Genome-Wide Investigation of 2,4-Diacetylphloroglucinol Protection Genes in *Arabidopsis thaliana*

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The compound 2,4-diacetylphloroglucinol (DAPG) is a well-known secondary metabolite produced by *Pseudomonas* spp. that are used as biocontrol agents. DAPG displays a remarkably broad spectrum of toxic activity against pathogens of plants. Yet high concentrations of DAPG may also have negative effect on plants, but the phytotoxicity of DAPG is not clearly understood. Here, we used genome-wide activation, tagging *Arabidopsis* plants as the model plant to investigate the plant response to DAPG. A total of 15 lines were selected as DAPG-tolerant plants from among 62,000 lines investigated. The DAPG-responsible genes were then identified via thermal asymmetric interlaced PCR and quantitative reverse transcription PCR, and the gene ontology analysis showed the distribution of these genes having different biological processes, cellular regulations, and molecular functional properties. Collectively, these findings suggest that plants may rely on several pathways to prevent DAPG phytotoxicity.

Keywords: activation-tagging line, biological control, 2,4-diacetylphloroglucinol, genome-wide screening, phytotoxicity

Pseudomonas fluorescens, a soil-inhabitant and gram-negative bacterium, is well known as a biocontrol agent that inhibits plant pathogens (Kwak and Weller 2013; Weller et al. 2007). DAPG (2,4-diacetylphloroglucinol) has several antibiotic properties, such as antifungal, antibacterial, anthelmintic, and even phytotoxic effects (Haas and Keel 2003; Keel et al. 1992; Weller et al. 2007). The pathogen-suppressive activity associated with the production of DAPG reportedly inhibits various plant diseases, such as take-all, damping off, and root rot (Raaijmakers and Weller 2001). While DAPG evidently exhibits toxicity to many plants, its phytotoxic effects upon plants remain largely uninvestigated.

The physiological and biochemical responses of *Arabidopsis* to *P. fluorescens* FPT9601-T5, a producer of DAPG, were investigated using the microarray approach (Wang et al. 2005).

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Those microarray experiments identified approximately 200 genes that were expressed differently through the DAPG-producer bacterial treatment; particularly so with respect to metabolisms, signal transduction, and detected genes related to stress response. In the rhizosphere, working with a strain overproducing pyoluteorin and DAPG (CHA0/pME3090) Maurhofer et al. (1995) showed that it preserved tobacco roots significantly better than the wild-type strain against *Thielaviopsis basicola*, but the strain drastically reduced growth of tobacco plants and was toxic to sweet corn. The phytotoxicity of DAPG affects dicotyledonous plants more than monocotyledonous plants (Keel et al. 1992) and it has been hypothesized that DAPG inhibits the root proton secretion system and primary root development (Phillips et al. 2004). In tomato, phytotoxicity of DAPG was also reported (Brazelton et al. 2008), in that DAPG altered the architecture of the tomato root and was capable of interfering with auxin signaling in seedlings. In sum, based on the limited work to date, it seems DAPG activity in plants involves multiple molecular targets that contribute to plant defense as well as phytotoxicity.

To investigate the bioactive mechanism of DAPG, the *Saccharomyces cerevisiae* genome-wide mutant library has been employed (Kwak et al. 2011; Troppens et al. 2013). That work showed DAPG inhibits fungal growth in several ways, including through damage to the cell membrane, bursts of reactive oxygen, cytosolic acidification, and disturbance to cell homeostasis (Kwak et al. 2011). Another mechanism of DAPG antifungal activity has also been proposed, in which cellular-level inhibition of respiration and ATP synthesis contributes to growth inhibition as the primary mode of toxicity (Troppens et al. 2013). The above findings suggest that, in a eukaryotic cell, DAPG may act upon several and primary cellular metabolisms.

