Role of salicylic acid glucosyltransferase in balancing growth and defence for optimum plant fitness

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Abstract
Salicylic acid (SA), an essential secondary messenger for plant defence responses, plays a role in maintaining a balance (trade-off) between plant growth and resistance induction, but the detailed mechanism has not been explored. Because the SA mimic benzothiadiazole (BTH) is a more stable inducer of plant defence than SA after exogenous application, we analysed expression profiles of defence genes after BTH treatment to better understand SA-mediated immune induction. Transcript levels of the salicylic acid glucosyltransferase (SAGT) gene were significantly lower in BTH-treated Nicotiana tabacum (Nt) plants than in SA-treated Nt control plants, suggesting that SAGT may play an important role in SA-related host defence responses. Treatment with BTH followed by SA suppressed SAGT transcription, indicating that the inhibitory effect of BTH is not reversible. In addition, in BTH-treated Nt and Nicotiana benthamiana (Nb) plants, an early high accumulation of SA and SA 2-O-β-D-glucoside was only transient compared to the control. This observation agreed well with the finding that SAGT-overexpressing (OE) Nb lines contained less SA and jasmonic acid (JA) than in the Nb plants. When inoculated with a virus, the OE Nb plants showed more severe symptoms and accumulated higher levels of virus, while resistance increased in SAGT-silenced (IR) Nb plants. In addition, the IR plants restricted bacterial spread to the inoculated leaves. After the BTH treatment, OE Nb plants were slightly larger than the Nb plants. These results together indicate that SAGT has a pivotal role in the balance between plant growth and SA/JA-mediated defence for optimum plant fitness.

KEYWORDS
benzothiadiazole (BTH), defence, fitness, growth, immune induction, salicylic acid, salicylic acid glucosyltransferase
1 | INTRODUCTION

The trade-off between growth and defence in plants has been thought to be regulated by signal pathways that are mediated by plant hormones (Huot et al., 2014). The phytohormone salicylic acid (SA) is involved in plant development and responses to biotic and abiotic stresses (Rivas-San Vicente and Plasencia, 2011). Most SA in cells is glucosylated and/or methylated (Rivas-San Vicente and Plasencia, 2011). The conversion of methyl salicylate (MeSA) to SA can be catalysed by SA-binding protein 2 (SABP2) to induce systemic acquired resistance (SAR) (Furouhar et al., 2005; Park et al., 2007; Tripathi et al., 2010), a key mechanism in the innate immune system of plants (Canet et al., 2010). On the contrary, SA can be converted into SA-2-O-β-D-glucoside (SAG) (Pastor et al., 2013). The glucosylation of SA is mediated by uridine diphosphate (UDP)-glucosyltransferase, also known as SA glucosyltransferase (SAGT) (Vlot et al., 2009), which is generally not required to induce SAR (Loake and Grant, 2007). Seto et al. (2011) reported that SAGT of tobacco also catalyses the glucosylation of tuberic acid (12-hydroxyjasmonic acid; TA), which is a derivative of jasmonic acid (JA), and its expression can be induced by mechanical wounding. SAGT expression is induced by exogenous SA application or pathogen attack, indicating a crucial role for SAGT in regulating a balance between SA and SAG. Chivas and Carr (1998) previously reported that SA-induced resistance was greatly reduced by the expression of NahG, which converts SA to catechol, in a transgenic Nicotiana tabacum line (NahG Nt), indicating that the resistance depends on the concentration of endogenous SA. The effect of exogenously applied SA on plant growth is often affected by plant species and growth stage, and varies with frequent changes in endogenous SA levels controlled by SAGT (Vlot et al., 2009; Klessig et al., 2018).

Chemically induced immune responses have been well studied and documented for developing strategies to protect plants from pathogen attack. Although the effectiveness of resistance-inducing chemicals varies widely depending on the combination of host and pathogen, such immune induction is generally accompanied by up-regulation of defence genes associated with SAR (Ryals et al., 1996; Oostendorp et al., 2001; Gozzo and Faoro, 2013; Faoro and Gozzo, 2015; Dempsey and Klessig, 2017). For synthetic immune induction, benzoazidialazole (BTH), also known as 1,2,3-benzoazidialazole-7-thiocarboxylic acid-S-methyl-ester (ASM), is one of the most commonly used chemical inducers. BTH can induce host resistance against a wide range of pathogens, including plant viruses (Ishii et al., 1999; Narusaka et al., 1999a, 1999b; Anfoka, 2000; Pappu et al., 2000; Oostendorp et al., 2001; Cools and Ishii, 2002; Smith-Becker et al., 2003; Mandal et al., 2008; Lin and Ishii, 2009; Takeshita et al., 2013; Frąckowiak et al., 2019). This immune induction by BTH is correlated with an increase in gene expression of resistance-related genes, including the pathogenesis-related protein 1 gene (PR1), a well-known marker of SA-induced SAR. Tripathi et al. (2010) reported that SABP2 catalyses conversion of BTH into acibenzolar and that acibenzolar is required for SAR induction in tobacco.

