Plant Biotechnology 27, 205–209 (2010)

Metabolomics Note

Transient increase in salicylic acid and its glucose conjugates after wounding in Arabidopsis leaves

Takumi Ogawa[1,2], Takeshi Ara[2], Koh Aoki[2], Hideyuki Suzuki[2], Daisuke Shibata[1,2,*]

[1] Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 980-8577, Japan; [2] Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan

* E-mail: shibata@kazusa.or.jp Tel: +81-438-52-3947 Fax: +81-438-52-3948

Received January 29, 2010; accepted February 24, 2010 (Edited by K. Yazaki)

Abstract We analyzed metabolic changes after mechanical wounding in Arabidopsis leaves to reveal wound effects on metabolism. *Arabidopsis thaliana* leaves were wounded by rubbing the leaf surface with silicon carbide particles (carborundum), as for experimental viral infection, and left up to 48 hours. We analyzed the metabolites of five replicate samples at each time point after wounding by ultra performance liquid chromatography time-of-flight mass spectrometry. Jasmonic acid production was induced immediately after wounding, as reported previously. Interestingly, we found that the amount of salicylic acid started to increase significantly 6 hours after wounding, followed by transient increases in salicylic acid glucoside and salicylic acid glucose ester 24 hours after wounding. The expression patterns of genes in salicylic acid biosynthesis pathway, which are available at public transcriptome databases, also support the observation of metabolic changes in salicylic acid and its glucose conjugates. These results indicate activation of salicylic acid metabolism after wounding, suggesting a role of salicylic acid in wound healing. The metabolome data obtained from this study is available from the MassBase metabolome database (http://webs2.kazusa.or.jp/massbase/).

Key words: Jasmonic acid, metabolic profiling, PR protein, salicylic acid, wounding.

The abiotic stress of mechanical wounding often occurs in natural environments, which leads to damage that stunts plant growth, and also creates sites for bacterial or viral invasion, which may cause vital diseases. Thus, immediate recovery from wounding is crucial for survival. Jasmonate and its conjugated forms are induced immediately after wounding, and activate a set of genes that play a role in defense against pests and pathogens (Balbi and Devoto 2008; Wasternack 2007; Wasternack et al. 2006). Recently, involvement of amino acid conjugates of jasmonic acid, especially jasmonate-Ile, has been rigorously documented as a signal transducer (Chini et al. 2007; Thines et al. 2007). It is generally observed that jasmonates are immediately induced after mechanical wounding, reach a peak around 60 minutes afterward, and then decline to the basal level. More recently, metabolic changes in jasmonates after wounding of Arabidopsis leaves were detected by metabolome analysis (Glauser et al. 2008; Grata et al. 2007). Following the initial response to wounding, accumulation of lignin and suberin at the wound site occurs to seal the wounded surface to impede further invasions by pathogens and fluid loss (Almagro et al. 2009). Up-regulation of genes involved in the phenylpropanoid pathway, which leads to synthesis of lignin and the anti-pathogenic compounds phytoallexins, has also been reported in many plants (Ferrer et al. 2008; Grace and Logan 2000). However, most of the biochemical processes of wound healing remain to be elucidated.

Despite extensive research on the salicylic acid-mediated signaling pathway for defense against pathogens (Delaney et al. 1994; Malamy et al. 1990), much less information is available on metabolic changes of salicylic acid in the wound healing processes. Lee et al. (2004) reported that the amount of salicylic acid in rice (*Oryza sativa*) leaves, which contain a high level of salicylic acid (approximately $10\,\mu g\,g^{-1}$ fresh weight), decreases rapidly after mechanical wounding. The change in salicylic acid immediately after wounding was inversely correlated with a burst of jasmonic acid, suggesting cross-talk between jasmonic acid and salicylic acid signaling pathways. However, in dicotyledonous plants such as tobacco, no metabolic changes in salicylic acid after wounding have been reported (Malamy et al. 1990; Sano et al. 1994). Interestingly, Yamada et al. (2004) reported that wound inducible expression of an Arabidopsis gene encoding

Abbreviations: SAG, *o*-*O*-β-D-glucosylbenzoic acid; SGE, 1-*O*-*o*-hydroxybenzoyl-D-glucose; UPLC-TOF-MS, ultra performance liquid chromatography time-of-flight mass spectrometry.

This article can be found at http://www.jspcmb.jp/

γVPE (a vacuolar processing enzyme with cysteine protease activity) in local and systemic leaves was attenuated in the salicylic acid deficient mutant *pds4-1* and also in transgenic plants expressing the NahG gene, in which salicylic acid accumulation is diminished. They suggested an involvement of endogenous salicylic acid in the wound induction of γVPE, although no evidence of metabolic changes in salicylic acid after wounding had been obtained.

