

# Modulating plant hormones by enzyme action

## The GH3 family of acyl acid amido synthetases

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**P**lants respond to developmental cues and environmental stresses by controlling both the level and activity of various hormones. One mechanism of modulating hormone action involves amino acid conjugation. In plants, the GH3 family of enzymes conjugates various amino acids to jasmonates, auxins and benzoates. The effect of conjugation can lead to activation, inactivation or degradation of these molecules. Although the acyl acid and amino acid specificities of a few GH3 enzymes have been examined qualitatively, further in-depth analysis of the structure and function of these proteins is needed to reveal the molecular basis for how GH3 proteins modulate plant hormone action.

Plant hormones control the interplay of a host of growth and developmental processes. Although the exact mechanisms of plant hormone sensing and signal response vary widely,<sup>1-5</sup> the auxin and jasmonate hormone response systems share similar mechanisms of action. Auxins, primarily indole-3-acetic acid (IAA), are involved in cell division, elongation and differentiation. The jasmonates (JA) play roles in seed germination, fertility, root growth and pathogen responses.<sup>1-3</sup> Perception of IAA and JA by either the SCF<sup>TIR1</sup> ubiquitin ligase complex<sup>6-9</sup> or the SCF<sup>COI1</sup> ubiquitin ligase complex,<sup>10-12</sup> respectively, recruits different classes of transcriptional modulators (i.e., Aux/IAA proteins and JAZ proteins, respectively) to the complex for ubiquitination. Degradation of Aux/IAA proteins modulates expression of auxin-responsive genes

and degradation of JAZ proteins alters transcription of jasmonate-responsive genes. A critical component in these plant hormone responses is the pre-receptor action of various enzyme systems that maintain both the levels and forms of small molecule signals.

Changes in cellular hormone concentrations and the chemical structure of a hormone will affect interaction with cognate receptors and alter developmental processes and responses. Biochemically, the levels and molecular structure of plant hormones are modified by the rates of their biosynthesis, catabolism, and/or modification. For example, in *Arabidopsis thaliana*, less than 5% of auxins are found in the active free form with the remaining 95% primarily conjugated to either amino acids or peptides.<sup>13</sup> Enzymatic conversion of unconjugated JA and IAA into the corresponding amino acid conjugates drastically alters hormone action (Fig. 1). Addition of isoleucine to JA results in synthesis of the bioactive jasmonate hormone (JA-isoleucine) that binds to the COI1 receptor to trigger JA-mediated responses.<sup>14</sup> In contrast, formation of IAA-aspartate and IAA-glutamate marks auxin for subsequent metabolic degradation and conjugation of IAA with either alanine or leucine maintains a ready pool for formation of the active free acid form of auxin.<sup>15,16</sup> Thus, metabolism of IAA and JA provides a simple means of modulating plant hormone receptor occupancy and controlling gene responses. Some GH3 family of proteins catalyze the conjugations of various amino acids to plant hormones, including JA and IAA; however,

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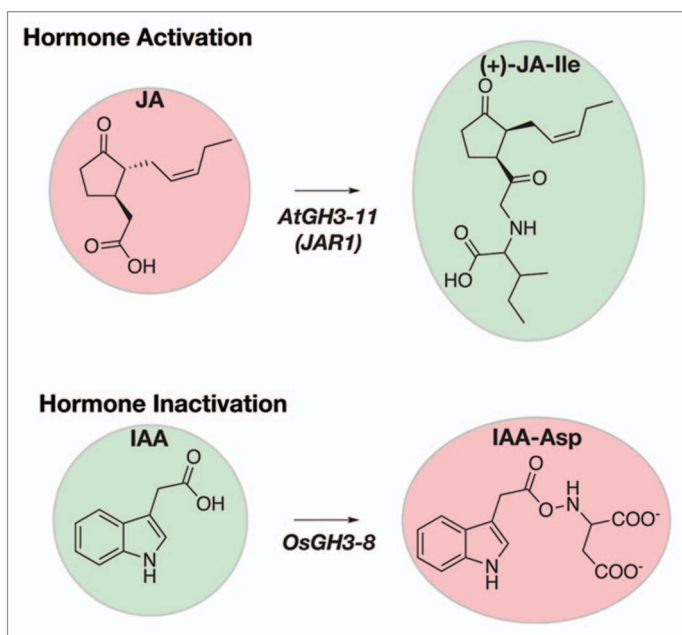
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**Figure 1.** Pre-Receptor Enzyme Action by GH3 Enzymes. Modification of jasmonic acid (JA) and indole-3-acetic acid (IAA; auxin) directly effects plant hormone potency. Conjugation of JA with isoleucine by AtGH3-11 leads to formation of the active jasmonate hormone (+)-JA-Ile (top part). Conjugation of IAA with aspartate by OsGH3-8 yields IAA-Asp, which leads to degradation of the active hormone (bottom part). Red and green correspond to inactive and active hormone forms, respectively.

a majority of GH3 proteins in plants remains uncharacterized.