Friedrich et al. (1996) showed that BTH increased the transcript level of PR1a in a dose-dependent manner, even in NahG Nt, and did not directly induce SA accumulation in the plants. These results indicate that BTH can elicit the resistance-related pathway downstream of SA accumulation. BTH can induce resistance to Peronospora tabacina and tobacco mosaic virus (TMV) in NahG Nt, suggesting that it can activate defence responses without SA accumulation (Friedrich et al., 1996). Lawton et al. (1996) first reported that BTH can induce host resistance in NahG Arabidopsis plants but not in the Nim1 (NPR1; NON-EXPRESSOR of PATHOGENESIS-RELATED GENES 1) mutant, suggesting that BTH can activate the SAR-associated pathway between SA accumulation and NPR1 expression. SA has been found to bind NPR1, which has a slightly higher affinity for BTH than for SA (Wu et al., 2012).

These findings raise the question: How can BTH induce the immune response more effectively than SA? We first analysed an inhibitory effect of BTH on viral infection and then analysed the changes in transcript level of the genes associated with host basal resistance. We eventually focused on the changes in transcript level of salicylic acid glucosyltransferase (SAGT) as the potential cause of the difference in action between BTH and SA. Here, we found that BTH suppressed SAGT expression to enhance immune induction and that SAGT can discriminate between SA and BTH to induce resistance. In addition, we propose that SAGT regulates not only SA but also JA, two major plant hormones controlling plant defence. We conclude that SAGT is a key factor that modulates the balance (trade-off) between plant growth and defence.

2 | RESULTS

2.1 | Prior treatment with BTH suppresses CMV-inducing symptoms

When wild-type (Wt) N. tabacum (Nt) and Nicotiana benthamiana (Nb) plants were treated with BTH 2 days before inoculation with cucumber mosaic virus (CMV), the plants developed very mild symptoms (Figure 1a,c). CMV accumulated to a much lower level in the BTH-treated Nt plants than in the control at 9 days post-inoculation (dpi) (Figure 1b), and to less than one-third the level of the control in BTH-treated Nb plants at 5 dpi (Figure 1d). These results suggest that BTH is certainly effective in suppressing viral spread to upper leaves. Treatments with BTH did not induce any abnormal development in the Nt and Nb plants.

2.2 | BTH and SA differ in their mechanism of action in immune induction

To characterize the chemical induction of immunity by BTH, we analysed the expression of several marker genes involved in SA-, jasmonic acid (JA)-, and ethylene (ET)-signalling, SA accumulation, and RNA silencing in BTH-treated leaf tissues of Wt Nt and NahG Nt plants. The transcript levels of these genes were measured using quantitative reverse transcription PCR (RT-qPCR) between 6 hr post-treatment (hpt) and 288 hpt. Accumulation of PR1a and PR1b transcripts in BTH-treated plants was significantly higher than in
water-treated plants at 12 hpt (Figure S1a,b), but other host genes (SAGT, Coi1, PDF 1.2, EREBP1, EREBP2, ERF1, PAL, and ICS) were not up-regulated by the BTH treatment compared with those in the water-treated controls (Figures 2 and S1c–i). Furthermore, the relative levels of the RDR1 and RDR6 transcripts were not consistently elevated by the BTH treatment (Figure S1j,k). Obvious differences in gene expression were not found between the NahG Nt and the Wt Nt plants (Figure S2a–l). These results suggest that the host responses activated by BTH in the SA-mediated signal transduction pathway are regulated downstream of SA biosynthesis.