Here we analyzed changes in metabolites after mechanical wounding in Arabidopsis leaves by ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS), and found that the amount of salicylic acid increased significantly after wounding, followed by increases in its glucose conjugates, suggesting a possible role of salicylic acid signaling in wound healing.

Arabidopsis thaliana Col-0 plants were grown at 22°C with a light/dark interval of 16 h/8 h for 4 weeks, and then the leaves were mechanically wounded. Wounding was carried out by rubbing the leaf surface with silicon carbide particles (carborundum), as for experimental viral infection (Takahashi et al. 1994). To wound the tissue reproducibly, we performed this treatment in strictly the same manner. Five independent wounding treatments were done for each sampling point. The small standard errors in values measured for metabolites in the present study indicated that the wounding was highly reproducible. The wounded leaves were kept at 22°C under continuous light for up to 48 hours. The leaf specimens were harvested and immediately frozen in liquid nitrogen and stored until metabolite extraction.

A UPLC ACQUITY system coupled with a Micromass LCT premier mass spectrometer (Waters Co., MA, USA) was used for UPLC-TOF-MS. The data were acquired with the MassLynx data processing tool of the supplier. A methanol extract was applied to a reverse phase column (ACQUITY UPLC BEH-C18, 2.1×150 mm, 1.7 μm, Waters Co.). For each sample, 5 μL was injected. The mobile phase consisted of 0.1% (v/v) aqueous formic acid (solvent A) and 0.1% (v/v) formic acid acetonitrile (solvent B). The gradient program was as follows: from 3 to 95% solvent B over 28 min, 95% solvent B for 5 min, and 3% solvent B for 2 min. The flow rate was set to 0.3 mL min^{-1} and the column oven temperature was kept at 40°C. The mass spectra of the extracts were obtained in negative ion electrospray ionization (ESI) mode. ESI conditions were optimized and set as follows: capillary voltage of 2.5 kV and sample corn voltage of 40 (for targeted analysis) or 120 (for non-targeted analysis) V. Desolvation and source temperature were set to 320 and 120°C, respectively. The desolvation and corn gas (N_2) flows were set at 50 and 600 mL min^{-1}, respectively. Detection was performed in negative ion mode in the *m/z* range 100 to 600 Da. For quantitative analysis, the V mode was selected, and the dynamic range was set to extended mode to maintain mass signal linearity within the dynamic range. Leucine enkephalin was used as a lock mass.

Salicylic acid and jasmonic acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich Japan Co. (Tokyo, Japan), respectively. Authentic samples of salicylic acid glucoside (SAG; *o-O-β*-D-glucosylbenzoic acid) and salicylic acid glucose ester (SGE; 1-*O-o*-hydroxybenzoyl-D-glucose) (Figure 1) were kindly provided by Professor Shigeo Tanaka of Tokyo University of Agriculture. These compounds in the samples extracted from plant leaves were identified through co-chromatography with the authentic standards.

To determine whether our experimental setting deals properly wounding-specific responses as reported previously, we measured jasmonic acid, a typical wound-response metabolite, in the wounded leaves (Figure 2A). An immediate induction of jasmonic acid with maximum accumulation 60 to 90 minutes after wounding, followed by a decline to the basal level, was seen, which is the typical induction pattern of the compound reported in Arabidopsis (Glauser et al. 2008; Grata et al. 2007). Thus, the specimens treated with silicon carbide particles appear to be suitable for wounding research. Furthermore, the standard errors of the values for metabolites measured from five replicates were relatively low, illustrating the reproducibility of the metabolite analysis.

We found that the amount of salicylic acid increased significantly 6 hours after wounding, then decreased to near the basal level at 24 hours, and then increased again at 36 hours, followed by a reduction by 48 hours (Figure 2B). The level of SAG had increased at 24 hours, and then declined to the basal level by 48 hours (Figure 2B). The level of SGE showed a similar pattern to that of SAG (Figure 2B). The increase in the total amount of salicylic acid, SAG and SGE (Figure 2B) indicates activation of salicylic acid metabolism after wounding. We also observed similar metabolic changes in salicylic acid, SAG and SGE in a transgenic plant expressing the *RCY1* gene for viral resistance (Sekine et al. 2008) (unpublished data). Induction of salicylic acid glucose

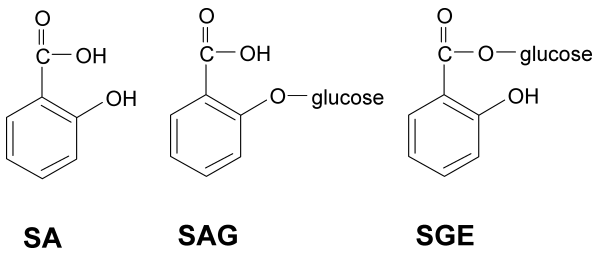

Figure 1. Structures of salicylic acid and its glucose conjugates. SA, salicylic acid; SAG, *o-O-β*-D-glucosylbenzoic acid; SGE, 1-*O-o*-hydroxybenzoyl-D-glucose.

