GH3-mediated Auxin Homeostasis Links Growth Regulation with Stress Adaptation Response in Arabidopsis*

Received for publication, November 13, 2006, and in revised form, January 8, 2007. Published, JBC Papers in Press, February 1, 2007, DOI 10.1074/jbc.M610524200

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Plants constantly monitor environmental fluctuations to optimize their growth and metabolism. One example is adaptive growth occurring in response to biotic and abiotic stresses. Here, we demonstrate that GH3-mediated auxin homeostasis is an essential constituent of the complex network of auxin actions that regulates stress adaptation responses in Arabidopsis. Endogenous auxin pool is regulated, at least in part, through negative feedback by a group of auxin-inducible GH3 genes encoding auxin-conjugating enzymes. An Arabidopsis mutant, wes1-D, in which a GH3 gene WES1 is activated by nearby insertion of the 35S enhancer, exhibited auxin-deficient traits, including reduced growth and altered leaf shape. Interestingly, WES1 is also induced by various stress conditions as well as by salicylic acid and abscisic acid. Accordingly, wes1-D was resistant to both biotic and abiotic stresses, and stress-responsive genes, such as pathogenesis-related genes and CBF genes, were up-regulated in this mutant. In contrast, a T-DNA insertional mutant showed reduced stress resistance. We therefore propose that GH3-mediated growth suppression directs reallocation of metabolic resources to resistance establishment and represents the fitness costs of induced resistance.

Plants are frequently exposed to diverse biotic and abiotic stresses throughout their life cycle. A number of stress signaling genes have been identified through molecular genetic studies and genome-wide screens, and a range of stress signaling pathways have been elucidated. However, the underlying molecular and biochemical mechanisms remain poorly understood in most cases, primarily due to the complexity of interactions between multiple signaling pathways (1–3).

Diverse growth hormones are involved in mediating stress responses and signalings. Salicylic acid (SA)² is a primary growth hormone that mediates plant disease resistance and abiotic stress responses. Several key components of SA signaling have been functionally characterized, among which the NPR1 (nonexpressor of PR1) and TGA transcription factors are the best understood (4, 5). Furthermore, the SA signaling pathways are interconnected with other growth hormone signaling pathways (1, 2, 6). The interplays of SA with jasmonic acid (JA) and ethylene have been extensively studied (6, 7). Biotic stress as well as abiotic stress also promotes abscisic acid (ABA) biosynthesis, further suggesting that growth hormones interact with one another in stress signaling and stress tolerance. Notably, recent reports have shown that light plays a crucial role in both biotic and abiotic stress responses. SA biosynthesis, PR-1 (pathogenesis-related 1) induction by SA, and hypersensitive response all require functional phytochromes (8, 9). Similarly, light is also essential for cold-induced CBF expression (10).

Commonly observed symptoms of infected or stressed plants include growth retardation and reduced metabolism, which may be caused by the reallocation of metabolic resources between different physiological pathways in order to maximize plant survival under stress conditions (11–13). Auxin has been implicated in such adaptive responses, a notion that is further supported by genome-wide analysis. It has been reported that the endogenous indole-3-acetic acid (IAA) level substantially increases upon pathogen infections (14), and expression of some auxin-regulated genes is altered in infected plants (15). In addition, most pathogens of Pseudomonas syringae and other bacterial pathogens produce a large amount of IAA (16). Furthermore, it has been demonstrated that enhanced antibacterial resistance is intimately related to repression of auxin signaling (17), strongly suggesting that auxin modulates plant responses to pathogen infections.

One mechanism by which plants coordinate auxin-mediated processes is to maintain the endogenous pool of auxins at an appropriate level. This can be achieved by regulating auxin

* This work was supported by the BK 21, Biogreen 21 (20050301034456) and National Research Laboratory programs, a grant from the Plant Signaling Network Research Center, Korea Science and Engineering Foundation Grant R02-2003-000-10011-0, Korea Research Foundation Grant 2005-070-C00129, and Plant Diversity Research Center of 21st Century Frontier Research Program Grant PF0330404-02 (to J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.
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² The abbreviations used are: SA, salicylic acid; JA, jasmonic acid; ABA, abscisic acid; IAA, indole-3-acetic acid; RT, reverse transcription; SAG, SA glucoside; MS, Murashige and Skoog.
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biosynthesis and distribution among different organs and by conjugate formation with sugars, peptides, and amino acids (18–20). Although the physiological importance of conjugates in auxin homeostasis is not yet fully understood, it is generally accepted that conjugate formation plays a critical role in auxin action.