Despite confirmations of the DAPG mode of action, such results alone are insufficient to establish the plant response at the physiological and cellular levels. To fill this knowledge gap, in this study, the genome-wide activation tagging of *Arabidopsis* lines was screened to discover genes interacting and plant tolerance to DAPG. Activation tagging of *Arabidopsis* lines is a powerful tool for genome-wide functional studies of bioactive compounds or other biological factors (Tani et al. 2004; Weigel et al. 2000). Specifically, it relies on multimerized 35S transcription enhancers from the cauliflower mosaic virus that are incorporated by T-DNA into the plant genome, thereby improving the expression of certain genes. The activation-tagging lines are appropriate materials for genome-wide analysis of DAPG-responsible genes to obtain accurate information. The objective of this study was to classify the response of plants to DAPG and to select lines tolerant to DAPG. We hypothesized that i) certain genes in *Arabidopsis* can regulate DAPG toxicity;

ii) functional gene overexpression increases plant tolerance to DAPG; iii) the genes involved can be investigated by a genome-wide screen with activation-tagged *Arabidopsis*; and iv) the plant response to DAPG could be predicted by analyzing the gene network and ontology of the *Arabidopsis* lines selected for tolerance.

RESULTS

DAPG inhibited the seed germination of *Arabidopsis thaliana* Col-2.

Assays for inhibition of the seed germination were evaluated in Murashige Skoog (MS) media with different concentrations of DAPG (Supplementary Fig. S1). In the control, seed rupture was very high, at 99.7% after 3 days, as was the cotyledon emergence at 6 days (99.2%). The percentage of rupture in 40 μ M DAPG was 9.3% (6 days), 28.92% (7 days), 66.6% (8 days), 75% (9 days), and 78.9% (10 days), and cotyledons appeared in 7.8% (7 day), 28.8% (8 day), 36.8% (9 day), 72.5% (10 day) of the samples. The rupture and emergence of cotyledons at concentrations >60 μ M DAPG was not observed until 10 days had elapsed (Supplementary Table S1).

A total of 15 activation-tagging lines showed tolerance against DAPG toxicity.

The activation-tagging pools were screened for DAPG tolerance, using two steps, and each test result was followed (Supplementary Table S2). The first screening was performed with 50 μ M DAPG, and this identified a total of 260 lines. The second screening was carried out with the T₄ seeds collected from the first screening lines and was conducted with 60 μ M DAPG, from which 15 lines from the 260 lines were further screened. The seed rupture and germination of the final selected 15 homozygous lines were reconfirmed at 50, 60, and 70 μ M of DAPG (Fig. 1). In particular, line [15]-1 was the most tolerant of DAPG, having rupture percentages of 58.23% at 50 μ M, 29.67% at 60 μ M, and 6.3% at 70 μ M in the DAPG-containing MS media.

Phenotype of the selected activation-tagging lines.

Phenotypic variation was observed in the 16 selected activation-tagging lines, relative to Col-2, at the 15-day growth stage (Supplementary Fig. S2A). On the MS agar medium, the seeds were incubated vertically for 15 days. The primary root length of Col-2 was 52.32 mm, while lines [1]-3 (68.28 mm), [1]-4 (71.48 mm), [1]-7 (65.10 mm), [12]-9 (68.11 mm), [14]-1 (66.15 mm), [15]-1 (69.11 mm), [15]-5 (74.96 mm), and [16]-8 (65.23 mm) all showed longer primary root length than the wild-type (Supplementary Fig. S2B). The Col-2 leaf area was 2.20 mm, which was similar to those of the selected lines (no significant differences were detected [Supplementary Fig. S2C]).

Identification of activation tag-flanking loci in the chromosomes.

To identify the place of insertion of the 15 selected lines, thermal asymmetric interlaced PCR (TAIL-PCR) amplified the flanking position adjacent to the left border of the pSKI015 vector. Of the 22 AD primers used in the experiment, selected line templates were amplified with eight AD primers (AD1, AD2, AD4, AD9, AD19, AD20, AD21, AD22) (Supplementary Table S3). The PCR conditions used for the primary, secondary, and tertiary TAIL-PCR procedures are outlined in Supplementary Table S4. At each stage, nested PCR products were reduced by a left border primer (LB) designed to have a distance between LB0 and LB1 primers of 54 bp and the distance between LB1 and LB2 primers of 201 bp, based on the pSKI015 vector (Supplementary Fig. S3). The tertiary template

