


Reply: Complexity in Camalexin Biosynthesis

In Su et al. (2011), we reported that accumulation of multiple glutathione S-transferases (GGTs), elevation of GST activity, and consumption of GSH coincides with camalexin production; GSTF6 overexpression increased and GSTF6 knockout reduced camalexin production; Arabidopsis thaliana GSTF6 expressed in yeast cells catalyzed GSH(IAN) formation; GSH(IAN); CysGly, and γ-GluCys(IAN) are intermediates within the camalexin biosynthetic pathway; and, finally, γ-glutamyl transpeptidases (GGTs) and phytochelatin synthase are involved in the catabolism of GSH(IAN). Based on these data and the expression patterns of these genes, we suggest that (1) GSH is the Cys derivative used during camalexin biosynthesis, (2) the conjugation of GSH with IAN is catalyzed by GSTF6, and (3) GGTs and phytochelatin synthase are involved in camalexin biosynthesis. The Letter to the Editor by Møldrup et al. (2013) and another report by the same group (Geu-Flores et al., 2011) argue that γ-glutamyl peptidases (GGPs), and not GGTs, are the γ-glutamyl cleaving enzymes in the camalexin pathway in Arabidopsis.

Møldrup et al. (2013) speculate that reduction of camalexin in our experiment by treatment with inhibitor acivicin (Su et al., 2011) may reflect inhibition of GGPs in addition to GGT activity. However, acivicin has been widely used as a specific inhibitor of GGT activity for in vitro and in vivo experiments of GGT (Wada et al., 2008). Crystal structure of GGT-acivicin complexes clearly revealed that acivicin binds to GGTs through several key amino acid residues in substrate binding pocket. These residues are highly conserved in GGTs from bacteria and animal cells (Wada et al., 2008). For example, Escherichia coli GGT binds acivicin through the formation of a covalent bond between the Oγ atom of its Thr-391 and the imino carbon of the dihydroisoxazole ring of acivicin and also through the formation of hydrogen bonds between acivicin and the GGT residues Arg-114, Asn-411, Gin-430, Asp-433, Ser-462, Ser-463, and Gly-484 (Wada et al., 2008). To show if Arabidopsis GGTs and GGPs also contain these conserved residues, we compared amino acid sequences of GGT1, GGT2, GGP1, and GGP3 with S-subunits of GGTs from bacteria and animal. The results reveal that GGT1 and GGT2 share 31.1 to 37.3% sequence identities with the selected bacteria and animal GGTs, whereas GGP1 and GGP3 share only 4.8 to 9.8% sequence identities with those GGTs. Figure 1 shows that all the conserved residues involving in the interaction of GGTs with acivicin are present in Arabidopsis GGPs, but not GGPs. Therefore, it is reasonable to suppose that binding of acivicin with Arabidopsis GGPs inhibits GGT activity. However, experimental evidence is needed to support the hypothesis of Møldrup et al. (2013) that acivicin interacts with GGPs and thereby inhibits GGT’s activities. For example, we are unaware of any data showing acivicin binding with the GGPs, the binding affinity, and/or the crystal structure of GGP-acivicin complex.

Møldrup et al. (2013) also raise an alternative explanation for reduced camalexin production following acivicin treatment, suggesting that “termination of all GGT activity could arrest glutathione and glutathione conjugates in the vacuole and
extracellular space” (Møldrup et al., 2013). However, experimental evidence to support this idea is lacking.

Møldrup et al. (2013) also questioned the liquid media system that we used for seedling growth and camalexin assays in our experiments (Su et al., 2011). They note that camalexin reduction in liquid media after inhibition of GGT activity or elimination of GGT gene expression is possibly due to the reduction of GGT-mediated camalexin secretion. In fact, the liquid media system has been demonstrated as a reliable system for camalexin assay by our group (Xu et al., 2008; Su et al., 2011) and by other groups (Bednarek et al., 2005; Ren et al., 2008; Mao et al., 2011). Currently, no evidence shows that GGTs are functional in the camalexin secretion. Most recently, ABCG37 and ABCC5, two ABC transporters in Arabidopsis, are reported to be functional in camalexin secretion (Badri et al., 2012).

