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Tat proteins as novel thylakoid membrane anchors organize a biosynthetic pathway in chloroplasts and increase product yield 5-fold

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ABSTRACT

Photosynthesis drives the production of ATP and NADPH, and acts as a source of carbon for primary metabolism. NADPH is also used in the production of many natural bioactive compounds. These are usually synthesized in low quantities and are often difficult to produce by chemical synthesis due to their complex structures. Some of the crucial enzymes catalyzing their biosynthesis are the cytochromes P450 (P450s) situated in the endoplasmic reticulum (ER), powered by electron transfers from NADPH. Dhurrin is a cyanogenic glucoside and its biosynthesis involves a dynamic metabolon formed by two P450s, a UDP-glucosyltransferase (UGT) and a P450 oxidoreductase (POR). Its biosynthetic pathway has been relocated to the chloroplast where ferredoxin, reduced through the photosynthetic electron transport chain, serves as an efficient electron donor to the P450s, bypassing the involvement of POR. Nevertheless, translocation of the pathway from the ER to the chloroplast creates other difficulties, such as the loss of metabolon formation and intermediate diversion into other metabolic pathways. We show here that co-localization of these enzymes in the thylakoid membrane leads to a significant increase in product formation, with a concomitant decrease in off-pathway intermediates. This was achieved by exchanging the membrane anchors of the dhurrin pathway enzymes to components of the Twin-arginine translocation pathway, TatB and TatC, which have self-assembly properties. Consequently, we show 5-fold increased titers of dhurrin and a decrease in the amounts of intermediates and side products in *Nicotiana benthamiana*. Further, results suggest that targeting the UGT to the membrane is a key factor to achieve efficient substrate channeling.

1. Introduction

Metabolic engineering has become an attractive alternative to synthetic chemistry due to the promise of producing a range of compounds, from high-value specialty compounds such as therapeutics to bulk commodities including plastics and biofuels, in a cheap and renewable manner. Many such specialty compounds are plant natural products whose biosynthesis requires enzymes termed cytochromes P450 (P450s). These are heme-containing monooxygenases that catalyze regio- and stereo-specific hydroxylations often difficult to perform by chemical synthesis. In eukaryotes, P450s localize to the endoplasmic reticulum (ER) and require a NADPH-dependent reductase to provide electrons as reducing power (Lindberg Møller, 2014; Nielsen et al., 2016; Lassen et al., 2014a).

Dhurrin (*D*-glucopyranosyloxy-(S)-*p*-hydroxymandelonitrile) is a cyanogenic glucoside used as a defense compound by *Sorghum bicolor* and has served as a model pathway to study the coupling of heterologously expressed P450s to photosynthetic electron transport (Nielsen et al., 2013; Gnanasekaran et al., 2016a; Wlodarczyk et al., 2016; Lassen et al., 2014b; Gangl et al., 2015). The pathway consists of two ER membrane-bound cytochrome P450 enzymes (CYP79A1 and CYP71E1) and a soluble UDP-glucosyltransferase (UGT85B1), which catalyzes the step-wise conversion of tyrosine to dhurrin (Fig. 1a). CYP79A1 converts L-tyrosine to *(Z)*-*p*-hydroxyphenylacetaldoxime (oxime), which is converted into the cyanohydrin *p*-hydroxymandelonitrile (nitrile) by CYP71E1. Lastly, UGT85B1 stabilizes the nitrile by glucosylation to yield dhurrin (Sibbesen et al., 1995; Kahn et al., 1997; Jones et al., 1999). Since the nitrile is labile at neutral and alkaline pH it dissociates into HCN and *p*-hydroxybenzaldehyde (aldehyde) if not rapidly glucosylated (Gleadow and Møller, 2014). These enzymes were recently shown to form a dynamic metabolon together with the cytochrome P450 oxidoreductase (POR), that efficiently