was eluted from the agarose gel and was sequenced by using the primer LB2. Of the 15 lines tagging *Arabidopsis*, TAIL-PCR confirmed the flanking loci of 13 lines, but two lines ([9]-4, [9]-5) failed to obtain the flanking loci. The TAIR database was searched for the location and direction of the pSKI015 vector in the genome. Figure 2 shows the schematic position of the pSKI015 vector inserted into the exon of the disrupted gene (black box) and intron (white box) regions. Distance is shown as a brown line between LB, right border, and the adjacent gene. Evidently, for some of the lines ([12]-9 and [16]-8) the pSKI015 vector was placed between two genes. For the [12]-9 line, the pSKI015 vector was situated 519 bp to the right of the *MCA1* and 2,917 bp to the left of the F-box family protein. The [16]-8 line was found positioned 6,969 bp to the right of the transposable element gene and 1,511 bp left of *GDSL1*. Corresponding physical maps of the insertion position of pSKI015 on chromosome 1 ([1]-3, [1]-4, [1]-11, [15]-5, [16]-8, [16]-10), chromosome 2 ([1]-7, [9]-2), chromosome 3 ([9]-1, [14]-1), chromosome 4 ([1]-8, [12]-9), and chromosome 5 ([15]-1) were drawn (Supplementary Fig. S4).

Expression level of the activated genes.

Genes that could be activated by the pSKI015 vector were examined in terms of their relative expression level (Fig. 3). The *ubc* and *sand* genes served as housekeeping reference genes (Supplementary Table S5) and the predicted genes in the selected lines were compared with the relative level of expression, in terms of fold changes. Among the lines, the gene expression pattern was up-regulated in 12 lines. Representatively, the [16]-10 line coiled-coil protein (CP) expressed 2.251-fold (*ubc* gene) and 2.1314-fold (*sand* gene) higher than the Col-2. The [15]-5 line cell wall-associated kinases (WAKs), overexpressed 3.112-fold (*ubc* gene) and 2.842-fold (*sand* gene) than the wild type. Unexpectedly, in the [16]-8 line, the gene encoding protein of the Gly-Asp-Ser-Leu family of esterases/acylhydrolases (*GDSL1*, AT1G29670) underwent 0.783-fold (*ubc*) and 0.641-fold (*sand*) downregulation.

Functional group of DAPG tolerance genes.

The GeneMANIA network was used to investigate interactions between the 12 genes (Fig. 4). The genes conferring DAPG tolerance in plants showed networked interactions, namely in terms of a physical interaction (51.91%), predicted protein interaction (10.87%), coexpression (9.87%), shared

Fig. 1. Seed germination percentages of *Arabidopsis thaliana* Col-2 and of the selected 15 activation-tagging lines containing 2,4-diacetylphloroglucinol (DAPG) (50, 60, 70 μ M) on Murashige Skoog medium. Germination proportions were verified, by seed rupture, at 15 days postincubation. All plants were grown in a growth chamber at 22°C, under 16-h light and 8-h dark conditions. Bars with differing letters indicate a significant difference ($P < 0.05$) and error bars represent standard deviations of the means.

protein (2.32%), genetic interaction (2.22%), and colocalization (0.4%). Nevertheless, four genes (*TP*, *BSDP*, *FRO5*) were left ungrouped from the specified query genes. In the hierarchy of gene functioning with protein domain attributes, Interpro (22.40%) identified the coannotation genes. Epidermal growth factor (EGF)-like calcium-binding domain (*WAKL2*, *WAKL4*, *WAKL5*, *WAKL11*, *WAKL17*, *WAKL8*, *WAK1*, *WAK3*, *VSR3*, *VSR4*, *WAKL18*), zinc finger UBP type (*BRIZ1*, *BRIZ2*), ADC synthase (*ASA1*, *ASA2*, *ICS1*, *ICS2*, *ADCS*, *AT3G55870*), hydroxyacylglutathione hydrolase (*GLX2-1*, *GLX2-2*, *GLX2-4*, *GLX2-5*), and fatty acid hydroxylase (*SBH1*) were included as feature-annotating classes. Based on gene ontology (GO) enrichment, tolerance of DAPG could be linked to the following biological functions: cellular aldehyde metabolic process, thiolester hydrolase activity, organic hydroxyl compound metabolic process (*GLX2-1*, *GLX2-2*, *GLX2-4*, *GLX2-5*), integral component of plasma membrane (*VSR3*, *VSR4*, *MCA1*), monocarboxylic acid metabolic process (*GLX2-1*, *GLX2-2*, *GLX2-4*, *GLX2-5*, *ADCS*), aromatic amino acid family biosynthetic process (*ASA1*, *ASA2*, *ADCS*), establishment of protein

localization to vacuole (*VSR3*, *VSR4*), tryptophan metabolic process, and tryptophan biosynthetic process (*ASA1*, *ASA2*) (Table 1). These results revealed the influence of DAPG phytotoxicity on multiple biological processes in *Arabidopsis* (Table 2).

