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Preference of *Arabidopsis thaliana* GH3.5 acyl amido synthetase for growth versus defense hormone acyl substrates is dictated by concentration of amino acid substrate aspartate

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ABSTRACT

The GH3 family of adenylating enzymes conjugate acyl substrates such as the growth hormone indole-3-acetic acid (IAA) to amino acids via a two-step reaction of acyl substrate adenylation followed by amino acid conjugation. *Arabidopsis thaliana* GH3.5 was previously shown to be unusual in that it could adenylate both IAA and the defense hormone salicylic acid (SA, 2-hydroxybenzoate). Our detailed studies of the kinetics of GH3.5 on a variety of auxin and benzoate substrates provides insight into the acyl preference and reaction mechanism of GH3.5. For example, we found GH3.5 activity on substituted benzoates is not defined by the substitution position as it is for GH3.12/PBS3. Most importantly, we show that GH3.5 strongly prefers Asp as the amino acid conjugate and that the concentration of Asp dictates the functional activity of GH3.5 on IAA vs. SA. Not only is Asp used in amino acid biosynthesis, but it also plays an important role in nitrogen mobilization and in the production of downstream metabolites, including pipecolic acid which propagates defense systemically. During active growth, [IAA] and [Asp] are high and the catalytic efficiency (k_{cat}/K_m) of GH3.5 for IAA is 360-fold higher than with SA. *GH3.5* is expressed under these conditions and conversion of IAA to inactive IAA-Asp would provide fine spatial and temporal control over local auxin developmental responses. By contrast, [SA] is dramatically elevated in response to (hemi)-biotrophic pathogens which also induce *GH3.5* expression. Under these conditions, [Asp] is low and GH3.5 has equal affinity (K_m) for SA and IAA with similar catalytic efficiencies. However, the concentration of IAA tends to be very low, well below the K_m for IAA. Therefore, GH3.5 catalyzed formation of SA-Asp would occur, fine-tuning localized defensive responses through conversion of active free SA to SA-Asp. Taken together, we show how GH3.5, with dual activity on IAA and SA, can integrate cellular metabolic status via Asp to provide fine control of growth vs. defense outcomes and hormone homeostasis.

1. Introduction

Plant hormones regulate development and response to their environment (Jaillais and Chory, 2010; Robert-Seilaniantz et al., 2011a). Indole-3-acetic acid (IAA – an auxin) and salicylic acid (SA) are plant hormones that predominantly promote development and defense, respectively (Dempsey et al., 2011; Spoel and Dong, 2012;

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Vanneste and Friml, 2009; Woodward and Bartel, 2005). Auxin regulates plant developmental processes such as organogenesis through its accumulation in organ primordia where it binds to its receptor, resulting in the degradation of transcriptional repressors of auxin-associated genes and the transcription of a myriad of auxin-associated genes (Kepinski and Leyser, 2005; Vanneste and Friml, 2009). SA synthesis is induced in response to (hemi)biotrophic pathogens such as the powdery mildew fungus *Golovinomyces orontii* (Dewdney et al., 2000; Wildermuth et al., 2001), the bacterium *Pseudomonas syringae* (Rasmussen et al., 1991), and tobacco mosaic virus (Malamy et al., 1990). When sufficient SA accumulates, the master plant immune regulator NPR1 is stable, active, and properly localized, resulting in the transcription of a suite of genes that mediate a robust local defense (Fu et al., 2012; Wu et al., 2012). Even higher levels of SA accumulate when a pathogen induces a hypersensitive response (HR) with programmed cell death (PCD) (Dempsey et al., 2011).

To control amplified downstream effects of hormones, hormone cellular concentrations are tightly regulated both spatially and temporally. For example, high local levels of SA accumulate and cause cell death in tobacco in response to tobacco mosaic virus or a fungal elicitor. Neighboring cells accumulate moderate levels of SA and mount a local defense response, and more distal cells accumulate minimal SA and mount no defense (Dorey et al., 1997; Huang et al., 2006). For auxin, spatial control of concentration and associated downstream impacts is mediated to a large extent by auxin transport and catabolism (Adamowski and Friml, 2015; Mellor et al., 2016). Furthermore, developmental and environmental context and inputs are integrated to coordinate and fine-tune cellular responses. For example, the atypical E2F transcription factor DEL1, which is only expressed in dividing tissue, promotes cell division by inhibiting endoreduplication, SA accumulation and defense (Chandran et al., 2014; Vlieghe et al., 2005).

Given their opposing roles in promoting growth versus defense, IAA and SA have long been known to act antagonistically (Denancé et al., 2013; Robert-Seilaniantz et al., 2011a). Exogenous auxin can suppress SA-dependent defense (Park et al., 2007a; Robert-Seilaniantz et al., 2011a, 2011b), while exogenous SA treatment decreases *Arabidopsis* biomass in an auxin-dependent manner (Canet et al., 2010). However, a sophisticated understanding of the variety of mechanisms by which IAA and SA modify each other's accumulation, activity, and function with cellular resolution remains limited (Denancé et al., 2013; Robert-Seilaniantz et al., 2011a).

