Universitat Autònoma de Barcelona

Feedback regulation of the methylerythritol 4-phosphate (MEP) pathway

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PhD thesis

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Dissertation presented by Xueni Di for the degree of Doctor of Plant Biology and Biotechnology at Autonomous University of Barcelona. This work was developed in the Centre for Research in Agricultural Genomics and Institute for Plant Molecular and Cellular Biology.

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Acknowledgements

My four years PhD life is like four seasons. At the beginning of the PhD, it is like the winter that working in a totally new country and with so much unfamiliar knowledge makes me nervous. While, my supervisor Manuel, post doctor Jordi and my kind lab mates like fire warm me and build my confident. During my second year of PhD, I keep a lot of hope just like spring. Even though the experiments didn't work at most time. I know that this is the problem that every PhD student need to overcome and I tried my best to win. Then the hot summer is coming. The students with 'ice-cream' (published papers) make me jealous and antsy. Luckily, the work goes successfully, and I start the virtuous circle. Successful work giving me passion to think and work hard, and then the hard work bring more good results. How time flies, after sowing in spring, carefully cultivate during summer, harvest autumn is coming!

During my undergraduate and master study, I joined a lot of national and international activities and won many awards, excepted for doing experiments. Therefore, I want to calm down and only do the research during the PhD. And the I do enjoy a lot during my phd life. I can clearly feel my progress. The third year of my PhD is the most enjoyable time in my life. I happen can come up new idea for the work and find the method to solve the problem. The meeting with Manuel is the happiest time for me. I always can get positive response from Manuel, and it makes me more confident to thinking. Furthermore, Manuel's suggestion always brings me to the new level. I totally lost myself in the research that year. And I sincerely thank the help from Manuel. He makes me confident to research and more determined to be a scientist!

Research integrity is always the important thing for the researchers. As a PhD student at very early stage of research, I am very careful about it. When I worked on the experiments of chapter 3, I got totally opposite results as we expect. My heart was

struggled, should I consider these opposite results was because of the mis order of samples and then continue prove our hypothesis or admit our expect is wrong. With a restless heart, I carefully repeated the experiment and got the same results as before. Our hypothesis was wrong. I checked relative article and thought about it day and night and finally fond possible reason. When I showed the data to Manuel, his words made relax. He told me that I did right, and not all experiments is same as we expect. In this case, what we need to do is to understand/explore why it happen like this. In the end, we build a very nice model by using different methods. Maybe it is very little story, but it cheers me to respect the experiment results. I hope in the future, when I read this story again, I am still the people respect all experiment results.

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Summary

Isoprenoids are one of the largest families of metabolites and they have a huge interest in the pharma, biofuel and agrofood industries. All isoprenoids derive from isopentenyl diphosphate (IPP) and its highly electrophilic double-bond isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP are derived from the mevalonic acid (MVA) pathway in archaea and the cytosol of eukaryotic cells, whereas most bacteria and the plastids of plant cells produce IPP and DMAPP by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Compared with the MVA pathway, the MEP pathway has a higher theoretical capacity to produce isoprenoid precursors, which has stimulated extensive research on further improving MEP pathway flux for the biotechnological production of isoprenoids of interest in microbial and plant biofactories. However, feedback regulation of the MEP pathway flux by IPP and DMAPP slows down the pathway flux as soon as IPP and DMAPP levels increase. IPP/DMAPP were reported to decrease the levels and activity of the first enzyme of the MEP pathway, deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS), but the specific mechanism was unknown. On the other hand, the possible inhibition of other MEP pathway enzymes by IPP/DMAPP was unexplored. The main objective of this thesis was to address these open questions using *Escherichia coli* and tomato (*Solanum lycopersicum*) as bacterial and plant models, respectively.

Unlike *E. coli*, which only has a single DXS-encoding gene, three genes encode DXS-like proteins in tomato. In the first part of the thesis we determined that isoforms SlDXS1 and SlDXS2 are true DXS enzymes that localize together in plastids and can form heterodimers whereas SlDXS3 lacks DXS activity possibly due to impaired binding of the cofactor thiamine diphosphate (TPP). In the second chapter of the thesis, we demonstrate that IPP and DMAPP allosterically inhibit the activity of bacterial (*E.coli*) and plant (tomato) DXS enzymes EcDXS and SlDXS1 by directly binding away from the active site of the enzyme. IPP and DMAPP binding was found to promote monomerization of the active DXS dimer. DXS monomers expose hydrophobic domains that are otherwise hidden in the dimer and can cause their aggregation and eventual degradation. We propose that DXS monomerization might be a relatively fast and reversible response to high IPP/DMAPP levels, whereas monomer aggregation and removal would represent a slow and irreversible response when excess IPP/DMAPP persists. In the third and last chapter part of the thesis, we investigate the possible effect of IPP and DMAPP on the activity of the next enzyme of the MEP pathway, DXS reductoisomerase (DXR), and a DXR-like (DRL) enzyme that replaces DXR in some bacteria. We found that IPP/DMAPP also feedback inhibit DXR activity (but not DRL). Similar to that described for DXS, regulation of DXR activity by IPP/DMAPP might have two phases. Competition of IPP/DMAPP with the DXR cofactor NADPH cofactor for binding to the active site of the enzyme would define the first phase, whereas the observation that DMAPP may also allosterically inhibit DXR by monomerization of the active dimer might represent a second (slow and non-reversible) phase. These results help us to understand the complexity of the feedback regulation network that operates at several steps of the MEP pathway and represent a solid foundation to eventually improving MEP pathway flux.

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List of abbreviations

AACT	Acetyl-CoA acetyltransferase
ATP	Adenosine triphosphate
CASP8	Critical Assessment of techniques for protein Structure Prediction
CDP-ME	4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol
CDP-MEP	4-Diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate
CFP	Cyan fluorescent protein
CLZ	Clomazone
CMK	CDP-ME kinase
CRTISO	Prolycopene isomerase
DLS	Dynamic light scattering
DMAPP	Dimethylallyl diphosphate
dpi	Days post-inoculation
DRL	DXR-like
DXP	deoxyxylulose 5-phosphate
DXR	DXP reductoisomerase
DXS	DXP synthase
e.g.	Exempli gratia (“for example”)
FAD	Flavin-adenine dinucleotide
FPP	Farnesyl diphosphate
FSM	Fosmidomycin
GAP	Glyceraldehyde-3-phosphate
GAP	Glyceraldehyde-3-phosphate
GFP	Green fluorescent protein

GGPP	Geranylgeranyl diphosphate
GMQE	Global Model Quality Estimate
GPP	Geranyl diphosphate
HDR	HMBPP reductase
HDS	HMBPP synthase
HMBPP	4-hydroxy-3-methylbut-2-en-1-yl diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
hpi	Hours post-infiltration
i.e.	Id est (‘that is’)
IDI	Isopentenyl diphosphate isomerase
IP	isopentenyl phosphate
IPK	IP kinase
IPP	Isopentenyl diphosphate
ITC	Isothermal titration calorimetry
kDa	Kilodalton
LB	Luria-Bertani
MCT	MEP cytidylyltransferase
MDS	MEcPP synthase
MEcPP	2-C-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	Methylerythritol 4-phosphate
MVA	Mevalonic acid
MVD	MVPP decarboxylase
MVK	MVA kinase
MVP	5-phospho-MVA
MVPP	5-diphospho-MVA

NF	Norflurazon
OD	Optical density
PMK	MVP kinase
PMVD	MVP decarboxylase
PYR	pyruvate
RFP	Red fluorescent protein
rpm	Revolutions per minute
SDGs	Sustainable Development Goals
TPP	Thiamine diphosphate

General introduction

General Introduction

0.1 Sustainable development

The United Nations Sustainable Development Goals (SDGs) were released in 2015, identifying 17 SDGs and 169 targets to drive progress towards a sustainable future for humanity and the planet for the next 15 years (Conference & Goals, 2015). In 2019, some achievements had been completed but others remain challenging, including the lack of food security. In this regard, an ambitious action has to be carried out, starting from 2020, to complete those goals in the rest decade (Fontana & Oldekop, 2020; Lalitha & Radhakrishnamurty, 2020).

0.1.1 Food nutritional quality

SDG2 and SDG3 are, respectively, 'Ending hunger, improving food security and nutrition, promoting sustainable agriculture' and 'Ensuring healthy lives and promoting life quality of well-being' (Assembly & Goals, 2015). Apart from the lack of food supply, a major problem is the issue of wide-scale malnutrition due to restricted access to healthy food, e.g., by the explosion of population or by unhealthy food habits (Tripathi et al., 2018). Food security is not only about the adequacy of food calories, but also about the availability of a proper combination and balance of carbohydrates, proteins and fats. In addition, diverse essential micronutrients, such as zinc, iron, iodine, vitamins, and minerals are required for people's health. Currently, 2 billion people in the world are suffering from micronutrient deficiencies, which could even lead to fatality (Singh et al., 2017; Godfray et al., 2010; Tripathi et al., 2018). Increased prevalence of overweight, obesity, coronary heart disease, stroke and diabetes, due to poor diet quality, results in an even higher death rate than alcohol, drug and tobacco abuse combined (Willett et al., 2019).