DISCUSSION

DAPG is a well-described bacterial secondary metabolite that is toxic to a wide range of plant pathogens, including many bacteria, fungi, and nematodes (Haas and Keel 2003; Raaijmakers and Weller 2001, 1998). High levels of DAPG are also harmful to plants, inhibiting their growth, and stalling the germination of seeds in a variety of plant species (Keel et al. 1992; Maurhofer et al. 1995). Further, DAPG was also hypothesized to suppress root proton secretion, thus inhibiting primary root growth (Phillips et al. 2004).

We selected the 15 *Arabidopsis* lines among 62,000 activation-tagging pools as DAPG-compliant lines. Adjacent genes of 13 lines were identified, and quantitative reverse transcription PCR

Fig. 2. Schematic representation depicting the insertion of the pSKI015 vector in selected *Arabidopsis* lines. The triangle is the pSKI015 vector in the flanking positions. Black bars indicate exons, white boxes represent introns, and lines show regions of the untranslated sequences. Each panel is labeled with the gene name and its TAIR ID. RB = right border and LB = left border.

(qRT-PCR) confirmed the expression levels of these genes. GO results suggested that different biological processes, cellular components, and molecular functions that jointly contributed to shaping plant tolerance traits to DAPG. These findings suggest that resistance to DAPG is not just a simple cellular function but, in fact, relies on a complex series of multiple cellular functions. The plasma membrane feature is the cellular interface that governs molecule communication and information shared between cells and their microenvironments (De Marcos Lousa et al. 2012). Integral membrane proteins consist of one or more hydrophobic transmembranes that span the lipid bilayer of the membrane. There are several other ions, hormones, and nutrients transporters that can function as an integral membrane. ABC transporters are responsible for the efflux or influx of a variety of compounds, including auxins, abscisic acid, and antimicrobials, as a representative example (Schulz et al. 2011). Transporters in membrane (*VSR3*, *VSR4*, *MCA1*) had improved tolerance to DAPG. This result suggested that membrane activity plays a critical and key role in protecting plants against the phytotoxicity posed to them by DAPG.

Genes for reactive oxygen regulation were also selected to improve tolerance to DAPG toxicity in our study. The network interaction map showed DAPG tolerance linked to aldehyde metabolism and thioester hydrolase (*GLX2-1*, *GLX2-2*, *GLX2-4*, *GLX2-5*). Aldehydes are compounds that are toxic to many organisms (LoPachin and Gavin 2014). The aldehydes were produced in plants when the latter were subjected to abiotic stress, and aldehyde dehydrogenases (*ALDH*) are known to play an important role in processes of detoxification (Kotchoni et al. 2006). Reduction in H_2O_2 and malondialdehyde (MDA) derived from cellular lipid peroxidation was followed by stress tolerance in the *ALDH3II*-transgenic plant. That finding suggested that *ALDH3II* acts not only as an aldehyde-detoxifying enzyme but also as efficient reactive oxygen species (ROS) scavenger, and the enzyme could prevent lipid peroxidation. Thioester hydrolases or thioesterases (TEs) form a large group of enzymes, which hydrolyze the thioester bond between a carbonyl group and a sulfur atom. For fatty acyl-acyl carrier

protein TEs (CpFATB), work has shown that constitutive expression by these enzymes activates an oxidative signal cascade and contributes to the regulation of peroxisomes levels (Zhang et al. 2012).

Plant tolerance to DAPG also improved in the *Arabidopsis* lines characterized by overexpression of amino acids biosynthesis. The aromatic amino acids (AAA) are the key molecules functioning in plant metabolism and their synthesis depends on the common precursor metabolite chorismate, of the shikimate pathway (Tzin and Galili 2010). The AAA also function as precursors of vital plant hormones, such as auxin and salicylate (Bartel 1997; Vogt et al. 2010). In this study, we established the biosynthetic process of the aromatic amino acid family (*ASA1*, *ASA2*, *ADCS*) in the DAPG-tolerance network. Likewise, the biosynthesis pathway for tryptophan is involved in the metabolism of indole-3-acetic acid (auxin), which is catalyzed from indole-3-glycerol phosphate by the tryptophan synthase alpha subunit. The tryptophan biosynthetic genes (*ASA1*, *ASA2*) identified in our study were found to enhance DAPG tolerance, based on their GO analysis. In other work, DAPG inhibited primary root growth and stimulated lateral root development in tomato seedlings (Brazelton et al. 2008). Exogenous DAPG applications inhibited the activation of an auxin-inducible GH3 promoter with a luciferase reporter gene in transgenic tobacco plants, and DAPG was inferred to alter root architecture by inhibiting the auxin-dependent signaling pathway.