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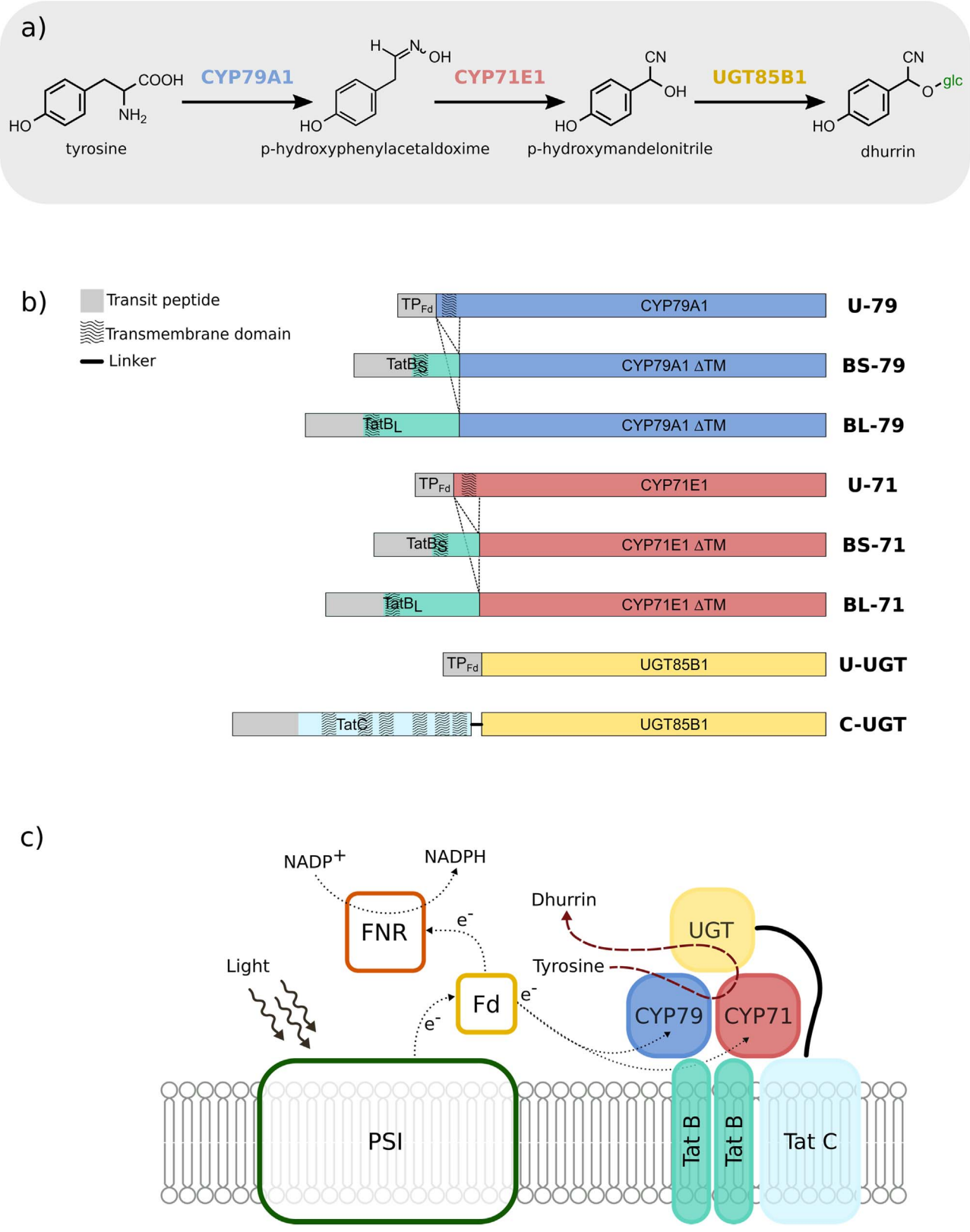

Fig. 1. Schematic representation of the dhurrin pathway, the fusion enzymes engineered and its organization in the thylakoids. **(a)** The dhurrin pathway consists of two membrane-bound P450s (CYP79A1 and CYP71E1) and a soluble UDP-glucosyl transferase (UGT85B1). **(b)** Construct design of fusion enzymes generated. The transit peptide from ferredoxin (TP_{Fd}) was fused to the cDNA encoding the native enzymes from *S. bicolor*: CYP79A1 (U-79), CYP71E1 (U-71) and UGT85B1 (U-UGT). Further the P450s without their transmembrane domains (CYP79A1: Δ1-105 and CYP71E1: Δ1-111) were fused to two different lengths of the cDNA encoding the membrane spanning and interacting part of the TatB protein from *A. thaliana:* TatB short – Δ151-260 (TatBS) and TatB long – Δ220-260 (TatBL). The UGT was fused to the cDNA encoding TatC from *A. thaliana* and a 15-GS linker was added between TatC and the UGT to ensure some flexibility. The natural N-terminal P450s transmembrane domain was removed in the fusion proteins since the Tat proteins contain endogenous transmembrane domains. **(c)** Schematic representation of the Tat-scaffolded dhurrin pathway in the thylakoid membrane in proximity of the highly abundant photosystem I complex. PSI, photosystem I; Fd, ferredoxin; FNR, ferredoxin-NADPH reductase.