Plants can be used as ‘green’ biological factories to produce food, feed, medicines, and biomaterials (Nogueira et al., 2018). Besides, micronutrients (vitamins, minerals), fibers and a wide array of phytochemicals in fruits and vegetables play a significant positive role in people’s health (Yahia et al., 2018). Indeed, epidemiological studies show that a plant-based diet helps to reduce the risk of diabetes, chronic diseases and cancer. People with high uptake of vegetables and fruits in their diet have only one-half of the cancer risk than those who take lower (Singh et al., 2009; Singh et al., 2014). In this regard, from the promotion of a healthy life’s point of view, enriching plant-derived food products with micronutrients becomes a promising solution to alleviate the malnutrition issue, and therefore reduce the risk of chronic diseases.

0.1.2 Sustainable and clean energy

With the fast development of society, the depleting natural resources and the degrading environmental quality put our planet at risk. Based on the abovementioned situation, SDG7 aims at ’ Ensuring access to affordable, reliable, sustainable and modern energy for all people’. Building an economic world that is free of reliance on petroleum derivatives and on hurtful arrangements and practices is an appealing topic (Haines, 2021). Bioethanol, the most utilized biofuel created by organisms, could be a promising solution (Azhar et al., 2017). However, most of the currently commercialized bioethanol is currently derived from corn or sugarcane feedstocks, which requires agrarian land and therefore undermines food security. Biodiesel, as the predominant renewable fuel for transportation in Europe, is derived from the transesterification of vegetable oils. Nevertheless, the production of such biodiesel also requires farming land that could somehow be utilized to develop food crops (Walls & Rios-Solis, 2020). In order to meet the goals of sustainable development, the development of new biofuels is a must. In this context, growing research interests have been focused on the investigation of natural isoprenoids with medium chain length and fatty acid-derived hydrocarbons as alternative biofuels (Jiménez-Díaz et al., 2017).

0.2 Isoprenoid nutrients and chemicals

Isoprenoids (also called terpenoids) are a family with more than 50,000 identified compounds, which makes them one of the largest and most structurally diverse groups of natural products (Kemper et al., 2017). They are present in archaea, bacteria, plants, animals and fungi, playing a wide diversity of biological functions. According to the number of carbons of their biosynthetic precursor, isoprenoids can be classified as hemiterpenoids (derived from precursors with 5 carbons), monoterpenoids (10 carbons), sesquiterpenoids (15 carbons), diterpenoids (20 carbons), triterpenoids (30 carbons) and tetraterpenoids (40 carbons). Lots of isoprenoids have a huge potential for practical applications in pharmaceutical treatments (paclitaxel and artemisinin), food, feed and cosmetics (carotenoids, tocopherols and coenzyme Q10), or biofuels (farnesene and pinene), among others (Vickers et al., 2017).

0.2.1 Isoprenoids benefit health

Isoprenoids, as natural products, can have biological activities in the counteraction and treatment of human diseases (Wang et al., 2005). They show biological activities such as anti-inflammatory, anti-aggregatory, anti-oxidative, anti-tumor, anti-coagulative effects, analgesic and sedative (Zhao et al., 2016). These compounds are able to balance blood coagulation and hemostasis, immunostimulants, and boost antioxidant activity to benefit human health by interacting with key molecular players in animal and human physiology (Nuutinen, 2018; Wagner & Elmadfa, 2003). The diterpenoid drug Taxol (used as an anticancer drug) and artemisinin (used as an antimalarial) are two well-known examples (Tetali, 2019).

In addition to its huge potential in the pharmaceutical industry, isoprenoids have great benefits for human health as health-promoting phytonutrients. Carotenoids like β-carotene, lycopene, lutein, β-cryptoxanthin, and zeaxanthin are natural pigments showing a color range from yellow to red, but they are also powerful natural antioxidants. Carotenoids with unsubstituted β-rings are also precursors of an important micronutrient: vitamin A. Moreover, dietary carotenoid products can also prevent diseases like type-2 diabetes, cancer, obesity and other age-related diseases, and help maintain cardiovascular health (Rodriguez-Concepcion et al., 2018). Tocopherols (vitamin E) are also a group of plant isoprenoids with antioxidant properties that are strongly recommended in the human diet. Therefore, improving the production of the abovementioned nutrients as well as other health-related isoprenoids in crop plants such as rice, maize, and tomato should substantially contribute to improve our diet and hence our life quality (Nogueira et al., 2018).

0.2.2 Isoprenoids as clean energy

Looking for advanced alternative biofuels is meaningful for sustainable development. The compact cyclic structures of isoprenoids give them great potential as high-density fuels (Walls & Rios-Solis, 2020). Being potential alternatives to petroleum-based fuels, some monoterpenes and sesquiterpenes have attracted a huge interest (Peralta-Yahya et al., 2012). However, the complex chemical structures of these compounds complicate the use of conventional organic synthesis strategies for their production at commercial scale with a competitive cost. On the other hand, the production of such compounds in plants requires a long growth cycle and is susceptible to environmental influences (Tippmann et al., 2013; Walls & Rios-Solis, 2020). Using microbial cell bio factories to produce heterologous isoprenoids with properties as renewable feedstocks can be a promising strategy to provide a sustainable solution at a relatively low cost.

0.3 IPP and DMAPP

0.3.1 Prenyldiphosphates

Isopentenyl diphosphate (IPP, $C5H12O7P2$) and its highly electrophilic double-bond isomer dimethylallyl diphosphate (DMAPP) (Figure 0.1) are the 5-carbon (C5) building blocks of all isoprenoids. Addition of different number of IPP molecules to a DMAPP core creates prenyldiphosphates of increasing size which are the starting points for the biosynthesis of isoprenoids (Mcgarvey & Croteau’, 1995; Luthra et al., 1999; Wang et al., 2019). Thus, one IPP and one DMAPP produce geranyl diphosphate (GPP), the precursor for the formation of C10 monoterpenoids. Two IPP and one DMAPP molecules form farnesyl diphosphate (FPP) and one more IPP results in geranylgeranyl diphosphate (GGPP). FPP and GGPP are the precursors of C15 sesquiterpenoids and C20 diterpenoids, respectively. Two FPP molecules condense head-to-head to form squalene, a precursor of C30 triterpenoids (including sterols). Phytoene is a precursor of C40 tetraterpenoids (carotenoids) formed by the head-to-head condensation of two molecules of GGPP.

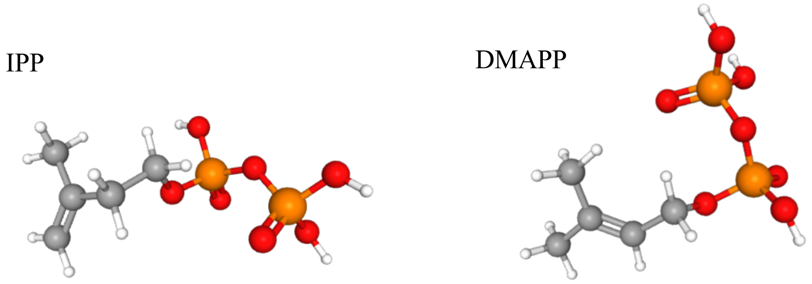

Figure 0. 1. Chemical structure model of IPP and DMAPP. The carbon atoms are shown as gray balls, the phosphate atoms are shown as orange balls, the oxygen atoms are shown as red balls and the hydrogen atoms are shown as white balls (PubChem).

0.3.2 IPP and DMAPP biosynthetic pathway

In archaea and the cytosol of eukaryotic cells, IPP is derived from the mevalonic acid (MVA) pathway, and then MVA-derived IPP is isomerized to DMAPP by isopentenyl diphosphate isomerase (IDI). In most bacteria and the plastids of plant cells, IPP and DMAPP are simultaneously produced by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Rodríguez-Concepción & Boronat, 2002; Rohdich et al., 2003). While archaea, fungi and animals normally use the MVA pathway to synthesize their isoprenoid precursors, plants use the MVA pathway in the cytosol and the MEP pathway in plastids (Figure 0.2) . Bacteria often use the MEP pathway, but there are many exceptions. A few bacteria use the MVA pathway instead of the MEP pathway, whereas others possess the two full pathways, and some parasitic strains lack both the MVA and the MEP pathways (probably because they obtain their isoprenoids from host cells). The astonishing metabolic plasticity of microorganisms has also led to shunt pathways and alternative enzymes to the canonical reactions of the MVA and MEP pathways (Erb et al., 2012; Pérez-Gil & Rodríguez-Concepcíon, 2013).