Plant sensitivity to DAPG was related to metabolic processes of organic hydroxy compounds (*GLX2-1*, *GLX2-2*, *GLX2-4*, *GLX2-5*). Glycolate, malate, and lactate are major hydroxyl acids found throughout the plant metabolism pathways (Maurino and Engqvist 2015). Some of them can be found as D- and L-stereoisomers. In particular, D-lactate is produced as a primary metabolism product in plants during methylglyoxal detoxification, from which a highly reactive cytotoxic compound formed (Mustafiz et al. 2014). Methylglyoxal is produced in plants under conditions of abiotic stress, such as salt, drought, and frost, and this metabolite assists in reducing the

Fig. 3. Relative expression level of the genes that accompanied the inserted loci pSKI015. Total RNA was extracted from 10-day-old plants on Murashige Skoog agar. SYBR Green, quantitative real-time PCR, and normalization by internal standard genes (*sand*, *ubc*) were used. Gene expression of the *Arabidopsis* wild-type Col-2 and the selected lines determined the transcription levels. The experiment was performed using three replications. Error bars represent the standard deviation of the mean. Asterisks indicate a significant difference ($P < 0.05$), according to the Student t test.

ROS damage (Hoque et al. 2012). D-lactate is metabolized by a cascade of glyoxalases consisting of the enzymes glyoxalase I (GLX1) and glyoxalase II (GLX2) (Mustafiz et al. 2014). Activity to glyoxalase in mitochondrial enhances the cell detoxification function. The mitochondrial electron transport system and oxidoreductase function were also found important in mitigating the toxicity of DAPG in *S. cerevisiae* (Kwak et al. 2011).

The vacuole in plant cells includes many components, such as protein, sugars, ions, and other secondary metabolites. The vacuolar ATPase is recognized as a key factor in controlling intracellular pH in vesicles, which is determined by the activity of H^+ pumps (Dettmer et al. 2006). Plant tolerance of DAPG was increased in the overexpression ([16]-10) line, which had the vacuolar proton transporting V-type ATPase complex (CP). Membrane-associated receptors, including the vacuolar sorting receptors (VSRs) family, mediate selective trafficking to the vacuole of soluble proteins (Kang et al. 2012). The vacuolar cargo trafficking system requires the VSRs to function well (De Marcos Lousa et al. 2012); both the pH controlling receptor and ligand interaction involves cargo transport to the vacuole (Reguera et al. 2015). Our results suggested the genes involved in vacuole localization of proteins (VSR3, VSR4) were part of the DAPG-tolerance gene network. Both VSR3 and VSR4 are important sorting receptors for storage cargo system in seed and vegetative tissue (Zouhar et al. 2010). The ability of the vacuole to regulate pH is essential to determining the toxicity of DAPG, which caused to cytosolic acidification in *S. cerevisiae* (Kwak et al. 2011).

DAPG-producing *Pseudomonas* spp. are relied upon as primary biocontrol agents in natural disease suppressiveness soils (Kwak and Weller 2013; Valente et al. 2020). Nevertheless, DAPG properties of a plant are not always obvious and several molecular targets are estimated to be involved. Our comprehensive set of results shows that DAPG can be toxic to a plant,

whose various mechanisms must operate jointly to prevent DAPG toxicity.

MATERIALS AND METHODS

Plant material and growing conditions.