Focusing on the genes with a significant change in transcript levels during the 12 days of observation, we noticed that only SAGT transcription was continuously down-regulated in the BTH-treated Wt Nt plants in the assay (Figure 2). We thus decided to elucidate the role of SAGT in the BTH-induced immunity. SAGT catalyses the conversion of SA to SAG, which does not stimulate plant defence responses (Lee and Raskin, 1998). The transcript level of SAGT in the BTH-treated Wt Nt plants compared to the level in the water-treated plants did not differ at 6 hpt (Figure 3a) but was lower at 12 and 48 hpt (Figure 3b,c). On
FIGURE 3  Transcript levels of several resistance-related genes in benzothiadiazole (BTH)-treated wild-type *Nicotiana tabacum* and *N. benthamiana* plants. (a)–(c), (g)–(i) Wild-type (Wt) *N. tabacum*, (d)–(f) WT *N. benthamiana* plants. (a)–(f) SAGT, (g)–(i) PR1a, (j)–(l) Coi1, (m)–(o) PDF1.2, (p)–(r) RDR6. Leaf samples were harvested at 6, 12, and 48 hr post-treatment (hpt) with BTH (0.12 mM) or salicylic acid (SA) (1 mM). Mean relative transcript levels (±SE) of the host genes were measured by quantitative reverse transcription PCR. Levels (in arbitrary units) are for individual plants (*n* = 4). Different letters denote statistically significant differences among the BTH-, SA- and water-treated leaves (Tukey–Kramer method; *p* < .05). Bars indicate standard errors (±SE).
the contrary, SAGT in the SA-treated Wt Nt plants was greatly up-regulated at 6 hpt (Figure 3a), then down-regulated to levels equivalent to those in the water-treated plants at 12 and 48 hpt (Figure 3b,c). We also used Wt Nb in the same experiments to determine whether the SAGT expression profile in response to BTH is similar in other Nicotiana species. The results showed that SAGT expression was very similar between the two species except that the SAGT transcript level in the BTH-treated Wt Nb plants was almost equivalent to that in the water-treated controls at 12 hpt, indicating that the initial effect of BTH in Nb can last longer than in Nt (Figure 3d–f). In addition to SAGT, we measured the transcript levels of PR1a, Coi1, PDF 1.2, and RDR6. The transcript levels of PR1a in the BTH-treated Wt Nt plants were equivalent to those in the water-treated controls at 6 hpt (Figure 3g) but were significantly higher at 12 and 48 hpt (Figure 3h,i). On the contrary, PR1a in the SA-treated Wt Nt plants was greatly up-regulated at 6 hpt (Figure 3g), then quickly down-regulated to the levels equivalent to those in the water-treated controls after 12 hpt (Figure 3h,i). These results suggest that the resistance induced by BTH lasts longer than that by SA. In the BTH- and the SA-treated Wt Nt plants, Coi1, PDF 1.2, and RDR6 had similar expression profiles (Figure 3j–r).
2.3 | BTH decreases the level of SAGT transcripts and SAG

We then conducted pre-BTH + subsequent-SA applications to investigate whether BTH can reduce the SAGT expression level compared to the SAGT induction by exogenously applied SA. As shown in Figure 4a,b, even a relatively low level of BTH (0.12 mM) efficiently cancelled the SAGT induction by a higher level of SA (1 mM). We therefore assume that BTH can directly and strongly control SAGT expression regardless of the concentration of endogenous SA.

Next, the levels of SA and SAG were measured in the Wt Nt and Wt Nb plants (Figures 5 and S3). In the Nt plants, both the SA and BTH treatments led to a temporal increase in SA at 6 hpt, but SA levels declined to the same levels as in the water-treated control by 12 hpt. On the contrary, only the SA-treated plants had much higher SAG levels than in the water-treated controls at 6, 12, and 48 hpt. The results in the Nb plants were essentially similar except that higher levels of SA seemed to persist longer than in the Nt plants (Figure S3). These results together suggest that the dynamics of SAGT regulation by BTH and SA are intrinsically different.