The MVA pathway is a six-step reaction pathway starting from acetyl-CoA (Figure 0.2). Acetyl-CoA acetyltransferase (AACT) is a class II thiolase that condenses two acetyl-CoA molecules into acetoacetyl-CoA. Then acetoacetyl-CoA with another acetyl-CoA molecule is converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase (HMGS). MVA is eventually produced via HMG-CoA in two reduction steps catalyzed by the enzyme HMG-CoA reductase (HMGR) with the participation of NADPH as the reductant. HMGR is considered the main rate-controlling enzyme of the MVA pathway (Rodríguez-Concepción & Boronat, 2015). Two different classes of HMGR have been described: class I HMGR enzymes are found predominantly in archaea and eukaryotes, whereas bacteria usually possess HMGR class II enzymes (Pérez-Gil & Rodríguez-Concepcíon, 2013). In the following steps of the pathway, MVA is then phosphorylated in two successive phosphorylation steps catalyzed by MVA kinase (MVK) and 5-phospho-MVA (MVP) kinase (PMK). IPP is produced by the ATP-dependent decarboxylation of 5-diphospho-MVA (MVPP) catalyzed by MVPP decarboxylase (MVD). IPP which can be further isomerized to DMAPP by IDI. In some organisms, these last steps of the MVA are different (Pérez-Gil & Rodríguez-Concepcíon, 2013). In particular, some archaea have an MVP decarboxylase (PMVD) enzyme that catalyzes the transformation of MVP to isopentenyl phosphate (IP), which then is converted into IPP by the enzyme IP kinase (IPK) (Grochowski et al., 2006). Two types of IDI enzymes are found to interconvert IPP and DMAPP. Type I IDI is present in bacteria and eukaryotic organisms (including plants) and it requires a divalent metal cofactor for activity, while type II IDI is found in archaea and bacteria and it requires reduced flavin and divalent metal cofactors for activity (Rohdich et al., 2004).

The MEP pathway involves seven enzyme-catalyzed steps (Figure 0.2). The C1 aldehyde group of glyceraldehyde-3-phosphate (GAP) and pyruvate are used to produce deoxyxylulose 5-phosphate (DXP) by DXP synthase (DXS) with the release of CO_2 (Lois et al., 1998; Lange et al., 1998; Sprenger et al., 1997). DXS is considered the main rate-controlling enzyme of the MEP pathway (Rodríguez-Concepción & Boronat, 2015b). In the following step of the pathway, DXP is intramolecularly rearranged and reduced to MEP in a reaction catalyzed by DXP reductoisomerase (DXR) (Lange & Croteau, 1999; Schwender et al., 1999; Takahashi et al., 1998). MEP is subsequently converted to 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME) and phosphorylated to 2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-MEP) by the enzymes MEP cytidylyltransferase (MCT) and CDP-ME kinase (CMK), respectively. CDP-MEP is subsequently converted to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) by MEcPP synthase (MDS). MEcPP is further reduced to 4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP) by HMBPP synthase (HDS). In the last step of the MEP pathway, the enzyme HMBPP reductase (HDR) transform

HMBPP into a mix of IPP and DMAPP with an approximate ratio of 5:1 (Rodríguez-Concepción & Boronat, 2002; Eisenreich et al., 2004; Bouvier et al., 2005;). Similar to that described for the MVA pathway, shunt pathways and alternative enzymes have been proposed for some steps of the MEP pathway (Pérez-Gil & Rodríguez-Concepcíon, 2013).For example, DXP can be produced from *S*-adenosylmethionine in the photosynthetic proteobacterium *Rhodospirillum rubrum* (Benjamin et al., 2012; Warlick et al., 2012) whereas some bacteria lack a canonical DXR enzyme and utilize a different protein named DXR-like (DRL) to catalyze the conversion of DXP into MEP (Sangari et al., 2010).

Compared with the MVA pathway, the MEP pathway has a higher theoretical capacity for the production of isoprenoid precursors, which has stimulated extensive research on further improving MEP pathway flux in microbial and plant systems (Rude & Schirmer, 2009; Vickers et al., 2015). However, the most successful approaches for the production of isoprenoid end-products have been achieved by improving the supply of IPP and DMAPP by using the MVA pathway whereas engineering the MEP pathway has provided only partial success. The production of industrially relevant compounds from MEP-derived precursors is still far from the predicted maximum yields of the pathway.

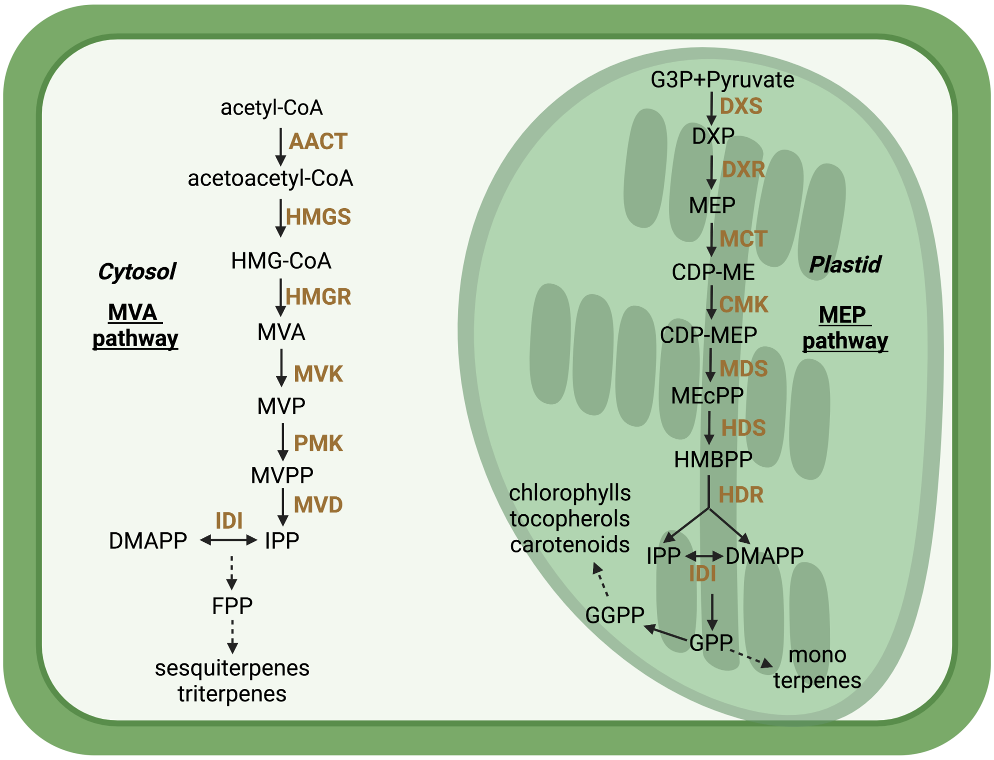

Figure 0. 2. Schematic representation of the main pathways that lead to the synthesis of isoprenoid precursors. Abbreviations are spelled out in the text.

0.4 Key enzymes in the MEP pathway

0.4.1 DXS

As the first enzyme in the MEP pathway, a variety of research indicates that DXS catalyzes the main rate limiting step of the pathway. Metabolic control analyses have shown that no other MEP pathway enzyme has a higher flux control coefficient in bacteria or plant systems (Volke et al., 2019; Wright & Phillips, 2014). In agreement with this central role in the regulation of the MEP pathway flux, the level of plastidial isoprenoid end-products can be changed by overexpression or reduction of DXS levels (Enfissi et al., 2005; Estévez et al., 2001; Morris et al., 2006). Endogenous DXS activity is regulated at multiple levels, including transcriptional, and post-translational (Rodríguez-Concepción & Boronat, 2015). When high MEP-derived IPP and DMAPP synthesis is demanded, e.g., for a peak production of carotenoids, the transcripts encoding DXS accumulate in deetiolating seedlings of *Arabidopsis thaliana* (Estevez et al., 2000), and ripening fruit of pepper and tomato (Bouvier et al., 1998; Enfissi et al., 2005). DXS activity is also tightly regulated at the post-translational level (Banerjee & Sharkey, 2014; Hemmerlin, 2013). The activity of the DXS enzyme is inhibited by IPP and DMAPP (Banerjee et al., 2013; Ghirardo et al., 2014). and the level of DXS protein increases when the levels of IPP and DMAPP decrease, e.g., after blocking of the MEP pathway (Guevara-García et al., 2005; Han et al., 2013) or consumption by downstream pathways (Ghirardo et al., 2014; Rodríguez-Villalón et al., 2009). In Arabidopsis, the J-protein J20 was found to bind to inactive forms of DXS and deliver them to degradation by the stromal Clp protease (Flores-Pérez et al., 2008; Pulido et al., 2013; Zybailov et al., 2009). However, whether the post-translational feedback inhibition by IPP and DMAPP influence DXS protein stability is still unknown (Rodríguez-Concepción & Boronat, 2015).

While bacteria typically contain a single DXS-encoding gene, plants usually have small gene families that produce several DXS proteins which can be grouped in three distinct classes: class 1, 2 and 3 (Cordoba et al., 2011; Saladié et al., 2014; Walter et al., 2002). However, not all plants have DXS isoforms from these three clases. For example, *Arabidopsis thaliana* contains three DXS-encoding genes (two of class 1 and one of class 3) but only the class 1 *DXS/CLA1* gene has been shown to encode an active DXS (Phillips et al., 2008). The tomato genome includes one gene per class (Walter et al., 2015). Enzymes belonging to class 1 are mostly classified as housekeeping. They include Arabidopsis DXS/CLA1 and tomato DXS1. Consistently, loss of these enzymes in Arabidopsis and in tomato cause a lethal albino phenotype (García-Alcázar et al., 2017; Mandel et al., 1996). In contrast, class 2 proteins are typically involved in the synthesis of specific (or secondary) isoprenoids involved in defense responses and signaling (Walter et al., 2002). Arabidopsis lacks a class 2 DXS enzyme, whereas the tomato DXS2 is mostly found in trichomes of leaves and petals (Paetzold et al., 2010). Class 3 proteins are considered to lack functional DXS activity because they miss critical amino acids required to bind thiamine diphosphate (TPP), a cofactor required for DXS activity. Phylogenetic studies suggest that class 3 DXS isoforms emerged later than class 1 and class 2, whereas co-expression analysis of the Arabidopsis *DXS3* gene showed association to genes involved in post-embryonic development and reproduction instead of metabolism or isoprenoid synthesis (Luna-Valdez et al., 2021).