Auxin functions to an extent by regulating a group of primary responsive genes: Aux/IAA genes, GH3 genes, and small aux-in-up RNAs (SALKIR) (19). Members of the Aux/IAA gene family have been studied in light regulation of auxin responses (18, 20). Although none of the SAUR genes are as yet functionally characterized, the SAUR proteins have been shown to bind to calcium/calmodulin (21), suggesting a role for the calcium ion in auxin signaling. Several GH3 genes have been studied using mutants with altered gene expression. GH3-overexpressing mutants, such as df1-D (22), df2-D (23), and ydk1-D (24), display reduced growth and altered light responses, suggesting a role for these GH3 proteins in light-auxin interactions. Recently, the GH3 proteins have been biochemically characterized. JAR1 has been found to activate JA by conjugating it to isoleucine (25). Several other GH3 genes encode enzymes that conjugate amino acids to IAA (26, 27). It is notable that the GH3.5 enzyme targets both IAA and SA, suggesting a role for this enzyme in IAA-SA interactions (26). The rapid induction of the GH3 genes by auxin may help to regulate auxin homeo-stasis by conjugating excess auxins to amino acids. However, their functional mechanisms may not be so simple, because only a fraction of the GH3 genes are induced by auxin (19).

Here, we demonstrate that both biotic and abiotic stress adaptation responses are mediated, at least in part, by auxin homeostasis governed through negative feedback regulation by a group of GH3 enzymes. Overproduction of an IAA-conjugating GH3 enzyme causes growth reduction, which is intimately linked to enhanced stress resistance. Stress-induced turnover of endogenous auxins by the GH3 enzymes is likely to be an adaptive strategy that coordinately modulates growth rate and stress resistance.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Conditions**—All Arabidopsis thaliana lines used were in the Columbia ecotype (Col-0) unless otherwise specified. Plants were grown in a controlled culture room at 23 °C with a relative humidity of 60% under illumination (110 μmol photons m−2 s−1) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea).

**Isolation of wes1-D and wes1—**Ecotype Col-0 was transformed with the activation tagging vector pSKI015, as previously described (28). To select herbicide-resistant transformants, T0 seeds were collected, sown in soil, and sprayed twice a week with a 1:1,000 dilution of Finale (AgrEvo, Montvale, NJ) containing 5.78% Basta. The herbicide-resistant seeds were further selected through 2–3 more generations, and homozygotic seeds were obtained. A dwarfed mutant (wes1-D) was chosen for further analysis in this work. wes1 was isolated from a pool of T-DNA insertion lines (SALK 151766:46.55X, Arabidopsis Biological Resource Center; Ohio State University).

The single T-DNA insertion event in wes1-D was verified by genomic Southern blot hybridization using the 35S enhancer sequence as a probe, followed by analysis of segregation ratios. The sequences flanking the insertion site were determined by a thermal asymmetric interlaced PCR (29).

**Analysis of Transcript Levels**—Transcript levels were ana-lyzed either by Northern blot hybridization or by reverse transcrip-tion (RT)-PCR-based Southern blot hybridization or by quantitative real time RT-PCR. Total RNA was isolated from aerial parts of 2-week-old plants, unless otherwise specified, or plant organs using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). For Northern blot hybridization, ~25–30 μg of total RNA samples were separated using 1.2% denaturing formaldehyde-agarose gel electrophoresis and hybridized to probes labeled with [32P]dCTP.

Semi-quantitative RT-PCR was employed to measure the transcript levels. Total RNA samples were pretreated extensively with RNase-free DNase I to remove any contaminating genomic DNAs. The first strand cDNA was synthesized from the above RNA samples using the 35S enhancer 5′ primer and random hexamer primer.

**Growth Hormone Treatments**—Plants were grown on Murashige and Skoog (MS)-agar plates containing 0.5×MS salts with vitamins, 0.5 g liter−1 4-morpholineethanesulfonic acid, and 0.8% Phytagar (Duchefa, Haarlem, The Netherlands) supplemented with IAA, JA, ABA (10 μM each), SA (0.1 or 1 mM unless otherwise specified), 1-amino cyclopropane-1-carboxylic acid (ACC) (50 μM), or 1-N-naphthylphthalaldehyde acid (20 μM). Whole plant parts of 2-week-old plants were used for total RNA extraction.

To accurately assess the effects of IAA on primary root growth and lateral root development, MS-agar plates were sup-
implemented with 0–10 \( \mu M \) IAA. Measurements from 30 roots were averaged and analyzed statistically using Student’s \( t \) test.