2.4 | SAGT stimulates biomass production and negatively regulates SA and JA levels

Because BTH treatment has been previously reported to lead to decreased plant biomass (Canet et al., 2010), we generated SAGT-overexpressing (OE1 and OE2) and SAGT-silenced (IR1 and IR2) Nb transgenic lines to examine whether the SAGT accumulation levels, which are suppressed by BTH, alter plant growth. After treatment with BTH, the OE1 and OE2 Nb lines produced higher levels of SAGT transcripts and SAGT (Figure 6a,b). As we expected, the transgenic lines also accumulated significantly lower levels of SA and higher levels of SAG than in Wt control plants (Figure 6c,d). Curiously, OE2 plants had less JA and TA and accumulated more tuberonic acid glucoside (TAG) than in the Wt control plants (Figure 6e–g). These observations suggest that JA is converted to TAG via TA by SAGT-mediated glucosylation, thus reducing JA accumulation (Figure 6e–g), in agreement with Seto et al. (2011), who described that SAGT can convert TA into TAG. OE1 plants sprayed with BTH also had slightly more biomass than the Wt control plants, and OE2 plants had significantly more (Figure 6h,i). In contrast, the biomass of IR1 and IR2 plants, which contained lower levels of SAGT transcripts (Figure S5), was not significantly greater than that of the Wt control plants after
Validation of SAGT-overexpression and SAGT-silenced *Nicotiana benthamiana* (Nb) lines. WT, wild-type Nb plants. (a) Mean relative transcript levels (±SE) of SAGT in SAGT-overexpression (OE1 and OE2) Nb lines were measured by quantitative reverse transcription PCR. Levels (in arbitrary units) are for individual plants (*n* = 4). *Significant difference between the OE1 (or OE2) and the WT Nb plants, according to Student’s *t* test (*p* < .05). (b) Western blot analysis of SAGT protein overexpressed in OE1- and OE2-transgenic Nb lines. SAGT was detected using anti-SAGT peptide antibody. (c)–(g) Mean relative levels of (c) salicylic acid (SA), (d) SA 2-O-β-D-glucoside (SAG), (e) jasmonic acid (JA), (f) tuberonic acid (TA), and (g) tuberonic acid glucoside (TAG) (±SE) in OE2 Nb plants. Levels (in arbitrary units) are for WT and OE2 plants (*n* = 4). *Significant difference between OE2 Nb line and the WT Nb plants, according to Student’s *t* test (*p* < .05). (h) Biomass of wild-type (WT), SAGT-overexpressing Nb lines (OE1 and OE2), and SAGT-suppressing Nb lines (IR1 and IR2) at 14 days post-treatment (dpt). Different letters denote statistically significant differences among the WT and the transgenic Nb lines (Tukey–Kramer method; *p* < .05, *n* = 7). (i) WT, OE lines and IR lines at 14 dpt. Whole plants were sprayed with benzothiadiazole (BTH) (0.12 mM).
treatment with BTH (Figure 6h,i). These results together indicate that SAGT has a key role in the trade-off between plant defence and biomass production.

2.5 | A role of SAGT in plant immunity

To confirm the direct involvement of SAGT in host defensive responses, we inoculated OE2 and IR2 plants with CMV. The OE2 plants developed chlorosis and yellow mosaic more consistently than the control Wt plants did (Figures 7a,c and S6), whereas the symptoms on IR2 plants were somewhat attenuated compared to the severe mosaic on the control at 21 dpi (Figures 7b,c and S6). Similarly, after the inoculation with Pseudomonas syringae, the IR1 and IR2 lines expressed a hypersensitive response in the infiltrated areas, indicating that SA-related host resistance had been activated in the IR plants (Figure 7d–f). CMV accumulation levels were approximately 3-fold higher in the OE2 plants than
those of WT, and one-quarter the level of WT in the IR2 plants (Figure 7g). We further confirmed that SAGT transcript levels were stably up-regulated in OE2 plants and down-regulated in IR2 plants at 21 dpi (Figure 7h,i). Other transgenic lines (OE1 and IR1) showed similar results (Figure S7). Taken together, these results demonstrate that SAGT has an important role in host defensive responses.

3 | DISCUSSION

3.1 | Induction of immunity by BTH does not induce SA synthesis and RNA silencing

In contrast to the strong induction of PR1a and PR1b gene expression by treatment with BTH, the other five JA/ET-mediated resistance-related genes, Coi1, PDF1.2, EREBP1, EREBP2, and ERF1, were not up-regulated (Figure S1). This result is consistent with the reports for Arabidopsis thaliana mutants that host resistance-related genes involved in the JA/ET signal transduction pathways are not key players in BTH-induced resistance (Lawton et al., 1996). In addition, the results from the comparative quantification of transcript levels of ICS, PAL, RDR, and RDR suggest that induction of immunity by BTH did not significantly induce biosynthesis of SA and RNA silencing (Figure S1). Then, what is the key factor for BTH-induced resistance? What is responsible for the difference between BTH and SA? We thus further analysed the role of SAGT in the BTH-mediated resistance, considering that SAGT may be responsible for the difference between BTH and SA (Figure 2).