One means by which hormone activity is directly regulated is via conjugation to amino acids. For example, IAA conjugation to Asp initiates auxin catabolism (Ostin et al., 1998), while conjugation to Ala stores IAA as an inactive form that is rapidly reactivated through hydrolysis by a dedicated enzyme (Rampey et al., 2004). The only SA-amino acid conjugate found in plants thus far is salicyloyl-aspartate (SA-Asp) (Bourne et al., 1991; Chen et al., 2013; Steffan et al., 1988). Similar to IAA-Asp, SA-Asp is not hydrolyzed back to SA (Chen et al., 2013). Furthermore, SA-Asp was unable to induce robust defense gene expression (Chen et al., 2013), suggesting SA-Asp, like IAA-Asp, is also an inactive form of the hormone dedicated to catabolism. However, an additional possibility is that it functions as a mobile form of SA involved in low level priming of defense (Chen et al., 2013).

Hormone-amino acid conjugation in plants is catalyzed by enzymes belonging to the GH3 (Gretchen Hagen 3) family which are members of the greater firefly luciferase family of adenylating enzymes (Staswick et al., 2005, 2002). GH3 enzymes are divided into three groups based on syntenic analysis and preferred substrates (Okrent and Wildermuth, 2011; Staswick et al., 2002). Generally, GH3s that conjugate JA are classified as Group I; GH3s that conjugate IAA are classified as Group II (Okrent and Wildermuth, 2011; Staswick et al., 2005); and Group III is less well characterized. In *Arabidopsis*, active acyl substrates are known only for one classic Group III member, GH3.12/PBS3, which prefers 4-substituted benzoates such as 4-hydroxybenzoic acid (4-HBA) and *para*-aminobenzoic acid (pABA) (Okrent et al., 2009; Okrent and Wildermuth, 2011).

Surprisingly, in addition to auxins, the Group II member GH3.5 (At4g27260) is also active on SA and is the only GH3 enzyme known with this activity (Chen et al., 2013; Staswick et al., 2005, 2002; Westfall et al., 2016). Endpoint assays indicated the possibility of GH3.5 conjugation of auxins to a variety of amino acids (Staswick et al., 2005; Wang et al., 2012), though *in planta* measurements point to Asp as the dominant amino acid conjugate (Park et al., 2007a; Zhang et al., 2007). As IAA-Asp and SA-Asp appear to be inactive or hypoactive non-hydrolyzable forms of these hormones, GH3.5 conjugation could play an important role in IAA and SA homeostasis and hormone cross-talk.

To better understand the function of GH3.5 in auxin and SA metabolism and response, we undertook a biochemical kinetic study of GH3.5 to accurately determine its acyl substrate preference for IAA, SA, and related substrates as well as its amino acid substrate preference (e.g. Asp). Kinetic parameters were recently reported for GH3.5 (Westfall et al., 2016). Our contemporaneous, independent examination of the kinetics of GH3.5 on a variety of auxin and benzoate substrates extends these findings. Most notably, we show that GH3.5 strongly prefers Asp as the amino acid conjugate and that the concentration of Asp dictates the functional activity of GH3.5 on IAA vs. SA. High levels of Asp can significantly modify GH3 reaction kinetics with the degree of inhibition dependent on the acyl substrate; therefore, kinetic parameters assessed at one high amino acid concentration may misrepresent GH3 K_m's and acyl substrate preference. Because IAA, SA, and Asp concentrations vary at the cellular level with developmental and environmental context, understanding GH3.5 activity and preference in the context of physiologically-relevant concentrations of these substrates allows us to specifically predict GH3.5 function in a context-dependent manner. These predictions are consistent with observed *GH3.5* gene expression and provide a mechanistic understanding for the dual function of GH3.5 in hormone homeostasis in growth and defense.

2. Results and discussion

2.1. Kinetic parameters of GH3.5 adenylation on auxin-like substrates

Hormone acyl substrate specificity for GH3.5 was initially explored using an endpoint PP_i Exchange Assay, which found GH3.5 to be active on a variety of auxins and SA (Staswick et al., 2005, 2002). To better understand the preference of GH3.5 for auxins, SA, and related compounds, we employed a high throughput kinetic assay of adenylation (Okrent et al., 2009), shown in Fig. 1. Similar to Staswick et al., 2005, we found GH3.5 was active on auxin-like compounds: IAA, indole-3-pyruvic acid (IPA), indole-3-butyric acid (IBA), indole-3-carboxylic acid (ICA), 2-phenylacetic acid (PAA) and the synthetic auxin 1-napthaleneacetic acid (NAA) (Fig. 2). GH3.5 exhibited the greatest affinity for IAA ($K_m = 45$ μM) and least for IBA ($K_m = 733$ μM). The V_{max} of all auxin-like substrates tested were very similar, 53–104 nmol * min^{-1} * μg^{-1} (Fig. 2B). The catalytic efficiency (k_{cat}/K_m) of GH3.5 was highest with IAA at 5.12 min^{-1} * mM^{-1}. Westfall et al. (2016) also found GH3.5 to be active on IAA, PAA, and NAA and to exhibit similar catalytic efficiencies.

As IAA is the dominant auxin, our further studies with GH3.5 focus on IAA as the auxin substrate. However, the ability of GH3.5 to act on a variety of naturally occurring auxin-like substrates is important, as they appear to play distinct roles in both plant