., Chan, Z., and He, C. (2015). Melatonin induces nitric oxide and the potential mechanisms relate to innate immunity against bacterial pathogen infection in *Arabidopsis*. *J. Pineal Res.* 59, 102–108. doi: 10.1111/jpi.12244

Siedlecka, A., Wlukld, S., Peronne, M. A., Micheli, F., Lesniewska, J., Sethson, L., et al. (2008). Pectin methyl esterase esterase inhibits intrusive and symplastic cell growth in developing wood cells of *Populus*. *Plant Physiol.* 146, 323–324. doi: 10.1104/pp.107.111963

Soito, A. J., Piippo, M., Allahverdiyeva, Y., Battchikova, N., and Aro, E. M. (2008). Light has a specific role in modulating Arabidopsis gene expression at low light. *BMC Plant Biol.* 8:13. doi: 10.1186/1471-2229-8-13

Song, Y., Chen, P., Xuan, A., Bu, C., Liu, P., Ingvarsson, P. K., et al. (2021). Integration of genome-wide association studies and co-expression networks reveal roles of *PtWRKY42-PtUGT76C1-1* in trans-zeatin metabolism and cytokinin sensitivity in poplar. *New Phytol.* 231, 1462–1477. doi: 10.1111/nph.17469

Spadoni, S., Zabotina, O., Di Matteo, A., Mikkelson, J. D., Cervone, F., Di, G., et al. (2006). Polygalacturonase-inhibiting protein (PGIP) interacts with pectin through a binding site formed by four clustered residues of arginine and lysine. *Plant Physiol.* 141, 557–564. doi: 10.1104/pp.106.076950

Sun, C., Lu, L., Yu, Y., Liu, L., Hu, Y., Ye, Y., et al. (2016). Decreasing methylation of pectin caused by nitric oxide leads to higher aluminium binding in cell walls and greater aluminium sensitivity of wheat roots. *J. Exp. Bot.* 67, 979–989. doi: 10.1093/jxberv514

Tabuchi, A., and Matsumoto, H. (2001). Changes in cell-wall properties of *Arabidopsis thaliana* roots under sodium chloride stress and sodium sensitivity in *Arabidopsis thaliana*. *J. Plant Physiol.* 158, 131–138. doi: 10.1016/s0176-1617(00)02232-1

Togninalli, M., Seren, Ü., Meng, D., Fitz, J., Nordborg, M., Weigel, D., et al. (2018). The AraGW AS catalog: a curated and standardized database of *Arabidopsis thaliana* transcription factors. *BMC Genomics* 9:1063. doi: 10.1186/s12864-018-4598-7

Tsutsui, T., Yamaji, N., and Ma, J. (2011). Identification of a cis-acting element of *ART1*, a C2H2-type zinc-finger transcription factor for aluminum tolerance in *Arabidopsis thaliana*. *Biomed. Res. Int.* 2011:647918. doi: 10.1155/2011/647918

Toginilalli, M., Seren, Ü., Meng, D., Fitz, J., Nordborg, M., Weigel, D., et al. (2018). The AraGW AS catalog: a curated and standardized database of *Arabidopsis thaliana* transcription factors. *BMC Genomics* 9:1063. doi: 10.1186/s12864-018-4598-7

Ohyama, Y., Ito, H., Kobayashi, Y., Ikka, T., Morishita, Y., Kurabayashi, A., et al. (2013). Characterization of AtSTOP1 orthologous genes in tobacco and other plant species. *Plant Physiol.* 162, 1937–1946. doi: 10.1104/pp.112.218958

Sadhuukkan, A., Agrahari, R. K., Wu, L., Watanabe, T., Nakano, Y., Panda, S. K., et al. (2020). Expression genome-wide association study identifies that phosphatidylinositol-derived signalling regulates ALUMINUM SENSITIVE3 expression under aluminium stress in the shoots of *Arabidopsis thaliana*. *Plant Physiol.* 46, 1020–1034. doi: 10.1104/pp.1903191

Ohya, Y., Ito, H., Kobayashi, Y., Ikka, T., Morishita, Y., Kobayashi, M., et al. (2013). Characterization of AtSTOP1 orthologous genes in tobacco and other plant species. *Plant Physiol.* 162, 1937–1946. doi: 10.1104/pp.112.218958

Sadhuukkan, A., Agrahari, R. K., Wu, L., Watanabe, T., Nakano, Y., Panda, S. K., et al. (2020). Expression genome-wide association study identifies that phosphatidylinositol-derived signalling regulates ALUMINUM SENSITIVE3 expression under aluminium stress in the shoots of *Arabidopsis thaliana*. *Plant Physiol.* 302:110711. doi: 10.1007/s11104-020-04777-3