DXS is a highly conserved dimeric protein in bacteria and plants. DXS protein monomers contains three domains (I, II, and III), which are homologous to the equivalent domains in related TPP-dependent enzymes such as transketolase and the E1 subunit of pyruvate dehydrogenase. However, DXS has a unique arrangement among these three domains, as its active site is located at the interface of domains I and II in the same monomer. The enzymatically active protein is a dimer in which domain I of one monomer is directly located above domains II and III of the same monomer and two monomers with the same structure are arranged side by side (Figure 0.3). In the dimer, the TPP cofactor is buried in the active site with the C-2 atom of its thiazolium ring exposed to a pocket that is the substrate-binding site (Xiang et al., 2007).

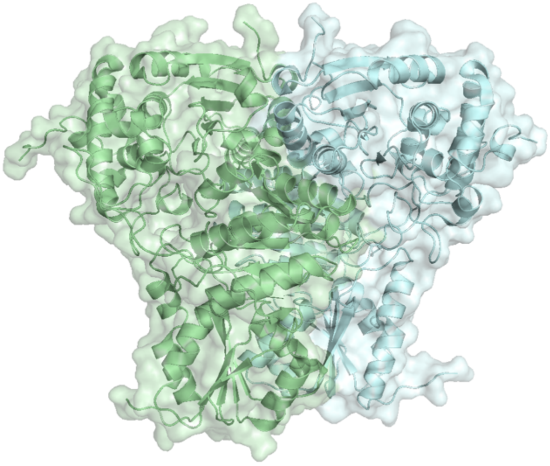

Figure 0. 3. Crystal structure of DXS from *E.coli.* The two monomers that form the active dimer are distinguished by color (green and blue). Image generated from structural data available at protein data bank.

0.4.2 DXR

DXR is the second enzyme in the MEP pathway, converting DXP into MEP (Figure 0.2). Because DXP is a precursor for the production of TPP and pyridoxal phosphate in bacteria (Julliard & Douce, 1991), DXR is actually considered the first committed step of the MEP pathway. However, the degree of DXR control over the metabolic flux of the MEP the pathway is still unclear. DXR acts as a rate-limiting enzyme in some plants and tissues but not in others. For instance, in *Arabidopsis thaliana*, overexpression of DXR increases the levels of plastidial isoprenoids (chlorophylls, carotenoids and taxadiene) (Carretero-Paulet et al., 2006). In peppermint, overexpression of DXR also increases essential oil isoprenoids (Mahmoud & Croteau, 2001). However, in tomato fruits, neither the transcription level nor the protein level of DXR is affected during carotenoid accumulation (Rodríguez-Concepción et al., 2001).

DXR protein is classified as a class B dehydrogenase that uses NADPH as a cofactor and contains three domains: (i) a large N-terminal NADPH-binding domain which exhibits an α/β topology with a seven-stranded parallel β-sheet and seven α-helices, (ii) a connective or linker domain which comprises five helices and a four-stranded β-sheet with one parallel and two antiparallel alignments, and (iii) a smaller C- terminal alpha-helical domain which is a four-helix bundle and is joined to the connective domain by an extended loop. The active enzyme is a dimer with crystal symmetry (Sweeney et al., 2005) (Figure 0.4).

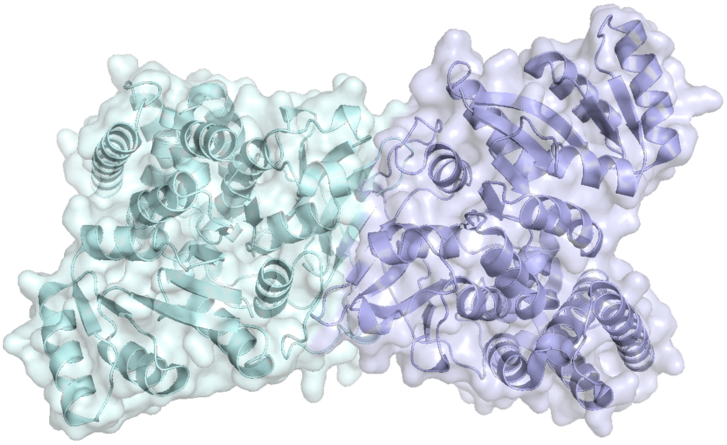

Figure 0. 4. Crystal structure of DXR from *E.coli.* The monomers in the active dimer molecule are distinguished by color (blue and purple). Image generated from structural data available at (Yajima et al., 2002).

A few bacteria (such as some *Brucella* and *Bartonella* strains) lack a canonical DXR enzyme and utilize a different protein named DXR-like (DRL)to catalyze the conversion of DXP into MEP (Sangari et al., 2010). The presence of DRL enzymes in bacteria lacking the MEP pathway suggests that their initial activity is independent of isoprenoid metabolism (Sangari et al., 2010). DRL belongs to an uncharacterized protein family predicted to have oxidoreductase features with sequence similarity to DXR only in the NADPH-binding domain. Furthermore, the active sites arrangement is different between DXR and DRL. As a consequence, the compound fosmidomycin (FSM) inhibits DXR but has little effect on DRL activity in vitro (Pérez-Gil et al., 2012; Pérez-Gil & Rodríguez-Concepcíon, 2013).

0.5 The research and industry value of tomato and *E.coli*

Tomato (*Solanum lycopersicum* L.) has a huge importance for the food industry because of its fruits, which contain several health-promoting compounds (including vitamins, carotenoids, and phenolics) (Martí et al., 2016) and can be commercialized in a variety of forms (e.g., as soups, juices, ketchup or in fresh) (Krauss et al., 2005; Y. Li et al., 2018). Besides, tomato is widely used as a model plant system due to features like fleshy fruit, compound leaves and sympodial shoots, which distinguishes it from other model plants (*Arabidopsis* or rice) (Kimura & Sinha, 2008). Furthermore, research in tomato can easily be applied to other Solanaceae plants (including potato, pepper, tobacco and eggplant) because they are closely related.

Escherichia coli (E.coli) is universally used as a model organism in molecular biology and biochemistry. Because of its rapid growth, easy genetic manipulation, and its relative metabolic simplicity, it is also popular as microbiological bio factory to produce isoprenoids of interest such as carotenoids or biofuels. Unfortunately, the supply of IPP and DMAPP via its native MEP pathway is typically insufficient for commercially-relevant applications because of the tight regulation of this pathway (Walls & Rios-Solis, 2020). Significantly improved isoprenoid synthesis has been achieved by using heterologous MVA pathway to supply IPP and DMAPP (Rodríguez-Villalón et al., 2008; Ward et al., 2018). However, the metabolic burden resulting from multigene expression needs to be considered (Liu et al., 2019). Hence, mechanisms such as the feedback regulation of the MEP pathway should be better understood to use the endogenous bacterial (and plant) pathway to overproduce isoprenoids meeting the industry requirements.

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Objectives

Objectives

IPP and DMAPP are the C5 building blocks required for the biosynthesis of all isoprenoids, a family of metabolites with high commercial value and health-related properties. In most bacteria and the plastids of plants, IPP and DMAPP are simultaneously produced by the MEP pathway. Biotechnological approaches directed towards increasing the production of isoprenoids of interest by up-regulating the MEP pathway to supply more IPP and DMAPP precursors have obtained results that are still far from the predicted maximum yields of the pathway. One of the main drawbacks to successfully increase MEP-derived supply of IPP and DMAPP is the self-repression of the pathway due to feedback regulation by its products, IPP/DMAPP. At the start of this PhD, it was known that this feedback regulation occurred at the level of DXS, the first and main flux-controlling enzyme of the MEP pathway. Specifically, it was reported that increased IPP/DMAPP levels were able to inhibit DXS enzyme activity and lead to reduced DXS protein levels. However, the specific mechanism connecting changes in IPP/DMAPP levels with altered DXS levels and activity was unknown. On the other hand, the possible inhibition of other MEP pathway enzymes by IPP/DMAPP had not been tested. The main objective of this thesis was to address these open questions using *Escherichia coli* and tomato (*Solanum lycopersicum*) as bacterial and plant models, respectively. To achieve this goal, we proposed two main objectives:

Objective 1: To reveal the molecular mechanism behind the feedback regulation of DXS by IPP/DMAPP. DXS is encoded by a single gene in *E. coli* but three genes in tomato. We proposed to systematically characterize the tomato DXS family and select the best candidate for subsequent studies with this protein and the bacterial enzyme to analyze the possible binding of IPP/DMAPP and the consequences eventually causing a reduced DXS activity *in vivo* and *in vitro*.