### The GH3 Family: A Common Gene Family in Plants

The first GH3 gene was identified in 1984 as an early auxin-responsive gene in *Glycine max* (soybean).<sup>17</sup> Since then, GH3-related genes have been found in multiple plant species; however, not all of these genes are auxin-responsive. In the model dicot species *Arabidopsis thaliana* (thale cress), there are 19 GH3 genes.<sup>18</sup> GH3 transcripts have also been reported in tobacco, pungent pepper and tomato.<sup>18-22</sup> In the genome of the model monocot *Oryza sativa* (rice) genome, there are 13 GH3 genes.<sup>23</sup> Transcripts have been detected, but not characterized, in other monocots, including wheat, maize, sorghum and barley.<sup>18,23</sup> In addition, GH3 genes are found in the gymnosperm *Pinus pinaster* and the moss *Physomitrella patens*.<sup>24-26</sup> EST analysis suggests that GH3 genes are found across multiple plants, mosses and algae.<sup>18,23</sup> Phylogenetic analysis of the GH3 families in *Arabidopsis* and rice show that these sequences fall into

three distinct groups (I–III), although rice lacks group III members,<sup>18,23</sup> as summarized in Table 1. To date, a number of GH3 genes from *Arabidopsis* and rice have been cloned, mainly through morphological screening of either loss-of- or gain-of-function mutants.

One of the two group I proteins in *Arabidopsis* is directly linked to JA hormone action. The *A. thaliana* GH3-11 (AtGH3-11) protein was first characterized by the Staswick group after identifying the *jar1* mutant, which showed insensitivity to JA treatment.<sup>14</sup> AtGH3-11 is essential for formation of the bioactive jasmonate JA-isoleucine<sup>14</sup> (Fig. 1). A different allele of this gene (i.e., *fn219*) was previously isolated from a screen to identify extragenic modifier mutations of *cop1*, a temperature-sensitive allele of the photomorphogenic development repressor COP1; however, no activity was assigned to the encoded protein.<sup>27</sup> The other group I GH3 in *Arabidopsis*, AtGH3-10, is associated with the *df2* mutant and is involved in red light-specific hypocotyl elongation, but no enzymatic activity has been reported for this protein.<sup>28</sup>

Characterization of multiple group II GH3 genes in *Arabidopsis* also demonstrates critical roles in plant growth and development primarily through the conjugation of IAA to amino acids. For AtGH3-2, a gain-of-function mutant *ydk1-D* was found through a short primary root, reduced lateral root number and reduced apical dominance phenotype.<sup>29</sup> AtGH3-5 appears to be involved in both IAA and salicylic acid (SA) action.<sup>14,30-31</sup> AtGH3-5 was first characterized in a promoter trap line that exhibited light-dependent reporter gene expression<sup>32</sup> and later by the loss-of-function mutant *wes1* and two gain-of-function mutants *wes1-D* and *gh3.5-1D*.<sup>14,33,34</sup> AtGH3-6 was isolated as the *df1* mutant, which negatively regulates shoot elongation and lateral root formation, but positively regulates the light response of hypocotyl length.<sup>35</sup> Later biochemical analysis of AtGH3-2, -5, -6 showed that these enzymes conjugate IAA to various amino acids.<sup>22</sup> In contrast to most group II GH3, AtGH3-9 expression is repressed by low concentrations of IAA and a loss-of-function mutant shows growth of longer primary roots.<sup>36,37</sup>

Three group II GH3 have been characterized in rice. The *O. sativa* GH3-8 (OsGH3-8) gene is auxin-responsive and functions in auxin-dependent development.<sup>38</sup> Overexpression of OsGH3-8 shows abnormal morphology, but enhanced resistance to the rice pathogen *Xanthomonas oryzae*.<sup>38</sup> The OsGH3-8 enzyme specifically conjugates IAA and aspartate to form IAA-aspartate.<sup>38-40</sup> Less is known about the other two identified rice GH3 genes. Overexpression of OsGH3-1 in rice causes dwarfism, significantly reduces free auxin levels and cell elongation, but increases resistance to fungal pathogens.<sup>41</sup> Similarly, a gain-of-function mutant, *ild1-D*, in the OsGH3-13 gene results in increased tillers, enlarged leaf angles, dwarfism and improved drought tolerance.<sup>42</sup>