Sadhuukkan, A., Enomoto, T., Kobayashi, Y., Watanabe, T., Iuchi, S., Kobayashi, M., et al. (2019). Sensitive to proton rhizotoxicity1 regulates salt and drought tolerance of *Arabidopsis thaliana* through transcriptional regulation of CIPK23. *Plant Cell Physiol.* 60, 2113–2126. doi: 10.1093/pcp/pcz120

Sadhuukkan, A., Kobayashi, Y., Nakano, Y., Iuchi, S., Kobayashi, M., Sahoo, L., et al. (2017). Genome-wide association study reveals that the aquaporin NIP1;1 contributes to variation in hydrogen peroxide sensitivity in *Arabidopsis thaliana*. *Mol. Plant* 10, 1082–1094. doi: 10.1016/j.molp.2017.07.003

Sakurai, N., Ara, T., Enomoto, M., Motoegi, T., Morishita, Y., Kurabayashi, A., et al. (2014). Tools and databases of the komics web portal for preprocessing, mining, and dissemination of genomics data. *Biomed. Res. Int.* 2014:948182. doi: 10.1155/2014/948182

Sawaki, Y., Iuchi, S., Kobayashi, Y., Kobayashi, Y., Ikka, T., Sakurai, N., et al. (2009). STOP1 regulates multiple genes that protect Arabidopsis from proton and aluminium toxicities. *Plant Physiol.* 150, 281–294. doi: 10.1104/pp.108.134700

Sawaki, K., Sawaki, Y., Zhao, C. R., Kobayashi, Y., and Koyama, H. (2016). Specific transcriptionic response in the shoots of *Arabidopsis thaliana* after exposure to Al rhizotoxicity: - potential gene expression biomarkers for evaluating Al toxicity in soils. *Plant Soil* 409, 131–142. doi: 10.1007/s11104-016-2960-8

Wang, Z., Yang, L., Wu, D., Zhang, N., and Hua, J. (2021). Polymorphisms in cis-elements confer SAUR26 gene expression difference for thermo-response
natural variation in Arabidopsis. New Phytol. 229, 2751–2764. doi: 10.1111/nph.17078
Watanabe, T., Urayama, M., Shinano, T., Okada, R., and Osaki, M. (2015). Application of ionomics to plant and soil in fields under long-term fertilizer trials. SpringerPlus 4:781. doi: 10.1186/s40064-015-1562-x
Wu, L., Sadhukhan, A., Kobayashi, Y., Ogo, N., Tokizawa, M., Agrahari, R. K., et al. (2019). Involvement of phosphatidylinositol metabolism in aluminum-induced malate secretion in Arabidopsis. J. Exp. Bot. 70, 3329–3342. doi: 10.1093/jxb/erz179
Yang, J. L., Zhu, X. F., Peng, Y. X., Zheng, C., Li, G. X., Liu, Y., et al. (2011). Cell wall hemicellulose contributes significantly to aluminum adsorption and root growth in Arabidopsis. Plant Physiol. 155, 1885–1892. doi: 10.1104/pp.111.172221
Yanhui, C., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., et al. (2006). The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol. Biol. 60, 107–124. doi: 10.1007/s11103-005-2910-y
Ye, Y., Chunyan, D., Laping, G., Yuan, Q., Xiaoyan, Y., Qi, C., et al. (2018). Distribution pattern of aluminum in Panax notoginseng, a native medicinal plant adapted to acidic red soils. Plant Soil 423, 375–384. doi: 10.1007/s11104-017-3510-8
Zhang, M., Ye, J., Xu, Q., Feng, Y., Yuan, X., Yu, H., et al. (2018). Genome-wide association study of cold tolerance of Chinese indica rice varieties at the bud burst stage. Plant Cell Rep. 37, 529–539. doi: 10.1007/s00299-018-2313-7
Zhao, C., Zayed, O., Yu, Z., Jiang, W., Zhu, P., Hsu, C. C., et al. (2018). Leucine-rich repeat extensin proteins regulate plant salt tolerance in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 115, 13123–13128. doi: 10.1073/pnas.1816991115
Zhou, Y., Xu, X. Y., Chen, L. Q., Yang, J. L., and Zheng, S. J. (2012). Nitric oxide exacerbates Al-induced inhibition of root elongation in rice bean by affecting cell wall and plasma membrane properties. Phytochemistry 76, 46–51. doi: 10.1016/j.phytochem.2011.12.004
Zhao, C., Zayed, O., Yu, Z., Jiang, W., Zhu, P., Hsu, C. C., et al. (2018). TRICHOME BIREFRINGENCE-LIKE27 affects aluminum sensitivity by modulating the O-acetylation of xyloglucan and aluminum-binding capacity in Arabidopsis. Plant Physiol. 166, 181–189. doi: 10.1104/pp.114.243808

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