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Title:

Characterization of a dynamic metabolon producing the defense compound dhurrin in sorghum

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One-sentence summary:
Functional characterization of a membrane associated dynamic metabolon catalyzing formation of the defense compound dhurrin in *Sorghum bicolor*

Abstract

Metabolic highways may be orchestrated by assembly of sequential enzymes into protein complexes, metabolons, to facilitate efficient channeling of intermediates and to prevent undesired metabolic cross-talk while maintaining metabolic flexibility. Here we report isolation of the dynamic metabolon catalyzing formation of the cyanogenic glucoside dhurrin, a defense compound produced in sorghum plants. The metabolon was reconstituted

in liposomes, which demonstrated the importance of membrane surface charge and presence of the glucosyltransferase for metabolic channeling. We used *in planta* Fluorescence Lifetime Imaging Microscopy (FLIM) and Fluorescence Correlation Spectroscopy (FCS) to study functional and structural characteristics of the metabolon. Understanding the regulation of biosynthetic metabolons offers opportunities to optimize synthetic biology approaches towards efficient production of high value products in heterologous hosts.

Main Text

Plants produce a plethora of specialized metabolites to fend off attack from herbivores and pests and to adapt to abiotic stresses. One class of specialized metabolites is the cyanogenic glucosides, such as dhurrin, which is present in *Sorghum bicolor* (*1*). Dhurrin is produced from the amino acid $_{L}$-tyrosine, synthesized by three membrane anchored proteins, the NADPH-dependent cytochrome P450 oxidoreductase (POR) and two cytochrome P450 enzymes (CYP79A1 and CYP71E1), and by a soluble UDP-glucosyl transferase (UGT85B1)(*2*)(Fig.1A). When cellular integrity is disrupted, such as by a chewing insect, dhurrin is hydrolyzed which results in release of toxic hydrogen cyanide (*1*).

The dhurrin content of three-day-old etiolated seedlings reaches 30% of their dry weight (*3*), even though the enzymes involved in dhurrin biosynthesis constitute less than 1% of the total membrane protein content (*4*). This efficiency may be governed by metabolon formation. Metabolon disassembly would result in release of an aldoxime intermediate, proposed to act as an antifungal agent (*5*). Several biosynthetic pathways have been proposed to involve metabolon formation (*2, 6-8*), but dynamic assembly and disassembly

have impeded accurate characterization of their various configurations. As a result, metabolons are often depicted as static entities composed of equimolar protein components (*2*, *6*). In this study we report protein-protein interactions and oligomer formation, protein dynamics and functional regulation of the metabolon catalyzing dhurrin synthesis.

Isolation of dynamic membrane-embedded metabolons is hampered by their destabilization and dissociation upon detergent solubilization of the lipid bilayer. Use of the styrene maleic acid (SMA) copolymer circumvents these issues. The SMA polymer spontaneously integrates into the lipid bilayer and carves out discrete lipid particles (SMALPs) containing the resident membrane proteins and the surrounding lipids (*9*). We employed the SMALP technology in combination with affinity chromatography to isolate the dynamic dhurrin metabolon (Fig.S1).

Application of the SMA polymer to microsomes prepared from etiolated sorghum seedlings resulted in formation of discrete SMALPs ranging from 10 to 25 nm in diameter (n=242) (Fig.1B). These particles were larger than previously reported SMA particles obtained from pure lipids or harboring a single protein (averaged 10 nm)(*9*). POR is the omnipotent electron donor to all microsomal P450s (*10*). The SMALPs were purified, by 2´,5´ADP-Sepharose affinity chromatography based on the NADPH cofactor requirement of POR, for co-purification of POR-associated proteins (Fig.S2). We analyzed the protein content in the course of SMALP purification by quantitative mass spectrometry (Fig.1C; Fig.S3; Database S1-S2)(*11*). As a control, the same purification was carried out using the anionic detergent cholate instead of the SMA polymer. The protein enrichment was determined based on the content of the three POR isoforms (POR2a-c), P450 proteins and other

enzymes including UGT85B1, dhurrinase, cytochrome b_5 (Cytb_5) and cytochrome b_5 reductase (Cytb_5 Red)(Table S1). Microsomal and SMA solubilized fractions displayed similar protein content (Fig.S3). Following affinity chromatography, 132 proteins were quantified in the SMALPs with the P450s CYP79A1 and CYP71E1 among the eight most abundant proteins (Fig.1C). In contrast, no P450 enrichment was observed using the cholate sample (Table S1). The soluble UGT85B1 was identified in all samples but could not be quantified in the purified SMALP sample most likely due to the extensive washing steps during microsomes preparation and affinity chromatography. The strong enrichment of the entire complement of membrane bound dhurrin pathway enzymes in the affinity purified SMALPs demonstrates that these enzymes are assembled in a metabolon.