3.2 | BTH-mediated immune induction is enhanced through the suppression of SAGT expression

Exogenous application of BTH not only induces resistance against viral infection, but also suppresses symptom development in the Wt Nt and Nb plants (Figure 1). To obtain a clue for the operation site of BTH in the resistance induction, we next analysed comparative changes in expression of the several defence-related genes in comparison with the case of SA. Among the examined host genes, PR1a showed a longer-lasting induction in the BTH-treated Wt Nt plants. The result was consistent with prominent up-regulation of PR1a in A. thaliana on the basis of microarray analyses (Gruner et al., 2013). We then noticed that BTH, but not SA, specifically suppressed SAGT transcription during plant immune induction (Figure 3), providing a feasible explanation for the phenomenon that BTH is more efficient than SA in induction of the SA-dependent host immune system. The inhibition of the conversion of SA into SAG could lead to high levels of SA accumulation under biotic and abiotic stresses. The SAGT transcript levels in NahG Nt plants treated with BTH were equivalent to those in the water-treated NahG Nt plants, implying that initial accumulation of SA may be necessary for the down-regulation of SAGT by BTH (Figure S2). As shown in Figure 5, SA seemed to be induced to some extent at 6 hpt by BTH, although BTH did not seem to induce SAG accumulation in either Wt Nt or Wt Nb plants (Figures 5 and S3). In support of our results, Friedrich et al. (1996) previously reported that their BTH treatment elicited host resistance pathways downstream of SA accumulation, not by directly enhancing SA accumulation. We here conclude that one of the crucial roles of BTH is the suppression of SAGT transcript levels to inhibit the conversion of SA to SAG. The observation that BTH is a stronger inducer of resistance than SA can be at least partially explained by the finding that BTH has a slightly higher affinity than SA for NPR1 (Wu et al., 2012); NPR1 is central to the activation of the SA-mediated signalling pathway. Our hypothesis based on SAGT provides an additional explanation for the BTH-mediated immune induction. We thus believe that BTH can function not only as a simple SA mimic, but also as an efficient suppressor of SAGT transcription.

3.3 | SAGT contributes to the trade-off between plant growth and defence

The effect of SA on plant growth varies among plant species. Exogenous application of SA promotes growth of soybean plants (Gutiérrez-Coronado et al., 1998) and larger ears of wheat (Shakirova et al., 2003). A low level (50 µM) of SA has a positive effect on growth of chamomile plants, but a high level (250 µM) has negative effect (Kováčik et al., 2009). In addition, the exogenous SA application (100 µM and 1 mM) suppressed the development of trichomes in A. thaliana (Traw and Bergelson, 2003). Exogenous SA also causes a change in hormonal balance to affect photosynthesis, transpiration, and opening and closure of stomata (Shakirova et al., 2003; Stevens et al., 2006; Abreu and Munné-Bosch, 2009). An appropriate level of SA may be indispensable to properly regulate plant growth rate; SA is even involved in flowering and senescence (Kuriana and Cleland, 1992; Morris et al., 2000). We here hypothesize that SAGT is a key player in the regulation of SA level and thus in plant growth.

The SAGT-overexpressing Nb lines, OE1 and OE2, had increased SAG levels and plant fresh mass, whereas the SAGT-silenced Nb lines, IR1 and IR2, did not (Figure 6). These results suggest that SAGT has a crucial role in optimizing plant fitness by allocating resources for resistance activation and biomass production by regulating SA and SAG levels. In a study of the effects of the SA-mediated signal transduction pathway on biomass production using BTH, Canet et al. (2010) reported that plant fresh mass of A. thaliana was reduced after BTH treatment, which they concluded to be due to the stimulation of the SA-mediated signal transduction pathway, not to BTH phytotoxicity. In addition, SA and BTH also inhibited the auxin-mediated signal transduction pathway, and AXR3, the auxin-inducible AUX/IAA transcription regulator, played a key role as the sensor for SA and BTH in controlling the balance between disease resistance and plant growth (Canet et al., 2010).