Arabidopsis thaliana Col-2 was used as the experimental control. To screen for DAPG-tolerant lines, the activation-tagging pools of CS31100 were used, i.e., 208 pools composed of 62,000 homozygote individual lines (the Arabidopsis Biological Resource Center, Columbus, OH, U.S.A.). Seed sterilization was performed by inserting 100 mg of seeds into a 1.7-ml tube and adding 1 ml of 1% NaOCl, then, vortexing the seed solution for 30 s. Using a pipette, the NaOCl was drained, and the washing process was carried out as follows. Distilled water (1 ml) was added, then, 30 s of a vortex mixer and centrifugation were applied to the mixture, with this washing cycle repeated five times. For seed vernalization, the disinfected seeds were stored at 4°C for 7 days in the dark. Each plant was grown on 0.7% MS agar medium (2.1 g of MS medium, 30 g of sucrose, 7 g of phyto agar per liter, pH 5.7) at 22°C under a 16-h light and 8-h dark photoperiod in a growth chamber (Seyoung Scientific, Bucheon, Korea).

DAPG sensitivity of *Arabidopsis thaliana* Col-2.

DAPG (Santa Cruz Biotechnology, Dallas, TX, U.S.A.) was dissolved (100 mM stock solution) in methanol and was placed in a glass bottle wrapped in foil and kept at -20°C to prevent light penetration. *Arabidopsis thaliana* Col-2 DAPG sensitivity was assessed on the MS agar medium for 10 days. Final DAPG concentrations of 0, 20, 40, 60, 80, and 100 μ M were amended in MS media, for which 100 to 200 seeds per DAPG concentration ($n = 5$ plates) were used. DAPG-untreated and methanol-only amended plates served as the controls. All the plates were incubated for 10 days under the same growth

Fig. 4. Network interactions between genes that induce plant tolerance to 2,4-diacetylphloroglucinol in *Arabidopsis*. Analysis of functional features—InterPro, coexpression, colocalization, physical interactions, and shared protein domains—was performed in the GeneMania system and was visualized using the R program. Each gene is depicted as a black circle whose attributes are represented as a black square. Larger circles represent the overexpressed genes. Interactions are defined as Interpro (dotted red), coexpression (yellow), colocalization (light green), predicted protein interactions (sea blue), and shared protein domains (pink).

chamber conditions described above. Seed germination including cotyledon development morphology, and signs of seed rupture were observed for 10 successive days.

Screening for plant DAPG tolerance in the activation-tagging pools.

In all, 62,000 activation-tagging lines were pooled in 21 groups, with eight to 10 stocks used for the first screening process (Supplementary Table S2). Seed sterilization and vernalization were performed as described above. The pools were screened on MS medium agar, which contained 50 μ M of DAPG. The plates were incubated in the growth chamber for

10 days. Each plate had 150 to 200 seeds, and the experiment consisted of 10 replications. DAPG-tolerant plants were transferred to bed soil (Hungnong, Pyeongtaek, Korea) and were cultivated for 8 weeks, to harvest the T₄ seeds.

For the second screening, the MS medium agar contained 60 μ M of DAPG. The selected lines were checked individually on a square plate (125 × 125 × 17.8 mm), having six seeds per square with 18 repeats per line. Germination based on seed rupture expressed as the average of the replications. The seeds were cultivated and examined over a 10-day period. The MS media contained 50, 60, or 70 μ M of DAPG, on which the 16 selected lines underwent final screening.

Table 1. Functional information of genes in the selected 2,4-diacetylphloroglucinol tolerance *Arabidopsis* lines