Functional regulation of dhurrin biosynthesis as a response to environmental stresses (*1*) likely involves dynamic assembly and disassembly of the metabolon. Therefore, we studied the effect of UGT85B1 on the channeling of $_L$-tyrosine towards dhurrin by *in vitro* reconstitution of the dhurrin enzymes in liposomes. Catalytic activities of P450s were determined based on the amounts of aldoxime (CYP79A1 mediated) and cyanohydrin (CYP71E1 mediated) produced following administration of radiolabeled $_L$-tyrosine substrate (Fig.2A). In the absence of UGT85B1, 50% of the produced aldoxime were further converted into the cyanohydrin, corresponding to $kcat^{CYP79A1}$=80 min^{-1} and $kcat^{CYP71E1}$=40 min^{-1}. When supplemented with UGT85B1 in the absence of UDP-glucose, UGT85B1 interacted with the two P450s (Fig.S4), increasing their catalytic properties ($kcat^{CYP79A1}$=100 min^{-1} and $kcat^{CYP71E1}$=80 min^{-1}) demonstrating an increased flux of $_L$-tyrosine through the P450s and improved channeling (80%; Fig.2A). Addition of UDP-

glucose did not further increase the catalytic efficiency of the two P450s or channeling of the aldoxime. Additionally, UGT85B1 did not stimulate CYP71E1 activity as observed by administration of aldoxime as a substrate (Fig.2A). Therefore, we propose that UGT85B1 binds specifically to both P450s and augments the flux and channeling of $_{L}$-tyrosine towards dhurrin.

The impact of membrane lipid composition on the catalytic activities of the dhurrin pathway P450s was examined by reconstitution experiments in liposomes made using different ratios of phospholipids guided by the enrichment found in the purified SMALP samples (Fig.S5, Database S3-S5). CYP79A1 activity was marginally influenced by the phospholipid composition. In contrast, the catalytic efficiency of CYP71E1 was highly sensitive to the lipid environment and dependent on the total concentration of negative charges derived from the lipid headgroups of PG, PS and PI with an optimum efficiency in liposomes containing 20% to 30% negatively charged phospholipids (Fig.2B). This matches the observed enrichment of PG in the purified SMALPs.

To study the organization of the dhurrin pathway enzymes *in planta*, CYP79A1, CYP71E1, UGT85B1, POR2b and different combinations of these, including control proteins, were transiently expressed in *N. benthamiana* leaf epidermal cells (*11*), the most suitable plant expression system for *in planta* FLIM and FCS. The expression levels of the heterologous proteins in *N. benthamiana* were quantitatively comparable to those in *S. bicolor* seedlings (Table S2; Database S6).

Upon co-expression of CYP79A1 and CYP71E1, several byproducts of the dhurrin pathway and some dhurrin were accumulated (Fig.3A). Formation of dhurrin reflects the

inherent capability of *N. benthamiana* to glycosylate exogenous compounds (*12*). Co-expression of UGT85B1 with the two P450s and without *Sb*POR2b resulted in accumulation of 263% more dhurrin and reduction of byproduct release to 9% of the level observed in the absence of the UGT85B1 (Table S3). The efficient channeling of intermediates, achieved upon co-expression of UGT85B1, supports the assembly of a metabolon *in planta* and confirms that endogenous *N. benthamiana* POR is sufficient to provide reducing equivalents to the P450s.

To further evaluate dhurrin metabolon formation, CYP79A1, CYP71E1, UGT85B1 and POR2b were expressed as fusion proteins with different fluorescent proteins suitable for *in planta* FLIM and FCS (Fig.S6-S8)(*11*). First, the functionality of the target enzymes after fusion was assessed. All possible combinations with eGFP fused to two of the three target proteins produced similar amounts of dhurrin and byproducts (Fig.3A; Table S3). No apparent difference between uses of N- or C-terminally tagged UGT85B1 was observed and in the following paragraphs we only show the data obtained for N-terminal fusion constructs. The tracking of fluorescent fusion constructs of the dhurrin enzymes with confocal microscopy techniques (Movies S1-S2) illustrated their fast movement in the plant cell along the ER network, as also observed in studies with other metabolons (*7, 8, 13*). Quantified by FCS, the average diffusion coefficients of individual dhurrin enzymes decreased upon co-expression of the two other partners, whereas the diffusion of the POR2b:eGFP fusion protein was not influenced by co-expression of dhurrin enzymes (Fig.3B-C; Fig.S9; Table S4-S5).