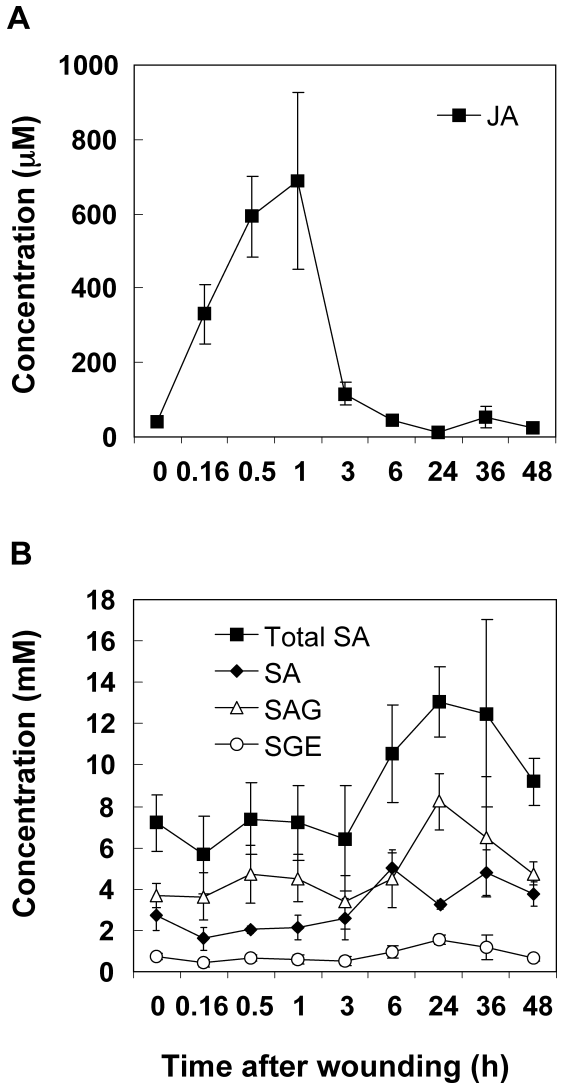

Figure 2. Metabolic changes after mechanical wounding in Arabidopsis leaves. At each time point, 5 replicates of leaves were subjected to UPCL-TOF-MS analysis of (A) JA, jasmonic acid (B) SA, salicylic acid; SAG, salicylic acid glucoside (*o*-*O*-*β*-D-glucosylbenzoic acid); SGE, salicylic acid glucose ester (1-*O*-*o*-hydroxybenzoyl-D-glucose). Total SA indicates the total amount of SA and its glucose conjugates (SA+SAG+SGE). Data indicate means±SE (n=5).

conjugates concomitantly with or slightly delayed relative to induction of salicylic acid is often seen in the response of plants infected with an avirulent pathogen (Delaney et al. 1994; Malamy et al. 1990). The conjugates seem to function to detoxify salicylic acid (Dean and Delaney 2008; Hennig et al. 1993; Lee and Raskin 1998; Lim et al. 2002; Quiel and Bender 2003; Ratzinger et al. 2009). The basal level of salicylic acid at 24 hours after wounding may reflect the highest accumulation of SAG and SGE, which are converted from salicylic acid, at this time point. These results demonstrate that mechanical wounding activates salicylic acid metabolism in Arabidopsis leaves. This study is the first, to our knowledge, to report increases in salicylic acid and its glucose conjugates after wounding in non-transgenic plants (see below).