Objective 2: To investigate the feedback regulation of other MEP pathway enzymes by IPP/DMAPP. Because DXR can be considered as the first enzyme completely exclusive of the MEP pathway, we used the *E. coli* and tomato DXR enzymes and the DXR-like protein from *Brucella abortus* to test their possible inhibition by IPP/DMAPP and eventually the underlying mechanism.

Chapter 1

Chapter 1. Exploring the function of the three tomato DXS isoforms

1.1 Introduction

The enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS) is the first enzyme of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which produces IPP and DMAPP precursors for plastidial isoprenoids such as monoterpenes, carotenoids, chlorophylls, tocopherols, phylloquinone and plastoquinone. In tomato, DXS is encoded by 3 different genes encoding isoforms SIDXS1 (Solyc01g067890), SIDXS2 (Solyc11g010850), and SIDXS3 (Solyc08g066950) with 719, 714 and 709 amino acid residues, respectively. They belong to DXS class 1 (SlDXS1), class 2 (SlDXS2) and class 3 (SlDXS3). Class 1 and 2 are thought to be true DXS enzymes. Class 1 are considered housekeeping isoforms required to produce essential isoprenoids such as photosynthetic pigments, whereas class 2 isoforms are also expressed in tissues harboring non-photosynthetic plastids and induced in response to environmental (biotic and abiotic) cues, being required for the synthesis of specific isoprenoids involved in defense responses and stress signaling (Walter et al., 2002). Class 3 proteins are considered to lack DXS function.

The primary structures of class 1 and 2 DXS enzyme are highly conserved between species and there is no biochemical difference in the function of the two isoforms (Walter et al., 2002). Complementation of *Escherichia coli* mutants defective in DXS activity demonstrated that both SIDXS1 and SIDXS2 are enzymatically active (Paetzold et al., 2010). However, they play very different physiological roles. Mutants defective in SlDXS1 exhibit albinism and are unable to develop, indicating a non-redundant role of SIDXS2 and SIDXS3 and supporting a major role for SlDXS1 in the production of essential, photosynthesis-related isoprenoids (García-Alcázar et al., 2017). SlDXS1 also supports the production of carotenoids that takes place during fruit ripening (Lois et al., 2000). *SlDXS2* transcripts show a much more restricted distribution than those encoding SlDXS1 as they are mainly found in trichome-rich organs, especially in leaves and fully open flowers (Paetzold et al., 2010). Mutants defective in SlDXS2 are not available yet, but the partial silencing of *SlDXS2* expression in tomato by RNAi resulted in decreased levels of the monoterpene β-phellandrene and increased level of two sesquiterpenes in trichomes (Paetzold et al., 2010). Because monoterpenes derive from the plastidial MEP pathway but sesquiterpenes usually derive from the cytosolic mevalonic acid (MVA) pathway, it was proposed that SlDXS2 might somehow alter ratios of MEP to MVA pathway allocation. No experimental data are available for SlDXS3.

Here we aimed at systematically characterizing the three tomato DXS isoforms, SlDXS1, SlDXS2 and SlDXS3, in the same set of experiments to better understand their function. In particular, we carried out a unified and complete analysis of their expression profiles, DXS activity and subcellular localization. In addition, we explored possible interactions between isoforms by forming heterodimers.

1.2 Results

1.2.1 Genes for tomato DXS isoforms are differentially expressed

To investigate the expression patterns of *SlDXS1*, *SlDXS2* and *SlDXS3* in tomato, the gene expression profiles of the three genes were obtained from the eFP browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi) (Fig. 1.1). *SlDXS1* is highly expressed in all tomato organs and tissues, including roots, leaves, flowers and fruits (particularly at the later stages of ripening). In contrast, *SlDXS2* is mainly expressed in flowers and leaves. *SlDXS2* transcripts are also present in fruit but only during the early stage of their development. *SIDXS3* maintains low expression level in all organs and tissues (Fig. 1.1a).

In order to further investigate the spatial expression of the three tomato DXS-encoding genes in fruit, data from different fruit tissues were obtained from the tomato expression atlas (http://tea.solgenomics.net/). The expression level of *SIDXS1* is similar to that of *SIDXS2* during early stages of fruit ripening, while the expression level of *SIDXS3* is much lower (Fig.1.1). The transcript level of *SIDXS1* increases in most fruit tissues but not in seeds during ripening (Fig.1.1b), whereas *SlDXS2* transcripts decrease in most fruit tissues but increase in seeds during ripening (Fig.1.1c). Unlike the other two isoforms, *SIDXS3* is expressed at similar level in seeds and other tissues during fruit ripening (Fig.1.1d). These results suggest isoform-specific roles of these isoforms in different tissues and particularly during fruit ripening and seed development.

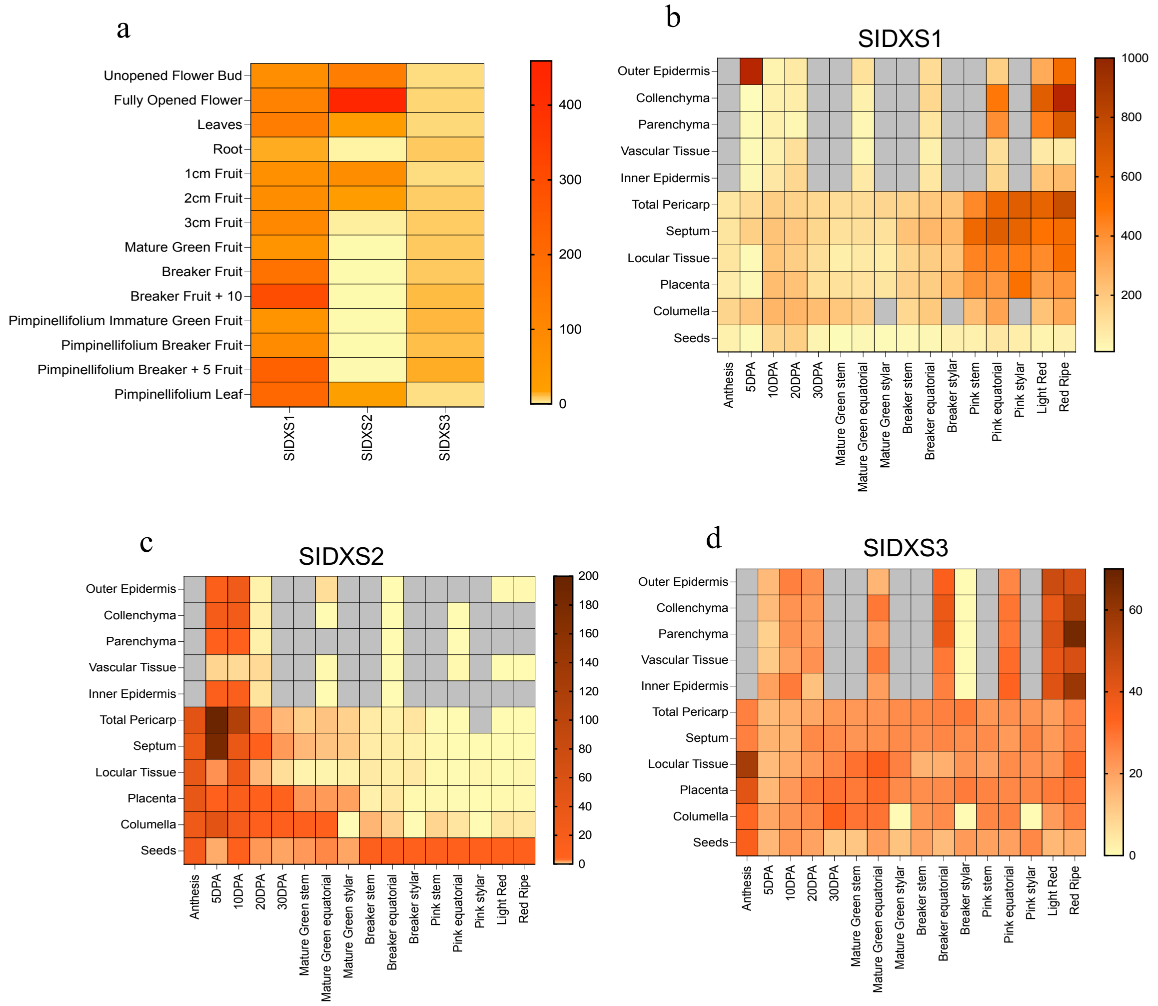

Figure 1. 1. Expression profiles of *SIDXS1, SIDXS2* and *SIDXS3* genes. **(a)** Transcript levels of the three SIDXS isoforms in different organs of the tomato cultivar M82. **(b) to (c)** Transcript levels of *SIDXS1* (b), *SIDXS2* (c) and *SIDXS3* (d) in different fruit tissues of the tomato cultivar M82.