Pathogen Inoculations, Trypan Blue Staining, and Determination of Bacterial Growth—Cells of \( P. syringae \) pv. tomato strain DC3000 were grown for 2 days at 28 °C in King’s B medium supplemented with 50 \( \mu g \) liter\(^{-1} \) rifampicin. Bacterial cells were harvested by centrifugation and resuspended at \( 10^7 \) colony-forming units ml\(^{-1} \) in 10 \( \mu M \) MgCl\(_2\) and 250 ppm Tween 80. Plants were inoculated by spraying with bacterial suspensions until the leaf surface was fully covered with fine droplets. Inoculated plants were incubated for 16 h at 25 °C and 100% relative humidity and then transferred to a growth chamber set at 25 °C and 80% relative humidity and grown under long day conditions. In order to visualize microscopic lesions, leaves were stained with trypan blue as previously described (30).

To determine bacterial growth in plants, whole leaves of 4-week-old plants were infiltrated with bacterial suspensions (10\(^5 \) colony-forming units/ml) of \( P. syringae \) pv. tomato strain DC3000 by applying a vacuum for 2 min followed by sudden release using a vacuum pump (model DOA-P104-AA; Sangwoo S & T, Seoul, Korea). Leaf discs of 0.5 cm in diameter were harvested, using a hole puncher, from the leaves at the indicated time intervals after infiltration, and bacterial growth was determined as previously described (31). The assays were repeated in three replicates, each consisting of five leaf discs from different inoculated leaves. Statistical significance of the measurements was determined using Student’s \( t \) test.

Abiotic Stress Tolerance Assays—Drought stress tolerance assays were carried out essentially as previously described (32) with minor modifications. Plants were germinated and grown for 10 days in pots filled with the mixture of perlite and vermiculite at a ratio of 1:1 under normal growth conditions (23 °C, 60% relative humidity). Each pot (9 cm in diameter) contained \( \sim 40 \) g of the mixture in dry weight. Drought stress was imposed by withholding water for 2 weeks, and survival rates were calculated for each plant group 5 days after rewatering. Three independent measurements, each consisting of 50 plants, were averaged and statistically analyzed using Student’s \( t \) test.

For high salinity tolerance assays, plants were germinated and grown vertically on MS-agar plates for 10 days and then transferred to MS liquid culture containing 200 \( \mu M \) NaCl and incubated under constant light for 3 days. To quantify damage from high salinity, leaf samples were soaked overnight in 95% EtOH at 65 °C, and the chlorophyll contents were measured spectrophotometrically (chlorophyll \( a/b \) (\( \mu g/\)ml) = OD\(_{664.3} \) \( \times \) 5.24 + OD\(_{648.6} \) \( \times \) 22.24). Eleven measurements were averaged, and statistical significance was determined using a student \( t \) test.

For freezing tolerance assays, \( \sim 30 \) plants grown for 3 weeks on MS-agar plates were incubated for 1.5 h at \(-7^\circ C\) and allowed to recover at 23 °C for 1 week. Three independent measurements of survival rates were averaged and statistically analyzed using Student’s \( t \) test.

Fully expanded rosette leaves from 2-week-old plants grown in soil were used to measure freezing-induced electrolyte leakage as previously described (33). One excised leaflet was placed in a 5-ml test tube containing 100 \( \mu l \) of deionized water, and the tube was placed in a circulator bath (PolyScience, Cheshire, UK) set at 0 °C. After equilibration for 1 h, ice chips were added to each tube. The temperature of the bath was programmed to decrease to \(-9^\circ C\) with a 1 °C decrement every 30 min. The tubes were removed from the bath when the designated temperature was reached and placed immediately on ice to allow gradual thawing overnight. The leaflets were then carefully transferred to a 50-ml tube containing 20 ml of deionized water and shaken overnight at 40 rpm. The conductivity of the solution was measured. The tubes with the leaflets were autoclaved, and the conductivity of the solution was measured again. The degree of electrolyte leakage was calculated as the percentage conductivity before autoclaving over that following autoclaving. Ten replicates were performed for each temperature treatment and statistically analyzed using Student’s \( t \) test.

Growth Hormone Measurements—Endogenous auxins were extracted from aerial tissues of 4-week-old plants as previously described (27), except that the final purification step by high pressure liquid chromatography was eliminated. Internal standards were \([^{13}C_6]\)IAA (Cambridge Isotope Laboratories, Andover, MA); \([^{13}C_6]\)IAA-Asp and \([^{13}C_6]\)IAA-Glu (kindly provided by J. Cohen); and \([^{13}C_6]\)IAA-Ala and \([^{13}C_6]\)IAA-Leu (synthesized as previously described (34)). Samples were derivatized using 2,3,4,5,6-pentafluorobenzyl bromide (35), purified by silica gel SPE chromatography, and dissolved in chloroform. Analyses were carried out on a Finnigan Trace gas chromatograph (inlet 280 °C), which was coupled to a DSQ mass spectrometer using negative chemical ionization. The reagent gas was methane with a source temperature of 200 °C, and the instrument was operated in the SIM mode. The ions collected for endogenous auxins were \( m/z \) 174, 245, 271, 285, and 287 for IAA, IAA-Ala, IAA-Asp, IAA-Glu, and IAA-Leu, respectively. Quantitative data were obtained from integrated peak areas as previously described (36).