Møldrup et al. (2013) regard the camalexin biosynthetic pathway to be cytosolic based on the cytosolic localization of some known enzymes in the pathway. Their point of view is that enzymes in a biosynthetic pathway usually are colocalized temporally and spatially (Møldrup et al., 2013). Because of possible apoplast localization of GGT1 and GGT2 (Martin et al., 2007; Ohkama-Ohtsu et al., 2007), they exclude these GGTs as the enzymes involved in camalexin biosynthesis. However, according to this viewpoint, the different localizations to the chloroplast for CYP79B2/79B3 (Hull et al., 2000), microsome for CYP71A13 (Nafisi et al., 2007) and CYP71A15 (Schuhegger et al., 2006), vacuole and cytosol for GSTF6 (Carter et al., 2004) (http://suba.plantenergy.uwa.edu.au/flatfile.php?id=AT1G02930.2), and cytosol for GGP1/GGP3 (Geu-Flores et al., 2011; Møldrup et al., 2013) will be difficult to explain, as these enzymes have been demonstrated as catalytic enzymes in the camalexin biosynthetic pathway. It is a generally accepted viewpoint that enzymes in a biosynthetic pathway might be located in different subcellular compartments and even different types of cells. Although temporal and spatial localization may be “important clues for assessing the

Figure 1. Multiple Sequence Alignment of Arabidopsis GGT1, GGT2, GGP1, GGP3, and GGTs from Other Organisms.

The coding sequences of all genes were aligned by ClustalW (MegaAlign program DNAStar). Sequences corresponding to the S-subunits of GGTs from bacteria and animals are presented. Identical residues in all GGTs are boxed. The residues that interact with acivicin in E. coli GGT are indicated by an asterisk above the alignment.
likelihood that a given enzyme is involved in a particular pathway in planta," as noted by Møldrup et al. (2013), the converse is not necessarily true, and the absence of colocalization cannot be taken as evidence that such enzymes do not work in the same pathway.

Another observation that led Møldrup et al. (2013) to argue that GPs instead of GGTs are involving in camalexin biosynthesis is that we did not show the accumulation of GSH(IAN) in GGT mutants in our previous work (Su et al., 2011). However, we have since measured the contents of GSH(IAN) in MKK9 hyperactive mutants (as MKK9DD), Columbia wild type, and GGT mutants undergoing camalexin biosynthesis. We found that GSH(IAN) accumulated in the wild type and in MKK9DD, ggt1, and ggt2 mutants undergoing camalexin biosynthesis. Figure 2 shows that GSH(IAN) accumulation is positively correlated with camalexin production. The result suggests that plants must produce more substrate [e.g., GSH(IAN)] in order to produce more product [e.g., camalexin].

As shown in Figure 2 of Møldrup et al. (2013), both the ggt1 and ggt2 mutants produced ∼40% less camalexin than the wild type after AgNO3 treatment. However, due to the larger variation between the biological repeats in their experiments, the authors suggest that there are no significant differences between GGT mutants and the wild type. By contrast, smaller variations were observed in the liquid culture system in our experiments. Using the liquid media system, we showed that GGT mutants produced 40 to 50% less camalexin than the wild type after pathogen treatment, and the differences were significant between mutants and the wild type (Su et al., 2011).

Most recently, Wang et al. (2012) reported that GH3.5, a multifunctional acetyl-amid synthetase, is involved in camalexin biosynthesis through catalyzing the conjugation of indole-3-carboxylic acid with Cys. This suggests an alternative camalexin biosynthetic pathway. Current data therefore suggest the existence of multiple complicated pathways in the control of camalexin biosynthesis, and the pathway chosen by Arabidopsis plants likely depends on different stimuli.

METHODS

Plant Growth and Camalexin and GSH(IAN) Measurement

Arabidopsis thaliana wild type (ecotype Columbia-0), ggt1-1 and ggt2-1 mutants, and MKK9DD transgenic seedlings were all grown and treated as described by Xu et al. (2008). The camalexin in liquid media and GSH(IAN) in seedlings were measured as described (Su et al., 2011).

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AUTHOR CONTRIBUTIONS

T.S., Y.L., and H.Y. performed the experiments. T.S., Y.L., H.Y., and D.R. wrote the article.

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