Canet et al. (2010) further described that the biomass of BTH-treated A. thaliana plants was reduced in a dose-dependent manner. Acibenzolar, a converted form of BTH, itself thus seems to be
maintained at a constant level without conversion to SAG in cells. Although endogenous SA in the BTH-treated plants was lower than in the SA-treated plants, the level had increased to some extent by 6 hpt (Figure 5). Because BTH suppresses transcription of SAGT in the BTH-treated plants (Figures 3 and 4), leading to an increase in endogenous SA, an additive effect of the elevated endogenous SA and exogenously supplied BTH may cause growth inhibition.

In contrast to an increase in SAG levels, the SA levels in the OE Nb lines were reduced by half by overexpression of SAGT. BTH treatment of SAGT-overexpressing Nb plants led to a slight increase in biomass compared with the Wt Nb plants (Figure 6). In the transgenic Nb plants, the inhibitory effect of BTH on SAGT expression must have been hampered by the overexpression of SAGT. On the contrary, the biomass of the Wt Nb plants will be reduced by the additive effect of BTH and endogenous SA. Considering the results together, we attribute the difference in biomass to the growth reduction in the Wt Nb plants as a result of low levels of SAGT, not to growth enhancement by high levels of SAGT in the SAGT-overexpressing Nb plants.

We found that BTH can down-regulate SAGT expression and thus reduce SAG accumulation, unlike SA, as illustrated in our hypothetical model in Figure 8a,b. In this model, we postulate that BTH can up-regulate endogenous levels of both SA and JA by suppressing SAGT synthesis, although a high level of endogenous SA eventually inhibits JA-mediated gene expression (Caarls et al., 2015) for approximately 10 days as estimated from Figure S1a,b, resulting in induction of the SA-related defence responses and subsequently lower biomass (Figure 8a). On the contrary, exogenously applied SA can up-regulate the expression level of SAGT, which converts SA to SAG, and also TA to TAG, leading to the inhibition of both SA- and JA-mediated resistance (Figure 8b) and eventually transient defence responses for approximately a couple of days as estimated from Figure 3g–i. Considering that SAGT is the key factor in the control of SA and JA levels, the use of BTH for studies on plant defence responses will be an excellent tool to understand the trade-off between plant defence and biomass production for optimum fitness.

Using the SAGT-overexpressing and -silencing Nb lines, we demonstrated that SAGT regulates the balance between plant defence and growth (Figure 8c,d). In this model, the constitutive overexpression of SAGT can simultaneously down-regulate both SA- and JA-mediated resistances, resulting in greater biomass production (Figure 8c). On the contrary, the constitutive silencing of SAGT can up-regulate SA-mediated resistance but reduce biomass production (Figure 8d). Although the involvement of JA in biomass production has not been intensively discussed before, unlike SA, there are still some findings in connection with this. For example, Campos et al. (2016) previously reported that attenuated growth of A. thaliana associated with anti-insect resistance could be caused by the JA-mediated signalling. Given the high relevance of SAGT to the SA/JA-mediated defence, it is possible that SAGT has roles not only in pathogen-induced but also in herbivory-induced resistance, both of which are perhaps associated with reduced plant growth. However, further information on the relationship between SAGT and JA would be necessary to clearly understand how SA (BTH)/JA controls the balance between plant growth and defence.

In the pathogen inoculation experiments, we found that the CMV accumulation levels were higher in the SAGT-overexpressing plants and lower in the SAGT-silencing plants, which can be explained in our model (Figure 7g–i). In addition, after Pseudomonas inoculation, expanding necrotic lesions developed on the inoculated leaves of Wt Nb plants, while necrotic lesions were not obvious on the SAGT-silenced Nb plants at 12 hpi (Figure 7d–f). Numerous papers support our observations. For example, Song et al. (2008) showed that transgenic A. thaliana plants overexpressing AtSAG1 were more susceptible to P. syringae than the wild type. Noutoshi et al. (2012) reported that inhibitors of SAGT actually enhanced resistance against P. syringae. Yao et al. (2007) also demonstrated that transgenic tobacco overexpressing the β-glucosidase gene from Butyrivibrio fibrisolvens H17c increased SA accumulation through the conversion of SAG into SA, leading to enhancement of host resistance to TMV.