1.2.2 SIDXS1 and SIDXS2 are true DXS enzymes

SlDXS1, SIDXS2 and SIDXS3 protein sequences were analyzed with *Target P (*https://services.healthtech.dtu.dk/service.php?TargetP-2.0*)*, a predictor of subcellular localization, to estimate the length of their putative plastid-targeting sequence. Sequences lacking the predicted plastid-targeting peptides were amplified from a tomato ripe fruit cDNA library using gene-specific oligonucleotides. Then the amplified *SlDXS1, SlDXS2, SlDXS3* cDNAs were cloned in a pBluescript vector under the control of the T7 promoter to test the enzymatic activity of the three isoforms by complementing the *dxs*-deficient *E.coli* strain EcAB4-2 (Sauret-Güeto et al., 2006). The genome of this strain harbors a synthetic mevalonate (MVA) operon that allows bypassing the lethal disruption of the DXS gene by producing IPP and DMAPP when MVA is supplied to the growth medium (Fig. 1.2). The transformed strains were grown in LB media with and without MVA. All transformed strains can grow when MVA is supplied because the MVA operon converts it into IPP and DMAPP, hence allowing the survival and growth of the cells. However, when MVA is not provided, the *dxs*-deficient strain can only grow when a functional DXS enzyme is present in the supplied construct (Fig. 1.2a). The EcAB4-2 strain transformed with the *E. coli* gene encoding DXS (*EcDXS*), *SlDXS1* and *SlDXS2* can survive when MVA is not supplied, while the strain transformed with *SlDX3* and empty vector cannot survive without MVA (Fig. 1.2b). These results show that EcDXS, SlDXS1 and SlDXS2 have DXS activity, but SlDXS3 doesn't.

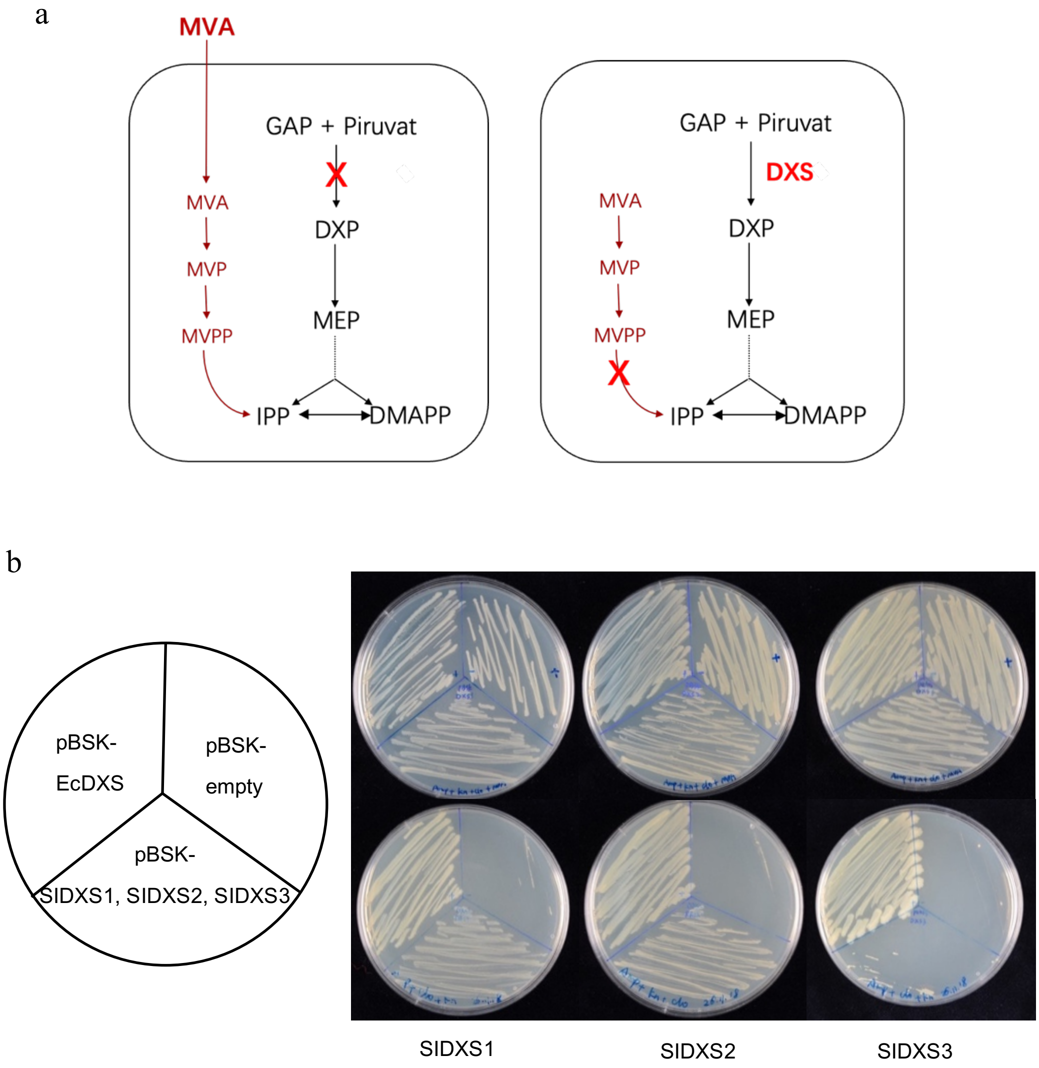

Figure 1. 2. DXS complementation analysis. (a) Model of complementation of the DXS deficient *E.coli* strain EcAB4-2. **(b)** Growth of EcAB4-2 cells transformed with the indicated constructs on LB plates supplemented either with or without MVA.

1.2.3 Modeling of SIDXS1, SIDXS2 and SIDXS3 protein structures

The class 3 DXS proteins are thought to lack functional DXS activity because key amino acid positions are not conserved in the thiamine pyrophosphate (TPP) binding pocket (Luna-Valdez et al., 2021). However, no experimental proof is available because it is difficult to obtain DXS protein crystals. Conformational changes during the catalytic cycle (Decolli et al., 2019; Zhou et al., 2017) and proteolytic degradation (Altincicek et al., 2000; Gierse et al., 2021; Song et al., 2007) led to a low number of available DXS crystal structures. We decided to exploit the available structural information for comparative protein structure modeling (Eswar et al., 2008; Pieper et al., 2006). We used the cryo-electron microscopy structure of the *A. thaliana* DXS protein (7bzx.1.A) (Yu et al., 2021) as a template to compare the 3-dimensional structures of SIDXS1, SlDXS2 and SlDXS3 using SWISS-MODEL (https://swissmodel.expasy.org/) based on the target-template alignment using ProMod3 (Table 1.1 and Fig 1.3). Similar coverage to the target sequence was observed for three SIDXS proteins: 0.92 (72-706) for SIDXS1, 0.91 (68-702) for SIDXS2 and 0.92 (69-696) for SIDXS3 (Fig 1.3a). However, the sequence identity of SIDXS1, SIDXS2 and SIDXS3 with the template is 88.75%, 73.93% and 58.24%, respectively, illustrating their diversity. The Ramachandran plot, which represents the energetically favored regions for backbone dihedral angles against of amino acid residues in protein structure, showed values of 88.63%, 87.28% and 86.18% for SIDXS1, SIDX2 and SIDXS3, respectively (Table 1.1 and Fig 1.3b). The Global Model Quality Estimate (GMQE), which evaluates the combined properties from the target-template alignment and the template structure, was 0.72, 0.70 and 0.65 for SIDXS1, SIDXS2 and SIDXS3, respectively, showing a good reliability of the prediction (Table 1.1).

Table 1. 1 Results of protein modeling by Swiss-model

Gene	Template	Sequence Identity	Coverage	Range	Ramachandran Favoured	QMEAN	QMEANDisCo Global
SIDXS1	7bzx.1. A	88.75%	0.92	72-706	88.13%	0.72	0.73±0.05
SIDXS2	7bzx.1. A	73.93%	0.91	68-702	87.28%	0.70	0.72±0.05
SIDXS3	7bzx.1. A	58.24%	0.92	69-696	86.18%	0.65	0.68±0.05

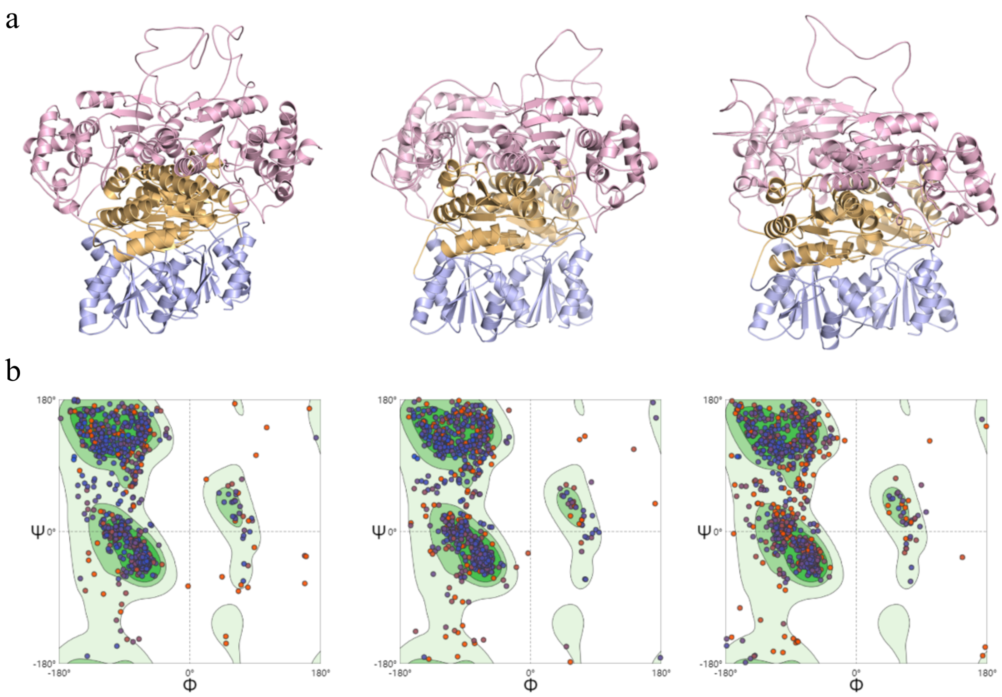

Figure 1. 3. Modeling of tomato DXS protein structures. **(a)** SlDXS1, SlDXS2 and SlDXS3 protein structures modeled by Swiss-Model. The three domains in the monomers are distinguished by colors: pink for domain I, orange for domain II and purple for domain III. **(b)** The Ramachandran plots of SlDXS1, SlDXS2 and SlDXS3.