Line (TAIR ID)	Gene	Biological progress	Cellular component	Molecular function
[1]-3 (AT1G70220)	RNA-processing, Lsm domain-containing protein	Unknown	Nucleus	Unknown
[1]-4 (AT1G69640)	Sphingoid base hydroxylase 1 (<i>SBH1</i>)	Oxidation reduction process, photomorphogenesis, sphingoid biosynthetic process	Golgi apparatus, endoplasmic reticulum, endoplasmic reticulum membrane, integral component of membrane	Iron ion binding, oxidoreductase activity, sphingosine hydroxylase activity
[1]-7 (AT2G31350)	Glyoxalase 2-5 (<i>GLX2-5</i>)	Methylglyoxal catabolic process to D-lactate via S-lactoyl-glutathione	Chloroplast, mitochondrion	Iron ion binding, zinc ion binding
[1]-8 (AT4G07825)	Transmembrane protein (<i>TP</i>)	Unknown	Integral component of membrane, mitochondrion	Unknown
[1]-11 (AT1G18870)	Isochorismate synthase 2 (<i>ICS2</i>)	Defense response, phyloquinone biosynthetic process, salicylic acid biosynthetic process	Chloroplast, plastid	Isochorismate synthase activity
[9]-1 (AT3G49800)	BSD domain-containing protein (<i>BSDP</i>)	Unknown	Nucleus	Unknown
[9]-2 (AT2G42160)	BRAP2 ring ZnF UBP domain protein 1 (<i>BRIZ1</i>)	Seed germination	Cytoplasm	Ubiquitin transferase
[12]-9 (AT4G35920)	MID1-complementing activity (<i>MCA1</i>)	Calcium ion transmembrane transport, calcium ion transport, carbohydrate homeostasis, cellular response to mechanical stimulus, osmosensory signaling pathway	Integral component of plasma membrane, nucleus	Calcium channel activity
[14]-1 (AT3G49190)	O-acyltransferase (WSD1-like) family protein (<i>ACAT</i>)	Triglyceride biosynthetic process	Nucleus, plasma membrane	Diacylglycerol O-acyltransferase activity
[15]-1 (AT5G23990)	Ferric reduction oxidase 5 (<i>FRO5</i>)	Ion transport, oxidation-reduction process	Integral component of membrane, plasma membrane	Ferric chelate reductase activity
[15]-5 (AT1G69730)	Wall-associated kinase family protein (<i>WAKs</i>)	Protein phosphorylation	Extracellular region, Integral component of membrane, plasma membrane	ATP binding, calcium ion binding, kinase activity, polysaccharide binding, protein serine/threonine kinase activity
[16]-8 (AT1G29670)	A member of the GDSL esterase/acylhydrolase family (<i>GDSL1</i>)	Lipid catabolic process	Apoplast, cell wall, chloroplast thylakoid, extracellular region, nucleus	Hydrolase activity
[16]-10 (AT1G20770)	Coiled-coil protein (<i>CP</i>)	Vacuolar proton-transporting V-type ATPase complex assembly	Extrinsic component of endoplasmic reticulum membrane, nucleus	Unfolded protein binding

Table 2. Gene ontology analysis of the functional network^a

Function	FDR	Genes in network	Genes in genome
Cellular aldehyde metabolic process	0.000355	4	21
Thiolester hydrolase activity	0.005169	4	47
Integral component of plasma membrane	0.097404	3	39
Monocarboxylic acid metabolic process	0.097404	5	251
Aromatic amino acid family biosynthetic process	0.097404	3	40
Aromatic amino acid family metabolic process	0.150161	3	49
Organic hydroxy compound metabolic process	0.221278	4	165
Establishment of protein localization to vacuole	0.538323	2	21
Tryptophan metabolic process	0.538323	2	21
Tryptophan biosynthetic process	0.538323	2	18

^a Each function of genes listed induced an increase in 2,4-diacetylphloroglucinol sensitivity. FDR = false discovery rates.

Phenotype observation of DAPG tolerance lines.

The Col-2 and 15 selected lines of *Arabidopsis* were compared for their phenotype characteristics on 1.2% MS medium (2.1 g MS medium, 30 g sucrose, 12 g phyto agar per liter). The growth chamber conditions were the same as before (discussed above), and the plants grew for 15 days in the vertical direction. Root length and leaf diameter of established seedling were measured using ImageJ software (64-bit java 1.8.0_112). The statistical analysis relied on two-sided Dunnett's multiple comparisons (set at $P < 0.05$).

TAIL-PCR and sequencing.