**FIGURE 8** Model for important roles of SAGT in plant responses to benzothiadiazole (BTH) and salicylic acid (SA). Bold vertical arrows indicate the direction of regulation. (a) Exogenous BTH treatment for plants. (b) Exogenous SA treatment for plants. (c) Constitutive SAGT expression in transgenic plants. (d) Constitutive SAGT silencing in transgenic plants.
On the contrary, this model does not seem to fit the case of rice. Umemura et al. (2009) previously documented that RNAi suppression of OsSGT1, a benzimidazole-responsive UDP-glucose:SA glucosyltransferase of rice, impaired benzimidazole-dependent disease resistance against blast disease in rice plants, indicating that the role of the ratio of SA/SAG in rice seems to be different from that in other plants. In fact, SAG in rice can induce resistance as SA does (Bundó and Coca, 2016). In rice, SA-mediated resistance is activated not only by SA but also by SAG, and SAG is differentially induced, suggesting that SAG is a key factor for SA-mediated rice resistance rather than SA per se (Bundó and Coca, 2016). We therefore presume that the role of SAG in rice is unique compared to other plants.

Gruner et al. (2013) previously reported that biologically induced SAR and the immunity induced by exogenously applied BTH shared a common signal transduction system for immune response but with some differences. According to their analyses, some UDP-glucosyltransferase (SGT synonym) genes of A. thaliana were up-regulated and others were down-regulated by either BTH or SA treatment; the individual genes were thus differentially expressed in response to BTH and SA. We here assume that a certain SAGT among the isoforms actively and specifically responds to pathogen infection; the induction of each SAGT gene would therefore differ depending on the pathogen. For example, Lee and Raskin (1999) demonstrated that tobacco SAGT was specifically induced after inoculation with avirulent viral and bacterial pathogens. Taken together, we conclude that the SAGT genes at least in two Nicotiana species play an important role in the trade-off between plant defence and growth.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plants, pathogens, and chemical treatment

N. tabacum and N. benthamiana plants were grown and maintained in an air-conditioned greenhouse at 25/20 °C (day/night) (Figures 2, S1, and S2) or in a growth chamber at 25 °C with a 12-hr photoperiod. The NahG-expressing transgenic N. tabacum line was generated essentially using the protocol of Bi et al. (1995), and the N. benthamiana line was described by Asai et al. (2010). Wild-type CMV-Y (Suzuki et al., 1991) was propagated and purified as described by Takeshita et al. (2012). The largest leaf on a plant of N. benthamiana was infiltrated with a bacterial suspension of P. syringae according to Krymowska et al. (2007), and kept at 25 °C.

The largest leaf on plants of N. tabacum and N. benthamiana was dipped into BTH (Syngenta Japan K. K., Tokyo, Japan) or SA (1.0 or 0.1 mM) for 5 s without a water rinse after treatment. In the biomass assay, the whole plants of the transgenic N. benthamiana lines were sprayed with BTH (Figure 6), BTH was used at 25 ppm (0.12 mM) because PR1 transcript accumulation in tobacco sprayed with 0.036 mM BTH can peak at levels equivalent to those with 1.2 mM BTH even at 7 days after treatment (Friedrich et al., 1996), and some of the tobacco leaves treated with BTH at 100 ppm (0.476 mM) hardened slightly in our preliminary experiments. SA, whose methylated derivative (MeSA) is volatile and is excreted from plants (Kumar, 2014), was also used at a higher concentration (1 mM) in some experiments (Figures 3 and 4) as done by Lewzey and Carr (2009), Naylor et al. (1998), and Zhou et al. (2014).

4.2 | RNA extraction and RT-qPCR analysis

Total RNA was extracted from leaf tissue with RNAiso Plus (Takara Bio) according to the user manual. Six biological replicates per experimental plot were used to validate gene expression by comparative reverse transcription-quantitative PCR (RT-qPCR). First-strand cDNA was synthesized using 50–200 ng of total RNA and ReverTra Ace qPCR RT Master Mix with gDNA Remover (ToyoBio), which contains oligo(dT) primer and random hexamer primer. A SAGT-specific primer was used for efficient reverse transcription of SAGT. Comparative qPCR targeting of a host gene and CMV RNA was performed by using THUNDERBIRD SYBR qPCR Mix (Toyobo) and the Thermal Cycler Dice RealTime System Single TP850 (Takara Bio) according to the procedure of the manufacturer and Takeshita et al. (2013). Each reaction mixture (total 20 μl) was prepared using 10 μl of THUNDERBIRD SYBR qPCR Mix, 1 μl of 10 pmol of each forward and reverse primer, 1 μl of cDNA template, and 7 μl of water. Primers used in RT-qPCR are listed in Table S1. Samples were analysed by relative RT-qPCR as done by Takeshita et al. (2013). Transcript accumulation of each target gene was normalized by the quantification of EF1α from N. tabacum and L23 from N. benthamiana. The EF1α and L23 were selected as normalizers after analyses by geNorm essentially according to Takeshita et al. (2013). These values were calculated according to the ΔΔC method (Pfaffl et al., 2002) and plotted with standard errors. The data were obtained from each assay repeated independently (n = 3–8).