Interaction of DXS proteins with their ligands (such as substrates or cofactors) is necessary for their enzymatic activity. In order to identify ligand-binding sites in SlDXS1, SlDXS2 and SlDXS3, the three protein models were uploaded to the 3D Ligand Site (https://www.wass-michaelislab.org/3dlig/index.html) web server, which is one of the top-performing for the prediction of ligand-binding sites according to the Critical Assessment of techniques for protein Structure Prediction (CASP8) (Wass & Sternberg, 2009). Based on the prediction, SlDXS1 and SlDXS2 have three binding sites which involve the thiamine diphosphate (TPP) cofactor, pyruvate (PYR) substrate and ion ligands (Fig. 1.4). However, SlDXS3 has four binding sites, which involve flavin-adenine dinucleotide (FAD), PYR and ion ligands. In particular, sodium (Na^+) and potassium (K^+) are predicted ligands at the first binding site of all three DXS proteins, whereas magnesium (Mg^{2+}), calcium (Ca^{2+}) and manganese (II) (Mn^{2+}) are ion ligands at the second binding site of SlDXS1. Only Mg^{2+} and Ca^{2+} binding was predicted with SlDXS2 at the second binding sites and SlDXS3 at the third binding sites, respectively. In contrast, FAD and PYR are ligands at the second binding site of SlDXS3 with Glu253, Pro249, Asn241, Tyr145, Val118, Thr117, Pro111 residues. Highly conserved residues participate in TPP binding (His, Asp, Ile, Glu, and Phe) in the active site of DXS enzymes from *E. coli*, *D. radiodurans*, and *A. thaliana* (Xiang et al., 2007). The same conserved residues are found in SlDXS1 (His145, Asp245, Ile447, Glu449 and Phe474) and SlDXS2 (His141, Asp241, Ile443, Glu445 and Phe470) at the third binding site, with TPP, PYR and sulfate (SO_4^{2-}) as ligands (Fig. 1.4). However, neither conserved residues nor predicted TPP binding site could be found in the SlDXS3 protein. Chloride (Cl^-) and PYR, rather than TPP, are the predicted ligands at the fourth binding site of SlDXS3 with Glu252, Arg549, Val251, Glu253, Glu420 and Lys250 residues. Based on the prediction of binding sites, the active site of SlDXS3 is not conserved and cannot bind the TPP ligand. This might be the main reason to explain SlDXS3 lacking DXS activity. Based on these results, we focused on SlDXS1 and SlDXS2 for the rest of experiments.

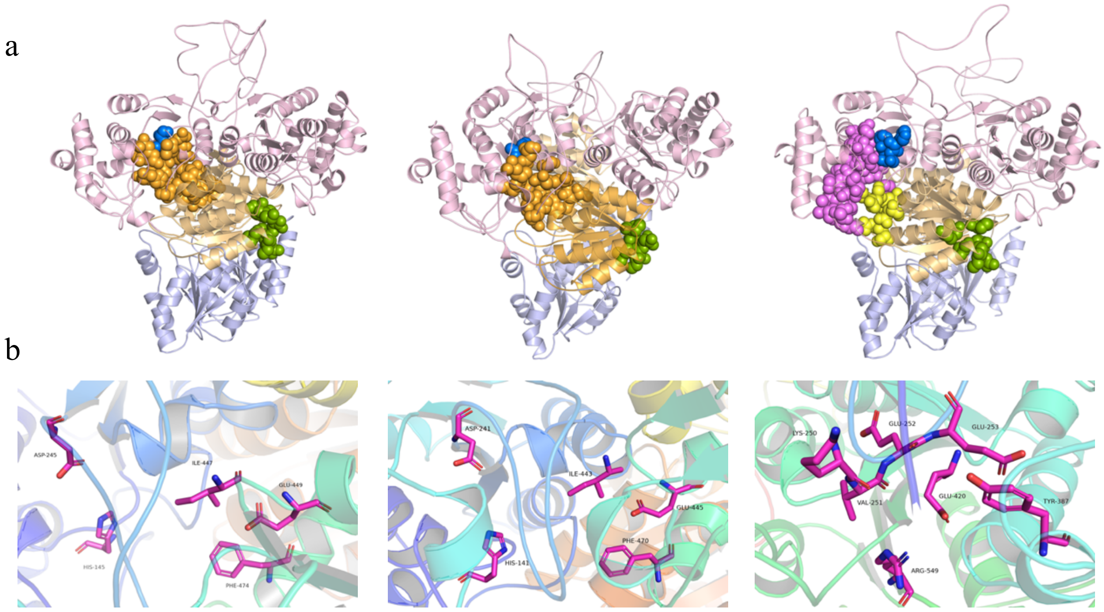

Figure 1. 4. Ligand binding sites predicted for DXS protein. **(a)** The binding sites are represented in different colors. The blue ball indicates the second cluster; The orange ball indicates the third cluster (TPP, PYR, SO_4^{2-} binding); The green ball indicates the first cluster (Na^+, K^+ binding); The purple and yellow ball from SlDXS3 indicates the second and fourth clusters (FAD and PYR binding). **(b)** The conserved residues at the TPP binding site of SlDXS1 and SlDXS2 and the residues at the PYR binding site of SlDXS3.

1.2.4 SlDXS1 and SlDXS2 can form heterodimers

To investigate the subcellular localization of SlDXS1 and SlDXS2, the full-length tomato DXS proteins fused to a RFP (*35S:SlDXS1-RFP*) or GFP (*35S:SlDXS2-GFP*) at the C-terminus were transiently expressed by agroinfiltration in *Nicotiana benthamiana* leaves (Fig. 1.5). Agroinfiltrated leaves were used for direct observation under a confocal microscope to identify the fusion proteins based on their fluorescence. Both fusion proteins were localized in chloroplasts and showed a spotted distribution similar to that of the GFP-tagged Arabidopsis DXS protein, DXS-GFP (Pulido et al., 2013, 2016). Furthermore, the fluorescence signals from SlDXS1-RFP and SlDXS2-GFP fusion proteins were observed to overlap (Fig. 1.5a), suggesting that they might be forming heterodimers.

To confirm whether SlDXS1 and SlDXS2 could physically interact, co-immunoprecipitation assays were next performed (Fig. 1.5b). Instead of fluorescent proteins that are known to dimerize themselves, for this experiment we fused the tomato isoforms to smaller tags that could be recognized using commercial antibodies. Constructs with SlDXS1 harboring a Myc tag (SlDXS1-Myc) and SlDXS2 with a hemagglutinin (HA) tag (SlDXS2-HA) at their C-terminal ends were co-expressed in *N. benthamiana* leaves. As a control, we used a Myc-tagged version of Arabidopsis phosphoribulokinase, a stromal enzyme of the Calvin cycle (PRK-Myc) (Barja et al., 2021). After immunoprecipitation with an anti-Myc antibody, samples were analyzed by immunoblot analysis using anti-Myc and anti-HA antibodies. The results conclusively showed that SlDXS2-HA could be co-immunoprecipitated using SlDXS1-

Myc but not with PRK-Myc, suggesting that SIDXS1 and SIDXS2 can form complexes *in vivo* (Fig1.5b)