Extraction and quantification of endogenous SA and SA glucoside (SAG) were performed using the leaf tissues from 2-week-old plants as previously described (37). ABA contents were determined using the Phytodetek ABA Immunoassay Kit according to the procedure provided by the manufacturer (Ide-tek, Sunnyvale, CA). Three independent measurements were averaged for both SA and ABA. Statistical significance was determined using Student’s \( t \) test.

RESULTS

WES1 Activation Causes Growth Retardation in wes1-D—From a screening of a pool of activation-tagged Arabidopsis mutants, we isolated a severely dwarfed mutant, wes1-D, which exhibited reduced growth and small plant organs. The genetic locus was designated WES1 (for WESO 1, meaning a dwarfed stature in Korean). Notably, the phenotypic alterations observed in wes1-D are quite similar to those of \( axr2-1 \), an auxin-resistant mutant with a mutation in domain II of IAA7 (supplemental Fig. 1A), and to those of \( dfl1-D, dfl2-D, \) and \( ydk1-1, \) in which auxin-inducible \( GH3 \) genes are overexpressed (22–24). In contrast, a T-DNA insertion mutant, \( wesI \), was phenotypically indistinguishable
from wild type plants but with slightly larger leaves (≈6–7% increase; Fig. 1A and supplemental Fig. 1B). It does not seem that the absence of discernible phenotypic changes in wes1 is simply due to functional redundancy among the GH3 proteins. It may be also attributable to the stress inducibility of the WES1 gene, since the wes1 mutant differentially responded to stress conditions (see below). These observations suggested that the wes1-D phenotype might be related to auxin action. However, unlike axr2-1, which exhibits short hypocotyls in both light and darkness, the dwarfed phenotype of wes1-D occurred only in the light (data not shown), suggesting a different molecular mechanism underlying the wes1-D phenotype.

Thermal asymmetric interlaced PCR was employed to map the T-DNA insertion site in the wes1-D genome (29). Together with analysis of segregation ratios (3:1/wes1-D/wild type basta-resistant plants/basta-susceptible plants) and genomic Southern blot hybridization (supplemental Fig. 2A), it revealed that a single copy of the 35S enhancer was inserted into the
region between At4g27250 and At4g27260. RT-PCR-based Southern blot hybridization indicated that, whereas the level of At4g27260 transcript is extremely low in wild type plants, it is present at a very high level in wes1-D (Fig. 1C and supplemental Fig. 2B), suggesting that overexpression of the locus may be the molecular cause for the wes1-D phenotype. Sequence analysis revealed that WES1 is identical to AtGH3/AtGH3.5, the expression profile of which has been studied using a promoter trap line (38). In contrast, the level of At4g27250 transcript was unaltered in wes1-D, and transgenic plants overexpressing the locus did not exhibit any phenotypic alterations (data not shown).

To confirm that overexpression of WES1 is associated with the wes1-D phenotype, WES1 was expressed under the control of the CaMV 35S promoter in Arabidopsis. Twenty-eight of the 36 transgenic lines (35S::WES1) obtained displayed a phenotype essentially identical to that of wes1-D (Fig. 1A). However, growth retardation and leaf curling were slightly less severe in the transgenic lines compared with wes1-D. This may be due to a gene dosage effect, since the level of WES1 transcript was lower in the 35S::WES1 transgenic plants than in wes1-D (supplemental Fig. 2B). The phenotypic changes in the remaining eight transgenic lines were only marginal, and the WES1 transcript level was only slightly induced (data not shown), further suggesting that overexpression of WES1 underlies the wes1-D phenotype.

WES1 encodes a polypeptide of 612 residues (supplemental Fig. 2C) that exhibits a high sequence identity with soybean GH3 (39) and Arabidopsis GH3 homologues (supplemental Fig. 3). Three sequence motifs (I–III) that are found in all of the known GH3 proteins (26) were also identified at the equivalent positions in WES1 (supplemental Fig. 2C).