The only characterized group III GH3 member is AtGH3-12.<sup>43-45</sup> Three *pbs3* mutants display increased disease susceptibility to virulent and avirulent *Pseudomonas syringae*.<sup>43</sup> The *gdg1* mutant is allelic to *pbs3*.<sup>45</sup>

**Table 1.** Summary of GH3 family members in Arabidopsis and Rice

GH3 protein	Group <sup>a</sup>	Mutant <sup>b</sup>	Acyl acid <sup>c</sup>	Amino acid <sup>c</sup>	Refs
AtGH3-1	II		?	?	22
AtGH3-2	II	<i>ydk1</i>	IAA	Met, Trp	22, 29
AtGH3-3	II		IAA	Asp, Met, Try, Trp	22
AtGH3-4	II		IAA	Asp, Met, Trp	22
AtGH3-5	II	<i>wes1</i>	IAA	Glu, Asp, Met, Tyr, Trp	14, 22, 30–34
AtGH3-6	II	<i>dfl1</i>	IAA	Asp, Met, Trp	22, 35
AtGH3-7	III		?	?	
AtGH3-8	III		?	?	
AtGH3-9	II		?	?	36–37
AtGH3-10	I	<i>dfl2</i>	?	?	28
AtGH3-11	I	<i>jar1/fin219</i>	JA	Ile	14, 27, 46
AtGH3-12	III	<i>pbs3/gdgl</i>	<b>benzoates</b>	<b>Glu, His, Leu, Met</b>	43–45
AtGH3-13	III		?	?	
AtGH3-14	III		?	?	
AtGH3-15	III		?	?	
AtGH3-16	III		?	?	
AtGH3-17	II		IAA	Glu, Met, Trp	22
AtGH3-18	III		?	?	
AtGH3-19	III		?	?	
OsGH3-1	II		?	?	41
OsGH3-2	II		?	?	
OsGH3-3	I		?	?	
OsGH3-4	II		?	?	
OsGH3-5	I		?	?	
OsGH3-6	I		?	?	
OsGH3-7	II		?	?	
OsGH3-8	II		<b>IAA</b>	<b>Asp</b>	38–40
OsGH3-9	II		?	?	
OsGH3-10	II		?	?	
OsGH3-11	II		?	?	
OsGH3-12	I		?	?	
OsGH3-13	II	<i>tdl1</i>	IAA	Glu, Asp, Phe, Leu, Ala	42

<sup>a</sup>Group assignments based on phylogenetic analysis.<sup>18,23</sup> <sup>b</sup>Mutant associated with GH3 gene. <sup>c</sup>Major acyl acid and amino acid substrates of the GH3 protein identified by substrate screening assays. Substrates in bold have been confirmed by steady-state kinetic analysis of purified protein. Question marks indicate unknown enzymatic activity and substrate specificity.

### GH3 Proteins: Acyl Acid Amido Synthetases

Although the GH3 proteins are widespread across the plant kingdom, relatively little is known about their metabolic roles and kinetic properties. The first insight on the biochemical function of the GH3 proteins was provided by analysis of the group I AtGH3-11 or JAR1, protein.<sup>14</sup> Sequence comparisons detected low amino acid similarity between AtGH3-11 and the firefly luciferase superfamily of

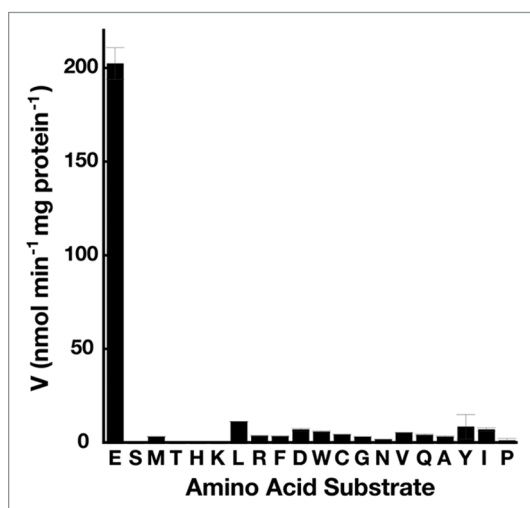
adenylating enzymes, suggesting a possible overall reaction for the GH3 proteins.<sup>14</sup> Adenylating enzymes catalyze the ATP-dependent activation of carboxyl groups on a variety of substrates by formation of an adenylated reaction intermediate.<sup>46</sup> Formation of the activated intermediate allows for subsequent reactions with other substrates.<sup>46</sup> Initial work on AtGH3-11 (JAR1) demonstrated that it catalyzes the formation of JA-Ile.<sup>47</sup> Subsequent studies of the Arabidopsis group II AtGH3-2, -3, -4, -5, -6 and -17 show that these enzymes