The Fluorescence/Förster Resonance Energy Transfer (FRET) efficiencies from pairwise combinations of all target fluorescent fusion proteins were calculated to gain a more detailed knowledge of the organization of the dhurrin metabolon (Fig.S10). The *in planta* FRET results demonstrated that CYP79A1, CYP71E1 and UGT85B1 all form homo- and hetero-oligomers with FRET values significantly higher than the controls (Fig.4A-E, Table S6-S10). FRET signals for CYP79A1-CYP79A1, CYP71E1-CYP71E1 and CYP79A1-CYP71E1 were unaffected by co-expression of UGT85B1. In contrast, UGT85B1-UGT85B1 oligomerization was enhanced by co-expression of either CYP79A1 or CYP71E1 with the highest FRET signal observed when the entire dhurrin pathway was expressed, suggesting recruitment of UGT85B1 by the P450s (Fig.4C). We therefore conclude that the soluble UGT85B1 interacts with both CYP79A1 and CYP71E1 but it is not necessary for CYP79A1-CYP71E1 complex formation (Fig.4E). CYP79A1, CYP71E1, CYP98A1 and POR2b are situated very close together at the ER surface, with comparable pairwise FRET values (Fig.4F; Table S11). All microsomal P450s require electron donation from POR, therefore it is not surprising that we find CYP98A1 to be in proximity with the dhurrin biosynthetic enzymes (Fig.4A,B,D). UGT85B1 was situated closely to the non-partner ER membrane proteins, CYP98A1 and POR2b when CYP79A1 and CYP71E1 were co-expressed (Table S12).

A prerequisite to understand how cells coordinate diverse metabolic activities is to understand how the enzyme systems catalyzing these reactions are organized and their possible enrollment as part of dynamic metabolons. Efforts to maximize product yield from genetically engineered pathways (*14-17*) would benefit from this information. In this study

we show that the dhurrin pathway forms an efficient metabolon. CYP79A1 and CYP71E1 form homo- and hetero-oligomers, which enable recruitment of the cytosolic soluble UGT85B1 (Fig.4G). UGT85B1 regulates the flux of $_{L}$-tyrosine and stimulates channeling between CYP79A1 and CYP71E1. Efficient metabolic flux and channeling requires an overall negatively charged lipid surface and may provide additional means for regulating the dynamic assembly necessary to respond swiftly to environmental challenges. Similar organization may characterize biosynthetic pathways of other specialized metabolites as well.

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Fig.1. Detergent-free isolation of the dhurrin metabolon including surrounding lipids

(A) The dhurrin biosynthetic pathway. **(B)** Negative stain transmission electron microscopy images of affinity purified SMALPs and distribution of particle sizes. (**C**) Quantitative mass spectrometry of the protein content of affinity purified SMALPs. Proteins related to dhurrin biosynthesis and other P450 enzymes are highlighted. Data is represented as the fraction of the total protein content and mean values of three independent measurements ±SD.

Fig.2. Protein-protein and protein-lipid interactions stimulate channeling.

(**A**) POR2b, CYP79A1, and CYP71E1 were reconstituted in liposomes of a total lipid extract from Sorghum microsomes. Catalytic activity of CYP79A1 and CYP71E1 was measured in the absence or following supplementation with UGT85B1 and/or UDP-glucose using radiolabeled $_{L}$-tyrosine or aldoxime as substrate. (**B**) POR2b, CYP79A1, CYP71E1 were reconstituted in liposomes with different lipid mixtures of POPC, POPG, POPS, POPE and PI. Catalytic activity of CYP79A1 and CYP71E1 was measured and compared

to the activity of the proteins reconstituted in a total lipid extract from Sorghum microsomes. Inset displays a titration of PG lipids and P450 activities. All functional data shows averages of biological triplicates and error bars indicate ±SD.

Fig.3. Functional fluorescence labeling of dhurrin biosynthetic enzymes and control proteins for *in planta* studies.

(A) LC-MS data of dhurrin biosynthesis in *N. benthamiana* upon expression of CYP79A1, CYP71E1 and UGT85B1. Metabolite profiles were monitored as Total Ion Chromatogram (TIC). Peaks 1-6 are byproducts of dhurrin intermediates. Prunasin was used as a primary internal standard and amygdalin as a secondary internal standard. The combination 79+71 was used as a reference (100%) for the total level of accumulated byproducts. Apparent diffusion of eGFP-labeled soluble proteins **(B)** and ER-proteins **(C)** measured by *in planta* FCS onto ER. CYP98A1:eGFP and free eGFP were used as controls. Letters indicate statistically significant similarities for the recorded values using t-test pairwise comparisons with $P<0.05$. Error bars indicate ±SD.

Fig.4. Protein-protein interactions *in planta* reveal formation of multi-enzyme clusters.

Pairwise protein association of the dhurrin enzymes was monitored in *N. benthamiana* by FLIM with eGFP and mRFP1-labeled proteins. FRET percentages reflecting the proximity and the frequency of association between protein constructs were calculated from the recorded eGFP lifetime. Error bars indicate ±SD. The effect of co-expression of the third dhurrin enzyme was determined by FRET measurements between **(A)** CYP79A1-CYP79A1, **(B)** CYP71E1-CYP71E1, **(C)** UGT85B1-UGT85B1, **(D)** CYP79A1-CYP71E1,

(E) CYP79A1-UGT85B1 and CYP71E1-UGT85B1 and **(F)** POR2b-P450. **(G)** Model of the dhurrin metabolon involving higher order clusters and enrichment of negatively charged phospholipids.

Supplementary Materials:

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Materials and Methods

Figs. S1-S10

Tables S1-S14

Movies S1-S2

Database S1-S6, as separate Excel files

References (18-30)

Author contributions.