WES1 Is Induced by Auxin and ABA—Several GH3 genes are known to be induced by auxin (22, 38). We examined the effects of auxin and other growth hormones on WES1 expression. IAA rapidly induced WES1 within 5 min after treatment (Fig. 1D). Interestingly, ABA also activated WES1 expression to a considerable level (Fig. 1D), suggesting a role for WES1 in ABA-regulated stress response. Since WES1 is auxin-inducible, an auxin transport inhibitor, 1-N-naphthylphthalamic acid, was also included in the assays. It did not confer any discernible effects on the WES1 expression (Fig. 1E). This may be because the basal level of the WES1 expression is extremely low under normal growth conditions.

Other growth hormones, such as 1-amino cyclopropane-1-carboxylic acid, GA, and methyl JA, did not confer any significant effects on WES1 expression (Fig. 1E), although WES1 was slightly induced by 1-amino cyclopropane-1-carboxylic acid but slightly repressed by methyl JA. The slight repression of WES1 by methyl JA may be related to the antagonistic roles of JA and SA in pathogen resistance (see below) (Fig. 4B).

High levels of WES1 transcript were detected in the shoot apex and flower tissues, and moderate levels were found in the stems (Fig. 1F). However, it was relatively lower in the leaves and roots. WES1 was expressed to a relatively high level in young seedlings and gradually decreased throughout the life cycle (supplemental Fig. 2D), although the overall transcript levels were low and could be detected only by RT-PCR.

Endogenous IAA Level Is Reduced in wes1-D—Examination of wes1-D roots grown on vertical MS-agar plates indicated that primary root growth was not discernibly affected (Fig. 2, A and B). However, the number of lateral roots was dramatically reduced in wes1-D (Fig. 2C), suggesting again a role for WES1 in auxin action. Consistent with this, a subset of Aux/IAA genes was suppressed in wes1-D and axr2-1 but induced to detectable levels in wes1 (Fig. 2D). ATHB2, which is involved in light regulation of auxin responses (e.g. shade avoidance response) (40), was also affected in a similar manner. These observations suggested that WES1 might play a role in auxin-mediated growth regulation.

It has been shown that GH3 enzymes inactivate IAA by forming conjugates with amino acids (27). Accordingly, the IAA-Asp level is elevated in dfl1-D (22, 27). We thus anticipated that the endogenous level of auxins would be altered in wes1-D and wes1. To examine this, we measured endogenous auxin levels using 4-week-old leaves. The IAA-Asp content was 7.2 times higher in wes1-D than in wild type plants, whereas it was decreased by about 50% in wes1 (Fig. 2E), suggesting that WES1 synthesizes IAA-Asp in planta. The amounts of free IAA were
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98.1 and 54.3 pmol g⁻¹ tissue fresh weight in wild type plants and wes1-D, respectively. In wes1-D, the 45% decrease in free IAA was found to be statistically significant in four replicate analyses (p < 0.05). In contrast, the difference in means between wes1 and wild type plants was not statistically significant, which might be due to functional redundancy among the GH3 enzymes. These data confirm the correlation between the reduction of active auxins and the wes1-D phenotype. It is also envisioned that the auxin-inducible WES1 gene may represent a negative feedback loop that maintains the endogenous level of active auxins at an appropriate level (see “Discussion”).

Primary root growth is inhibited by excess amounts of exogenous auxins and is frequently used as an assay system for auxin activity. We examined the sensitivity of wes1-D roots to exogenous auxin. Primary root growth in wes1-D was slightly but reproducibly resistant to exogenous IAA (supplemental Fig. 4), and this effect was most evident at an IAA concentration range of 10⁻⁸ to 10⁻⁶ M (p < 0.05). In contrast, wes1 was more sensitive to IAA (p < 0.05). This observation is consistent with the proposed biochemical role of WES1 in auxin metabolism and the reduction of free IAA in wes1-D (Fig. 2E).

Auxin feeding experiments strongly supported the above notion. When seedlings were grown on MS-agar plates supplemented with IAA, lateral root formation in wes1-D seedlings was efficiently recovered (Fig. 3). However, whereas lateral root formation was readily enhanced by IAA at a concentration as low as 10 nM in wild type seedlings, the IAA effects became evident at IAA concentrations of higher than 1 μM in wes1-D seedlings. In contrast, the IAA effects were higher in wes1 seedlings compared with those in wild type seedlings. Altogether, these observations indicated that the endogenous auxin level is lower in wes1-D.

WES1 Is Induced by SA and Pathogen Infections—WES1 is unique among the GH3 enzymes characterized so far in that it possesses dual substrate specificities. It is active on both IAA and SA (26). It was thus postulated that WES1 might play a role in SA-mediated stress responses.

We observed that PR-1 was significantly induced in wes1-D as well as in axr2-1 but was unchanged in wes1 (Fig. 4A). In addition, WES1 was induced by SA, being observable at 2 h and reaching a maximum of more than 5-fold increase by 4 h after SA application (Fig. 4B). These results indicated that WES1 is closely associated with SA-dependent responses.