accept IAA as a substrate to function as acyl acid amido synthetases.<sup>22</sup> Based on screening of amino acid preference using a thin-layer chromatography (TLC)-based assay, it was suggested that these GH3 proteins utilize a broad range of amino acid substrates,<sup>22</sup> as summarized in Table 1. Using a liquid chromatography-mass spectrometry (LC-MS) assay, OsGH3-13 also conjugates multiple amino acids to IAA.<sup>42</sup> The highest activity of OsGH3-13 was observed with glutamate, but aspartate, phenylalanine, leucine and alanine are also

**Table 2.** Comparison of kinetic parameters of AtGH3-12, OsGH3-8, and AtGH3-17

	Assay method	Kinetic parameter	Assay method	Kinetic parameter
AtGH3-12	HPLC <sup>a</sup>	$V_{\max} = 20.4 \text{ min}^{-1}$	coupled UV/Vis <sup>b</sup>	$V_{\max} = 13.2 \text{ min}^{-1}$
	HPLC <sup>a</sup>	$K_m^{4\text{-HBA}} = 450 \text{ }\mu\text{M}$	coupled UV/Vis <sup>b</sup>	$K_m^{4\text{-HBA}} = 63 \text{ }\mu\text{M}$
	adenylation <sup>a</sup>	$K_m^{\text{ATP}} = 791 \text{ }\mu\text{M}$	coupled UV/Vis <sup>b</sup>	$K_m^{\text{ATP}} = 99 \text{ }\mu\text{M}$
	none	not determined	coupled UV/Vis <sup>b</sup>	$K_m^{\text{Glu}} = 3.83 \text{ mM}$
OsGH3-8	mass spec <sup>c</sup>	$V_{\max} = 21.1 \text{ min}^{-1}$	coupled UV/Vis <sup>d</sup>	$V_{\max} = 20.6 \text{ min}^{-1}$
	mass spec <sup>c</sup>	$K_m^{\text{IAA}} = 123 \text{ }\mu\text{M}$	coupled UV/Vis <sup>d</sup>	$K_m^{\text{IAA}} = 187 \text{ }\mu\text{M}$
	mass spec <sup>c</sup>	$K_m^{\text{ATP}} = 50 \text{ }\mu\text{M}$	coupled UV/Vis <sup>d</sup>	$K_m^{\text{ATP}} = 36 \text{ }\mu\text{M}$
	mass spec <sup>c</sup>	$K_m^{\text{Asp}} = 1.58 \text{ mM}$	coupled UV/Vis <sup>d</sup>	$K_m^{\text{Asp}} = 3.91 \text{ mM}$
AtGH3-17			coupled UV/Vis <sup>b</sup>	$V_{\max} = 7.4 \text{ min}^{-1}$
			coupled UV/Vis <sup>b</sup>	$K_m^{\text{IAA}} = 58 \text{ }\mu\text{M}$
			coupled UV/Vis <sup>b</sup>	$K_m^{\text{ATP}} = 138 \text{ }\mu\text{M}$
			coupled UV/Vis <sup>b</sup>	$K_m^{\text{Glu}} = 0.54 \text{ mM}$

<sup>a</sup>Data summarized from Okrent, et al. 2009.<sup>45</sup> <sup>b</sup>Protein was expressed, purified, and assayed, as described in Chen, et al. 2010;<sup>40</sup> with errors of less than 15% for  $n = 3$ . <sup>c</sup>Data summarized from Chen, et al., 2009.<sup>39</sup> <sup>d</sup>Data summarized from Chen, et al. 2010.<sup>40</sup>



**Figure 2.** Amino Acid Specificity of AtGH3-12. Purified protein was assayed by coupling AMP formation to a UV/Vis assay, as previously described in reference 40. Substrate concentrations were 1 mM IAA, 1 mM ATP and 5 mM amino acid. Data is shown as a mean  $\pm$  SE. ( $n = 3$ ).

substrates for the enzyme.<sup>42</sup> Surprisingly, the kinetic parameters for only two GH3 proteins (OsGH3-8,<sup>39,40</sup> and AtGH3-12,<sup>45</sup>) have been determined to date.