However, our results appear to be inconsistent with previous research that has reported no significant increase in salicylic acid after wounding (Malamy et al. 1990; Sano et al. 1994). Although Yamada et al. (2004) showed an involvement of salicylic acid in wound induction of γVPE, they also reported that the amounts of salicylic acid in Arabidopsis leaves 72 hours after wounding were below the detection limit ($0.5\,\mu g\,g^{-1}$ leaves) of capillary electrophoresis, which they used for the analysis. However, because the basal level of salicylic acid is about $0.3\,\mu g\,g^{-1}$ fresh weight and its maximum amount $0.7\,\mu g\,g^{-1}$ fresh weight, as measured by UPLC-TOF-MS (Figure 2B), it may be difficult to monitor reliably such changes in salicylic acid using capillary electrophoresis. As the amount was declining at 48 hours in our experiments (Figure 2B), it is also likely that the level of salicylic acid at 72 hours would already have reached the basal level. If so, capillary electrophoresis would not be able to detect the compound. Yamada et al. also found no detection of *PR-1* gene transcript, which is known to be induced by salicylic acid accumulation, at 2, 4, 48 or 72 hours after wounding, and concluded that little if any salicylic acid accumulated in these tissues in response to wounding. However, as they did not measure the transcript at the time points of 6 to 36 hours, which was crucial for detecting increased amounts of salicylic acid and glucose conjugates (Figure 2B), it is possible that *PR-1* transcript is induced only during this short time period. Indeed, this seems to happen as discussed below.

In wild-type tobacco plants wounded with silicon carbide particles, no increase in salicylic acid was observed up to 6 days with analyses at one day intervals (Sano et al. 1994). The time points of the measurements of salicylic acid may not have been frequent enough to detect the short-term change (within 24 hours) we detected in Arabidopsis. Alternatively, tobacco may not respond to wounding by salicylic acid induction. Interestingly, Sano et al. (1994) reported that a transgenic tobacco line overexpressing a gene encoding a Ras-related small GTP binding protein showed salicylic acid induction after wounding. They suggested that overexpression of the protein somehow interferes with the normal wound signal pathway and results in abnormal cross-signaling between the wound- and pathogen-induced signal transduction pathways. If an increase in salicylic acid after wounding also occurs in a short time period in tobacco, which was not measured in previous studies, it is intriguing to speculate that the small GTP binding protein might function to sense a wounding signal in normal plants. This hypothesis remains to be tested.

We sought microarray data for salicylic acid

biosynthesis and responsive genes after wounding, which are available from the AtGenExpress database (http://pfg.psc.riken.jp/AtGenExpress/). This database provides gene expression data obtained from various microarray experiments with the Affymetrix ATH1 array of 22,746 probe sets covering about 23,700 genes (Kilian et al. 2007). We obtained a dataset of gene expression profiles after wounding from the database, calculated the mean intensity of all replicates in the dataset and generated expression ratios for all time points to the values of the corresponding control experiments. The expression profile shows that *AtSAGT1*, encoding salicylic acid glucosyltransferase 1, *AtBSMT1*, encoding benzoic acid and salicylic acid carboxyl methyltransferase 1, and the pathogenesis-related gene *PR1*, a typical salicylic acid inducible gene, are up-regulated at 12 hours after wounding (data not shown). As the wounding treatment was carried out by punctuation of the leaves with a custom made pin-tool consisting of 16 needles, the experimental conditions differed from ours. Nonetheless, this time frame of *PR1* expression is comparable with the time point of increased accumulation of salicylic acid and its conjugates shown in our experiments (Figure 2). Although Yamada et al. (2004) reported no obvious wound induction of *PR1*, it might be possible that, as they measured at the time points of 2 hours, 4 hours, 24 hours and 48 hours after wounding, *PR1* is expressed transiently only around 12 hours after wounding. In contrast, the genes *ICS1* and *ICS2*, encoding isochorismate synthase 1 and 2, respectively, which function upstream of salicylic acid synthesis, are not activated at these time points. The result also suggests that endogenous salicylic acid at the basal level may not affect the expression of *PR1*, but an increase in its amount does affect expression. Endogenous salicylic acid at the basal level could be sequestered in a cell organelle or other tissues that have no effect on expression of *PR1*. This hypothesis remains to be tested.

In conclusion, our results demonstrate increases in salicylic acid and its glucose conjugates SAG and SGE after wounding in Arabidopsis, indicating activation of salicylic acid metabolism after wounding. The metabolome data set for wounding processes has been deposited in the MassBase metabolome database (http://webs2.kazusa.or.jp/massbase/), which should facilitate further study of wound healing processes.

Acknowledgements

We thank Dr. Shigemi Seo (National Institute of Agrobiological Sciences, Tsukuba, Japan) and Professor Ikuko Nishimura (Kyoto University) for their advice on salicylic acid research, and Professor Toshiaki Umezawa (Kyoto University) for discussions of wound-inducible lignin. We thank Professor Shigeo Tanaka (Tokyo University of Agriculture) for a kind gift of *o*-*O*-*β*-D-glucosylbenzoic acid and 1-*O*-*o*-hydroxybenzoyl-D-glucose. This work was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO) of Japan as part of the "Development of Fundamental Technologies for Controlling the Material Production Process of Plants" and by Grant-in-Aid for JSPS Fellows (20-7609).