We next investigated the expression of defense genes in wes1-D following P. syringae infection. In wes1-D, the PR-1 transcript level was high even before infection. Following infection, it was further induced. The PR-1 transcript level returned to the basal level 2 days after infection in wild type plants. In contrast, the transcript level remained elevated in wes1-D, even after 3 days (Fig. 4C). PDF1.2 also displayed a distinct expression pattern in wes1-D. It was rapidly induced to a high level after infection, which was maintained at least for 3 days, in wild type plants. Although PDF1.2 expression in wes1-D initially appeared to be identical to that in wild type plants (Fig. 4C, time point 0), with rapid induction of a high level of transcription following infection, the transcript level gradually decreased after the peak expression was attained in wes1-D. This may be related to the primary role of PDF1.2 in the JA signaling pathway (41). In wild type plants, the expression of IAA1 gradually decreased following infection, an inversely proportional response to WES1 induction following SA application. This is probably due to a reduction in active auxins caused by the SA-mediated WES1 induction. These results strongly support a role for WES1 in SA-dependent stress responses.

wes1-D Is Resistant to Pathogen Infections—In infected plants, increased synthesis of endogenous SA or its conjugates triggers the induction of PR genes and the development of disease resistance symptoms. The level of PR-1 transcript is high in wes1-D, and WES1 is activated by SA and pathogen infections. Therefore, we hypothesized that wes1-D might be resistant to pathogen infections.

When plants were infected with P. syringae, disease symptoms were much less severe on the leaves of wes1-D (Fig. 5A). The wes1-D leaves exhibited greatly reduced wilting and necrosis, a result clearly demonstrated by trypan blue staining (Fig. 5B). Interestingly, enhanced pathogen resistance was also observed in axr2-1 but not observed in wes1. To quantitatively examine the enhanced resistance in wes1-D, plant leaves were infected with P. syringae, and bacterial growth was determined. Bacterial population was greatly reduced in wes1-D but slightly increased in wes1 (Fig. 5C). These results are consistent with the activation of PR-1 in wes1-D and the induction of WES1 by SA and pathogen infections. Notably, bacterial growth was also
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Auxin Regulates PR-1 Expression — PR-1 is induced by at least two SA-mediated signaling pathways, depending upon whether NPR1 is required or not (2). TGA genes play a central role in the NPR1-dependent pathway. Several genes, including AtWhy1 (42) and SS1 (suppressor of SA insensitivity) genes (41), are involved exclusively in the NPR1-independent pathway. Therefore, a question arises as to how WES1 regulates PR-1 expression.

To answer the question, expression patterns of several SA-regulated genes were examined in wes1-D. GST6 is an immediate early gene that is induced by SA and is frequently used as a marker for endogenous SA content. GST6 expression was unaltered in wes1-D and wes1 (Fig. 6A). Expression of NPR1, TGA genes, and AtWhy1 also remained unchanged. These results suggested that induction of PR-1 is caused by a WES1-mediated pathway independent of SA biosynthesis in wes1-D.

Direct measurements of endogenous SA levels further supported the notion. The level of free SA was elevated in wes1-D that conjugates amino acids specifically to JA (25, 26), may regulate auxin metabolism, similar to the role of WES1 in SA-mediated response.

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Auxin Regulates PR-1 Expression — PR-1 is induced by at least two SA-mediated signaling pathways, depending upon whether NPR1 is required or not (2). TGA genes play a central role in the NPR1-dependent pathway. Several genes, including AtWhy1 (42) and SS1 (suppressor of SA insensitivity) genes (41), are involved exclusively in the NPR1-independent pathway. Therefore, a question arises as to how WES1 regulates PR-1 expression.

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(~80 ng/g of tissue, fresh weight) compared with that in wild type plants (~20 ng/g of tissue, fresh weight) (Fig. 6B). However, the SA level in wes1-D is far below the level that is usually observed in infected plants (37). In addition, we also observed in our assay system that when measured 2 days after infection with P. syringae, the level of free SA was in a range of 0.8 – 1.4 µg/g of tissue, fresh weight (data not shown). These observations suggest that the elevated SA level may not significantly contribute to the PR-1 induction in wes1-D. The level of SAG was also elevated in wes1-D (~1 µg/g of tissue, fresh weight). However, the elevation does not seem to be physiologically significant, since the SAG level reached 15–20 µg/g of tissue fresh weight in infected plants in our assay system (data not shown).