4.3 | Measurement of phytohormones

Leaf tissues were weighed immediately after harvest, then frozen in liquid nitrogen and stored at −80 °C until measurement of SA and SAG using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MSMS). Leaf samples were prepared and analysed with UPLC-MSMS as done by Matsuura et al. (2009) and Fujiwara et al. (2016).

4.4 | Construction of SAGT-overexpressing and SAGT-silenced transgenic plants

For the SAGT-overexpressing transgenic Nb lines, the PCR-amplified SAGT ORF (1,436 bp) sequence was amplified using primer pair SAGT-F and SAGT-R (Table S1). The fragment was cloned into pENTR/D-TOPO (Thermo Fisher Scientific). The entry clone was integrated into the binary vector pBl-OX-GW (Inplanta Innovations, Inc.), using the GATEWAY system (Thermo Fisher
Scientific). The recombinant pBI-SAGT binary plasmid was transferred into Agrobacterium tumefaciens LBA4404. Nb plants were transformed by the conventional leaf disk transformation method and selected on kanamycin-containing Murashige-Skoog medium as done by Fukuzawa et al. (2018). Two SAGT-overexpressing lines (OE1 and OE2) were selected from 38 T₁ lines regenerated from T₀ generation. The T₁ lines of OE1 and OE2 were confirmed to be homozygous.

For the SAGT-silenced transgenic Nb lines, plasmid pBI-IR-SAGT (Figure S4) containing an inverted repeat of the 600-nt SAGT sequence was constructed. The PCR-amplified 600-nt SAGT sequence was amplified using primer pair SAGT-210F and SAGT-809R (Table S1). The fragment was then cloned into pENTR/D-TOPO (Invitrogen). The entry clone was integrated into the binary vector, pBI-sense, an-S1). The fragment was then cloned into pENTR/D-TOPO (Invitrogen).

Two independent SAGT-silenced lines (IR1 and IR2) were selected from 113 regenerated lines. Reduced transcript levels of SAGT in the two lines at the T₂ generation stage were verified by RT-qPCR (Figure S5). Seeds from the two selected T₂ transgenic lines were collected, and homozygous T₁ lines were used for the subsequent experiments.

### 4.5 | Nucleotide sequencing

The nucleotide sequences of the RT-qPCR product and transgene were verified using the Big Dye Terminator DNA Sequencing Kit v. 3.1 (Applied Biosystems) and the ABI Prism 310 Genetic Analyzer. The sequence was analysed using the program GENETYX-Win v. 10 (GENETYX Corp.).

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### DATA ACCESSIBILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**FIGURE S1** Mean relative transcript levels of resistance-related genes in BTH-treated and water-treated *Nicotiana tabacum* plants

**FIGURE S2** Mean relative transcript levels of resistance-related genes in BTH-treated and water-treated *NahG*-transgenic *Nicotiana tabacum* plants (NahG Nt)

**FIGURE S3** Mean relative levels of SA and SAG in BTH- (or SA-) treated wild-type *Nicotiana benthamiana* plants

**FIGURE S4** Schematic representation of pBI-IR-SAGT plasmid used to construct SAGT-silenced transgenic *Nicotiana tabacum* lines

**FIGURE S5** Validation of SAGT-silenced *Nicotiana benthamiana* lines

**FIGURE S6** CMV-Y-induced symptoms on SAGT-overexpressing (OE2) and -silenced (IR2) *Nicotiana benthamiana* lines

**FIGURE S7** Symptoms, mean relative viral RNA and transcript levels of SAGT in SAGT-overexpressing (OE1) and the SAGT-silenced (IR1) *Nicotiana benthamiana* lines infected with CMV-Y

**TABLE S1** Oligonucleotide primers used in RT-qPCR analyses and construction of transgenic plants

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