Recently, we used OsGH3-8, which specifically functions as an IAA-aspartate amido synthetase, for analysis of the chemical and kinetic mechanism of a GH3 protein.<sup>39,40</sup> These studies demonstrate formation of a chemically competent adenylation intermediate in the reaction sequence and define the order of substrate/product binding and release.<sup>40</sup> Using both a MS-based assay that directly monitored product formation and a spectrophotometric assay coupled to AMP release following

IAA-Asp formation, the kinetic parameters for the overall reaction catalyzed by OsGH3-8 were determined.<sup>39,40</sup> Each assay yields comparable kinetic values,<sup>39,40</sup> as summarized in Table 2. Importantly, these assays provide a quantitative assessment of GH3 protein acyl acid and amino acid specificities. OsGH3-8 is highly specific for IAA (150 nmolmin<sup>-1</sup>mgprotein<sup>-1</sup>) compared to JA (0.3 nmol min<sup>-1</sup> mg protein<sup>-1</sup>) and SA (1.1 nmol min<sup>-1</sup> mg protein<sup>-1</sup>). Other auxin-analogs, including phenylacetic acid, indole butyric acid and naphthalenec acid, were also substrates with catalytic efficiencies (i.e.,  $k_{\text{cat}}/K_m$ ) 1.4- to 9-fold lower than that observed for

IAA.<sup>40</sup> Similarly, the enzyme is also highly specific for aspartate. Asparagine, the next best amino acid substrate, shows a 45-fold reduction in  $k_{\text{cat}}/K_m$  compared to aspartate and other amino acids are more than 100-fold lower.<sup>40</sup> These results suggest that under biological conditions, it is highly unlikely that OsGH3-8 accepts amino acids other than aspartate as a substrate.

AtGH3-12 is the only biochemically studied group III enzyme. It favors 4-substituted benzoates, including 4-aminobenzoate and 4-hydroxybenzoate (4-HBA), as substrates, but accepts benzoate with moderate activity and is inactive with 2-substituted benzoates.<sup>45</sup> In this study, the authors used various assays for activity analysis, including a spectrophotometric adenylation assay, which monitors only the first half of the chemical reaction sequence, to determine the  $K_m$  value for ATP and an HPLC assay to determine  $V_{\max}$  and the  $K_m$  for product formation using 4-HBA as a substrate (Table 2). Although no kinetic information for amino acid specificity was provided, a TLC assay was used to show that 4-HBA was conjugated to various amino acids. These results suggest that glutamate, histidine, lysine and methionine are the major substrates with isoleucine, leucine, valine, threonine, threonine, serine and tryptophan as minor substrates.

Studies on the group II AtGH3, AtGH3-12 and OsGH3-8 raise the question—are these proteins promiscuous or specific for amino acid substrates? Addressing this point, will help to define

the possible biological roles of these proteins. For comparison, the OsGH3-8 spectrophotometric assay was used to re-evaluate AtGH3-12 and to determine the kinetic parameters for AtGH3-17 (Table 2). For AtGH3-12, the  $V_{\max}$  and  $K_m$  values for 4-HBA, ATP and glutamate were re-determined and are comparable to those originally reported for this protein and to OsGH3-8. Importantly, AtGH3-12 shows an 80-fold preference for glutamate compared to the next best amino acid substrate, indicating that this enzyme is highly specific and unlikely to accept other amino acid substrates in vivo (Fig. 2). Analysis of AtGH3-17 shows that this enzyme displays kinetics similar to OsGH3-8 and AtGH3-12 (Table 2). In comparison to earlier studies, these experiments emphasize the need to use assays that accurately determine substrate specificity for further studies on the possible biochemical functions of the GH3 proteins in plants.

## Summary

Plant development, growth and fitness are all determined by the complex integration of multiple signaling pathways and hormone responses. As part of the system that regulates plant hormone effects, GH3 enzymes play important, but ill defined, roles, as evidenced by the various mutant phenotypes associated with these genes. The GH3 proteins appear to control levels of major plant hormones, including IAA and JA, and modulate pathways responsible for plant growth and development, seed development, light signaling, drought response, thermogenesis and pathogen resistance.<sup>13-45</sup> Biochemically, the GH3 proteins are defined by their ability to catalyze the conjugation of acyl acids to amino acids via an adenylation reaction. Through a simple chemical modification, GH3 proteins can regulate levels of inactive and active forms of JA and IAA. For example, the ability of a GH3 protein to modify the chemical structure of multiple JA- and IAA-related substrates may allow for changes of metabolic pools across a biosynthetic pathway, in addition to modifying the final hormone product. Although initial work has qualitatively determined the substrate activity for a

handful of GH3 proteins, further detailed analysis of the acyl acid and amino acid specificities of the GH3 family is an essential step toward defining the possible biological function of these enzymes. In time, complete analysis of the temporal and spatial expression patterns, cellular localization and specificity of enzymatic activities will provide insight on how this diverse protein family modulates plant hormone function.

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