To obtain more direct evidence that WES1 induction of PR-1 is SA-independent, wes1-D was genetically crossed to the nahG transgenic plants. The resultant wes1-D × nahG cross exhibited the wes1-D phenotype (data not shown), and the level of the PR-1 transcript was lower than that in wes1-D but much higher than that in wild type plants (Fig. 6C), unequivocally demonstrating that the PR-1 induction is caused by a SA-independent pathway in wes1-D. It is also envisioned that full induction of PR-1 requires both the SA-dependent and SA-independent signals. The moderately altered levels of SA and SAG in wes1-D might be attributable to a WES1-modulated negative feedback regulation, similar to that proposed for auxin.

To further explore the molecular mechanism underlying the induction of PR-1 in wes1-D, wild type plants were pretreated with SA for 1 h and subsequently treated with SA, IAA, or 2,4-dichloro-phenoxyacetic acid in different combinations. When plants were treated with individual growth hormones, expression of PR-1 was strongly induced by SA but discernibly repressed by 2,4-dichloro-phenoxyacetic acid and IAA (Fig. 6D). When treated with SA and auxin together, the level of PR-1 transcript was much lower than that in plants treated with SA alone. These results indicate that auxin counters the inductive effects of SA on PR-1 expression. The negative effect of auxins on SA-regulated PR-1 induction might be related to the fact that numerous plant pathogens, including strains of P. syringae, produce large amounts of IAA upon infection of plants (16) (see “Discussion”).

WES1 Mediates Abiotic Stress Responses—Growth retardation is one of the symptoms commonly observed in infected and stressed plants. We observed that WES1 is strongly induced by SA and pathogen infections. Accordingly, the wes1-D mutant that is featured by reduced growth exhibits enhanced resistance to pathogen infections. Notably, WES1 is also induced by ABA (Fig. 1D). We thus hypothesized that WES1 would also be involved in ABA-regulated abiotic stress responses.

To test this, wild type plants were grown under various abiotic stress conditions. WES1 was indeed up-regulated by cold (4 °C), drought, and heat (37 °C) treatments (Fig. 7A). Consistent with this, wes1-D exhibited greatly enhanced resistance to drought (Fig. 7B). wes1-D was also resistant to freezing temperature, as evidenced by measurements of survival rate and electrolyte leakage after freezing treatment for 1.5 h at −7 °C (Fig. 7C). Spectrophotometrical measurements of chlorophyll contents demonstrated that wes1-D is also resistant to a high salt environment (600 mM) (Fig. 7D). Furthermore, wes1-D exhib-
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**FIGURE 8.** IAA effects on CBF expression (A), measurements of endogenous ABA contents (B), and a proposed working scheme for WES1 function (C). A, IAA effects on CBF expression. Transcript levels were examined by RT-PCR runs. B, endogenous ABA contents in wes1-D and wesi. An ABA biosynthetic mutant, aboa3-1, was also included in the assays for comparison. C, a proposed working scheme for WES1 function. Feedback regulation of endogenous auxin pool by a group of auxin-inducible IAA-conjugating enzymes (GH3s), which includes WES1, contributes to the auxin-mediated modulation of plant growth in response to environmental stresses. WES1 is activated in stressed plants, resulting in reduction of endogenous IAA as well as activation of stress-regulated genes (PR-1 and CBF genes).

**DISCUSSION**

**GH3 Enzymes in Auxin Homeostasis**—The GH3 enzymes have been proposed to regulate endogenous auxin content by conjugating amino acids to IAA (26, 27). In *Arabidopsis*, the major part of the endogenous IAA pool is present either in amide linkages with amino acids and peptides or in ester linkages with sugars (20). Only a small fraction is present as free IAA, indicating that conjugated forms play an important role in auxin homeostasis.

Conjugated auxins may be either inactive storage forms of IAA or intermediates that are destined for catabolic metabolism. IAA-Asp and IAA-Glu are examples of the latter (18, 20), whereas evidence indicates that IAA-Ala and IAA-Leu can be metabolized to release active IAA at certain developmental stages (44). *Arabidopsis* mutants, dfl1-D and ydk1-D, that over-express GH3 genes involved in IAA conjugation exhibit auxin-deficient phenotypes (22, 24). In one of these mutants (dfl1-D), the level of IAA-Asp was greatly elevated, and the level of free IAA was modestly reduced (27), similar to what we observed for wesi-D. In contrast, the levels of IAA-Ala and IAA-Leu were not affected in either of these mutants. Thus, it appears that a major function of WES1 and related GH3 enzymes is to direct IAA into the auxin catabolic pathway involving IAA-Asp, thereby decreasing the level of free IAA. However, it is still possible that the elevated level of IAA-Asp by WES1 overproduction may also contribute to the wesi-D phenotype, as has been discussed with a henbane (*Hyoscyamus muticus* L.) mutant that exhibits temperature-sensitive auxin auxotrophy (45). The mutant produced a wild type amount of IAA, but the half-life of endogenous IAA-Asp was significantly reduced in this mutant. As a result, the temperature sensitivity was rescued by exogenous IAA-Asp. However, it was not extensively examined whether IAA-Asp plays a physiological function or is metabolized to an unidentified auxin derivative. Direct measurements of turnover rates of IAA and IAA-Asp will help to clarify this uncertainty.