References

Almagro L, Gomez Ros LV, Belchi-Navarro S, Bru R, Ros Barcelo A, Pedreno MA (2009) Class III peroxidases in plant defence reactions. *J Exp Bot* 60: 377–390

Balbi V, Devoto A (2008) Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. *New Phytol* 177: 301–318

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM et al. (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448: 666–671

Dean JV, Delaney SP (2008) Metabolism of salicylic acid in wild-type, ugt74f1 and ugt74f2 glucosyltransferase mutants of *Arabidopsis thaliana*. *Physiol Plant* 132: 417–425

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K et al. (1994) A Central Role of Salicylic Acid in Plant Disease Resistance. *Science* 266: 1247–1250

Ferrer JL, Austin MB, Stewart C Jr, Noel JP (2008) Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiol Biochem* 46: 356–370

Glauser G, Grata E, Dubugnon L, Rudaz S, Farmer EE, Wolfender JL (2008) Spatial and temporal dynamics of jasmonate synthesis and accumulation in *Arabidopsis* in response to wounding. *J Biol Chem* 283: 16400–16407

Grace SC, Logan BA (2000) Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. *Philos Trans R Soc Lond B Biol Sci* 355: 1499–1510

Grata E, Boccard J, Glauser G, Carrupt PA, Farmer EE, Wolfender JL, Rudaz S (2007) Development of a two-step screening ESI-TOF-MS method for rapid determination of significant stress-induced metabolome modifications in plant leaf extracts: the wound response in *Arabidopsis thaliana* as a case study. *J Sep Sci* 30: 2268–2278

Hennig J, Malamy J, Grynkiewicz G, Indulski J, Klessig DF (1993) Interconversion of the salicylic acid signal and its glucoside in tobacco. *Plant J* 4: 593–600

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S et al. (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J* 50: 347–363

Lee A, Cho K, Jang S, Rakwal R, Iwahashi H, Agrawal GK, Shim J, Han O (2004) Inverse correlation between jasmonic acid and salicylic acid during early wound response in rice. *Biochem Biophys Res Commun* 318: 734–738

Lee HI, Raskin I (1998) Glucosylation of Salicylic Acid in *Nicotiana tabacum* Cv. Xanthi-nc. *Phytopathology* 88: 692–697

Lim EK, Doucet CJ, Li Y, Elias L, Worrall D, Spencer SP, Ross J, Bowles DJ (2002) The activity of *Arabidopsis* glycosyltransferases toward salicylic acid, 4-hydroxybenzoic acid, and other benzoates. *J Biol Chem* 277: 586–592

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic Acid: A Likely Endogenous Signal in the Resistance Response of Tobacco to Viral Infection. *Science* 250: 1002–1004

Quiel JA, Bender J (2003) Glucose conjugation of anthranilate by

the *Arabidopsis* UGT74F2 glucosyltransferase is required for tryptophan mutant blue fluorescence. *J Biol Chem* 278: 6275–6281

Ratzinger A, Riediger N, von Tiedemann A, Karlovsky P (2009) Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*. *J Plant Res* 122: 571–579

Sano H, Seo S, Orudgev E, Youssefian S, Ishizuka K (1994) Expression of the gene for a small GTP binding protein in transgenic tobacco elevates endogenous cytokinin levels, abnormally induces salicylic acid in response to wounding, and increases resistance to tobacco mosaic virus infection. *Proc Natl Acad Sci USA* 91: 10556–10560

Sekine KT, Kawakami S, Hase S, Kubota M, Ichinose Y, Shah J, Kang HG, Klessig DF, Takahashi H (2008) High level expression of a virus resistance gene, *RCY1*, confers extreme resistance to Cucumber mosaic virus in *Arabidopsis thaliana*. *Mol Plant Microbe Interact* 21: 1398–1407

Takahashi H, Goto N, Ehara Y (1994) Hypersensitive response in cucumber mosaic virus-inoculated *Arabidopsis thaliana*. *Plant J* 6: 369–377

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A et al. (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* 448: 661–665

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann Bot* 100: 681–697

Wasternack C, Stenzel I, Hause B, Hause G, Kutter C, Maucher H, Neumerkel J, Feussner I, Miersch O (2006) The wound response in tomato—role of jasmonic acid. *J Plant Physiol* 163: 297–306

Yamada K, Nishimura M, Hara-Nishimura I (2004) The slow wound-response of γVPE is regulated by endogenous salicylic acid in *Arabidopsis*. *Planta* 218: 599–605