Plant DNA was extracted, using the modified Edward buffer: 200 mM of Tris-HCl, pH 7.5, 250 mM of NaCl, 25 mM of EDTA, 0.5% sodium dodecyl sulfate (Edwards et al. 1991). The LB primer was designed using the known sequences of the pSKI015. The characteristics of the three nested primers were that LB1 has a distance of 54 bp to LB0 and LB2 has a distance of 201 bp to LB1 (Supplementary Table S2). As random primers, 21 arbitrary degenerate primers (AD1 approximately 21) were used (Wu et al. 2015). The PCR was performed using KOD-FX-Neo (Toyobo, Osaka, Japan) and Top DNA polymerase (Bioneer, Daejeon, Korea). The PCR reaction mixture conditions can be found in Supplementary Table S3. The primary TAIL-PCR program went as follows: 4°C for 2 min, 93°C for 1 min, 95°C for 1 min, then four cycles at 94°C for 30 s, 62°C for 1 min, 72°C 4 min, followed by 94°C for 30 s, 25°C for 3 min, ramp up for 4 min (0.2°C per second up to 72°C), 72°C for 2 min 30 s, 14 cycles at 94°C for 10 s, 68°C for 1 min, 72°C for 3 min, 94°C for 10 s, 68°C for 1 min, 72°C for 3 min, 94°C for 10 s, 44°C for 1 min, 72°C for 3 min. All the samples were incubated at 72°C for 10 min and were then held at 4°C. The secondary TAIL-PCR program was performed as follows: 4°C for 2 min, 11 cycles at 94°C for 10 s, 64°C for 1 min, 72°C for 3 min, 94°C for 10 s, 64°C for 1 min, 72°C for 3 min, 94°C for 10 s, 44°C for 1 min, 72°C for 3 min, and the final extension was performed at 72°C for 10 min and held at 4°C. The tertiary TAIL-PCR program consisted of 4°C for 2 min, then 19 cycles at 94°C for 10 s, 44°C for 1 min, and 72°C for 2 min. The PCR products were verified by gel electrophoresis (1.0% agarose), and the DNA fragments were extracted from the gel using the Expin Gel SV (GeneAll, Seoul, Korea). For the sequencing, a TAIL-PCR template was used with LB2 and amplified AD primers.

RNA extraction, RT, and qRT-PCR.

Arabidopsis (T₅) activation-tagging lines and Col-2 plants were grown on MS medium for 10 days and were homogenized (using liquid nitrogen). Their RNA was extracted from homogenized samples with the RiboEx kit (GeneAll) and was then stored at -80°C. The cDNA was synthesized using ReverTra Ace- α - (Toyobo), for which 2 μ g of total RNA was used per sample for cDNA synthesis as follows: 4 μ l of 5 $\times$ RT buffer, 2 μ l of dNTP mixture (10 mM each), 1 μ l of RNase inhibitor (10 U/ μ l), 1 μ l of Oligo (dT) 20 (10 pmol/ μ l), and 1 μ l of ReverTra Ace, with RNase-free H₂O added to attain the 20- μ l final volume. The PCR machine was set for 20 min at 42°C; after the synthesis process, the RNA polymerase was inactivated by a heat treatment (99°C for 5 min). The qRT-PCR was performed using SYBR Green Real-Time PCR master mix (Toyobo). The reaction volume (20 μ l) comprised 1 μ l of 50-fold diluted cDNA, 10 μ l of SYBR Green, 1 μ l of each forward and reverse primer, and 7 μ l of distilled water (Supplementary Table S4). Both *ubc* and *sand* were employed as housekeeping reference genes (Czechowski et al. 2005). The qRT-PCR was carried out in a thermocycler (Bio-Rad, Hercules, CA, U.S.A.). Its cycle conditions were as follow: 95°C for 1 min, then 44

cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s, followed by 95°C for 10 s and 55°C for 5 s. This experiment had three replications per gene. Relative gene expression levels were calculated by the cycle threshold method and, so, were quantified with respect to the internal control gene.

Mapping the insertion loci and chromosome.

Genes conferring plant tolerance to DAPG were searched for in The *Arabidopsis* Information Resource (TAIR) database. Their respective identification number was used to identify their locus on the *Arabidopsis* genome and place of insertion, for which the chromosome map tool (TAIR database) was used. To insert the flanking gene into the genome, the sequence was used and identified by TAIL-PCR amplicon sequence information; it was confirmed to be the start-end point and direction of pSKI015.

Network and GO.

Network and GO analyses were conducted to categorize the functional groups of DAPG tolerance-related genes. The functional network within the genes was grouped by GeneMANIA, a prediction tool for network weights based upon the relevance among the query genes (Montejo et al. 2014). Only overexpressed genes were identified as query values. The detailed options were confirmed by biological process-based network, and the maximum resultant gene value was 21 and the maximum resultant attributes adjusted to 15. Network data were obtained using default setting for attributes, coexpression, colocalization, genetic interactions, physical interactions, predicted, and shared protein domains. The raw data were used to visualize the network in the R computing platform. GO terms predicted via the functional enrichment analysis were obtained from the GeneMANIA.

AUTHOR-RECOMMENDED INTERNET RESOURCES

GeneMANIA online network: <https://genemania.org>
TAIR website: <https://www.arabidopsis.org>

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