T-DNA insertion mutants of the GH3 genes, including wesi, usually exhibit only marginal phenotypic alterations (22, 25, 27).
Auxin Regulates both Biotic and Abiotic Stress Adaptation Responses—Transgenic plants carrying disease resistance genes frequently produce less biomass and fewer siliques, possibly due to the fitness costs incurred by induced resistance (12, 43). This might explain why most attempts to develop crop plants with enhanced resistance to fungal and bacterial pathogens have been unsuccessful to date (12). Costs of induced cold tolerance have also been demonstrated in transgenic Arabidopsis plants overexpressing CBF genes (46). Several growth hormones play essential roles in stress adaptation responses; among these, SA, JA, ethylene, and ABA are the best understood (3). Of particular interest is the observation that some SA- or ABA-responsive genes and those induced by pathogen infections and abiotic stresses are also influenced by auxin (2, 6, 15).

Our results indicate that feedback regulation of active auxin content by WES1 correlates with enhanced resistance to pathogen infections. This view is not inconsistent with the previous reports. The increased level of endogenous IAA detected in infected cells upon pathogen infections (14) would be derived from pathogen secretion of IAA (16). It has been proposed that pathogen-secreted IAA enhances virulence, probably by weakening the SA-mediated defense responses in infected plants (47). It is thus likely that induction of WES1 or other related GH3 genes by pathogen infections is a host mechanism for regaining auxin homeostasis.

WES1 is also induced by various abiotic stresses. Consequently, wes1-D is resistant to abiotic stresses. Furthermore, a broad array of abiotic stress-related genes, including those encoding proline metabolic enzymes, are affected in wes1-D, suggesting that auxin plays a role in several abiotic stress signaling pathways. Consistent with the WES1 induction by various growth hormones and diverse stress conditions, scanning of cis-acting regulatory DNA elements within the WES1 promoter region (~2 kbp from the start codon) using a promoter signal scan program PLACE (available on the World Wide Web at www.dna.affrc.go.jp/PLACE/signalscan.html) revealed that the WES1 promoter contains numerous DNA elements that are predicted to be responsible to auxin, ABA, SA, and biotic and abiotic stresses. Examples include multiple copies of ACGTG (drought-inducible, ABRE-like element); TGTCCT (ARF-binding); TGACG (IAA/SA-inducible); TGTTGA, AAAGAT, and TTGACC (disease-inducible); CACGTG (ABA-inducible); and TTGAC (SA-inducible), although it remains to be functionally examined.

We believe that the enhanced resistance observed in wes1-D is not simply due to indirect effects caused by chronic stress as frequently observed in dwarfed, stressed mutants but is specific to WES1 function. The WES1 enzyme is biochemically unique among the GH3 enzymes in that it possesses an SA-conjugating activity (26). This might represent a negative feedback loop for SA, similar to that described for IAA, and provide an additional way of auxin-SA interactions. The different time courses for PR-1 and WES1 expression by SA (Fig. 4B) and the altered levels of endogenous SA and SAG in wes1-D (Fig. 6B) would be explained by such a feedback loop. In addition, although wes1 is phenotypically similar to wild type plants, it is discernibly more susceptible to pathogen infections and abiotic stresses.

Based on the previous observations as well as our own data, we propose that regulation of auxin homeostasis by WES1 and related GH3 enzymes represents a means of modulating auxin actions in stress adaptation response. However, it should be noted that GH3-mediated auxin homeostasis is one of the diverse molecular schemes for auxin actions in this process. It has recently been demonstrated that a flagellin-induced microRNA represses auxin signaling by targeting auxin receptor genes and makes host plants less susceptible to bacterial infection (17). Their results provide another mechanism for auxin action during stress adaptation response, in which TIR1-mediated auxin signaling is modulated by bacterial infection. We also observed that an auxin signaling mutant axr2-1 is resistant to pathogen infections (Fig. 5B). Further works are necessary to elucidate the genetic network of auxin responses and signaling cascades. Mutants in auxin response and signaling as well as those in SA and ABA signalings will be of great help to answer the question.

Acknowledgments—We thank Drs. N.-H. Chua and K. Shimamoto for scientific discussions.

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