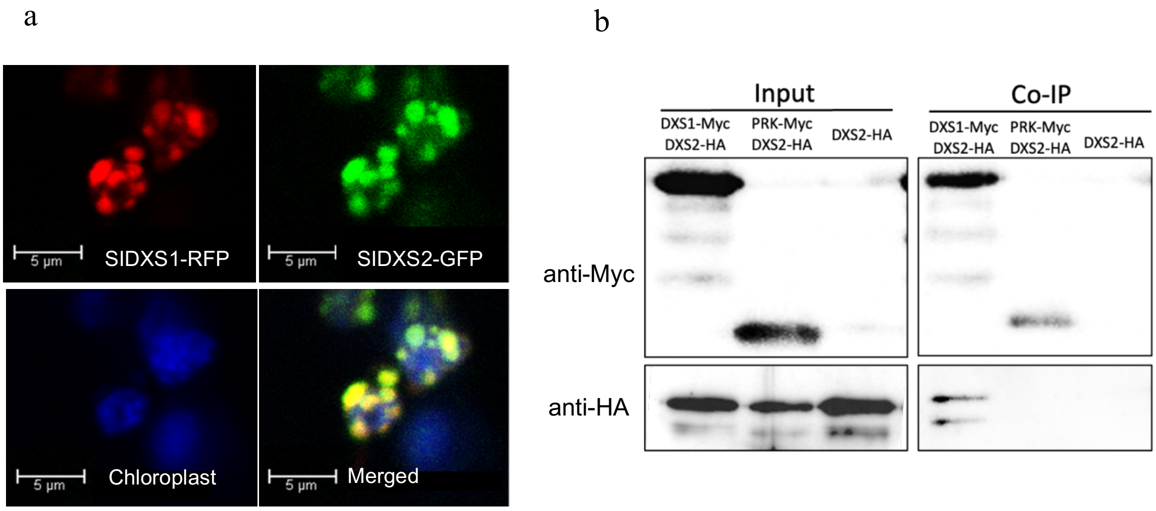

Figure 1. 5. Interaction of SIDXS1 and SIDXS2. (a) Representative confocal microscopy images of a *N. benthamiana* leaf cell transiently co-expressing SIDXS1-RFP and SIDXS2-GFP fusion proteins. Red autofluorescence from RFP, green fluorescence from GFP, blue autofluorescence from chlorophyll and merged channels are shown. **(b)** Co-immunoprecipitation of SIDXS1-Myc and SIDXS2-HA. *N. benthamiana* leaves were co-agroinfiltrated with the indicated proteins. A fraction of the protein extracts (Input) was used to test protein production using immunoblot analyses with antibodies against Myc or HA. After immunoprecipitation (Co-IP) of the remaining protein extracts using anti-Myc, samples were used for immunoblot analyses with anti-Myc (to confirm successful immunoprecipitation) and anti-HA (to detect the presence of co-immunoprecipitated HA-tagged proteins).

In order to confirm whether SlDXS1 and SlDXS2 might form enzymatically active heterodimers, the corresponding monomers were first computationally separated from their dimer structure by pymol and then they were used to create a virtual heterodimer with ZDOCK SERVER (https://zdock.umassmed.edu/). The interaction structure model with the highest docking score (2619.573) was selected and then further analyzed with Pymol 2.3.0 and LIGPLOT 2.2.4. According to the Zdock result, the monomers of SIDXS1 and SIDXS2 are arranged side by side to form a heterodimer that shows the same structure than SlDXS1 or SlDXS2 homodimers (Fig.1.6). The residues around the protein-protein interaction interface can form numerous hydrophobic interactions and hydrogen bonds that help stabilize the protein-protein complex (Fig.1.6).

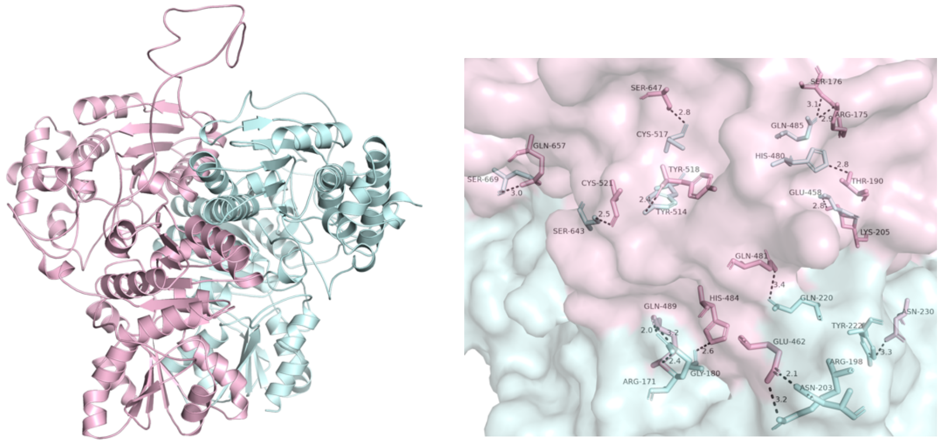

Figure 1. 6. SIDXS1 and SIDXS2 heterodimer model. SIDXS1 monomer is shown in pink and SIDXS2 monomer in blue. Strong hydrogen bonds forming at the interface of the two monomers are shown in the magnification.

1.3 Discussion

SIDXS1, *SIDXS2* and *SIDXS3* genes in tomato encode two DXS isoforms with specific features (SlDXS1 and SlDXS2) and another protein with unknown function (SlDXS3). SIDXS1 appears to act as a housekeeping isoform to contribute to phytohormone and photosynthetic pigment synthesis and hence it is expressed at higher levels than the other two genes in most tissues of the tomato plant (Fig. 1.1). Its strong upregulation in the pericarp and other fruit tissues besides seeds during ripening is consistent with a predominant participation of this isoform in the massive production of carotenoids that changes the fruit color from green to red (Lois et al., 2000). By contrast, the more restricted distribution of transcripts for SIDXS2 suggests more specialized roles for this isoform. Indeed, SlDXS2 was found to be required for the synthesis of specific isoprenoids involved in defense responses and signaling (Paetzold et al., 2010)(Walter et al., 2002). The increasing expression of *SlDXS2* in seeds as they mature (a process occurring concomitantly with fruit ripening) suggests that this isoform might also provide precursors for isoprenoid production in seed or/and embryonic tissues. SIDXS3 expresses at very low-level during tomato plant growing and ripening (Fig. 1.1). Consistent with our conclusion that SIDXS3 is not a true DXS (Fig. 1.2), DXS3 proteins from Arabidopsis, maize, and rice have also been found to lack functional DXS activity in complementation assays like the one used in our work (Luna-Valdez et al., 2021).

DXS enzymes convert glyceraldehyde-3-phosphate (GAP) and PYR to DXP with a loss of CO_2 in a reaction that requires a divalent cation (Mg^{2+} or Mn^{2+}) and uses TPP as the cofactor at the active site (Lois et al., 1998; Lange et al., 1998; Sprenger et al., 1997). The residues in the active site are strictly conserved in *E. coli/ D. radiodurans* and *A. thaliana* DXS sequences (His80/82/146, Asp152/154/208, Ile368/371/448, Glu370/373/450/418, and Phe395/398/475, respectively). We found that loss of TPP binding might explain the lack of DXS activity in SlDXS3 (Fig. 1.4), as previously suggested (Luna-Valdez et al., 2021). According to the 3D Ligand Site prediction, the TPP ligand binding site can be found only in SIDXS1 and SIDXS2 with the conserved residues of His 145/141, Asp 245/241, Ile447/443, Glu449/445, and Phe 474/470, respectively. Both SIDXS1 and SIDXS2 were confirmed to be located in chloroplasts, where the MEP pathway takes place (Fig. 1.5a). Moreover, the subplastidial localization of SIDXS1 and SIDXS2 was found to be the same as the fluorescence from fusion proteins to RFP and GFP showed a clear overlapping (Fig. 1.5a). Co-immunoprecipitation assays further showed that SIDXS1 and SIDXS2 can form complexes *in vivo* (Fig. 1.5b), whereas structure modeling confirmed that SIDXS1 and SIDXS2 monomers could form heterodimers similar to DXS homodimers hence catalytically active (Fig. 1.6). The potential biological function of such heterodimers is difficult to predict. In the tomato plant, SlDXS1 and SlDXS2 transcripts are found in many tissues, but sometimes they show opposite accumulation profiles (e.g. during fruit ripening and seed development). Interestingly, they are expressed at similar levels in young (1-2cm) fruit (Fig. 1.1), suggesting that SIDXS1-SIDXS2 heterodimers might be preferentially formed at phases of rapid growth. It is also possible that SIDXS3 may interact with SIDXS1 or SIDXS2 to form SIDXS1-SIDXS3 or SIDXS2-SIDXS3 heterodimers that might contribute to decrease the DXS activity of SlDXS1 and SlDXS2 isoforms. However, the virtually constitutive profile and low expression level of *SlDXS3* argues against a major regulatory role for this protein.

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Chapter 2

Chapter 2. Allosteric feedback inhibition of DXS involves monomerization of the active dimer

2.1 Introduction

Isoprenoids (also known as terpenoids or terpenes) are a vast family of natural compounds produced in all free-living forms of life. Despite their astonishing variety both at the structural and functional levels, all isoprenoids derive from the universal building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In nature, two different pathways are responsible for the synthesis of IPP and DMAPP (Rodríguez-Concepción & Boronat, 2015). The mevalonate (MVA) pathway is present mainly in eukaryotes and archaea and uses acetyl-CoA as an initial substrate whereas the methylerythritol 4-phosphate (MEP) pathway is found in most bacteria and produces IPP and DMAPP from pyruvate and glyceraldehyde 3-phosphate (GAP). In plants both pathways coexist but in different subcellular compartments. The MVA pathway produces IPP and DMAPP in the cytosol to synthesize sterols and sesquiterpenes, whereas the MEP pathway is located in plastids and produces the precursors of plastidial isoprenoids such as monoterpenes, carotenoids, and the side chains of chlorophylls, tocopherols, phylloquinones and plastoquinone.

The first step of the MEP pathway is catalyzed by the enzyme deoxyxylulose 5-phosphate (DXP) synthase (DXS), which uses pyruvate and GAP to generate DXP. DXP is converted into MEP by the next enzyme of the pathway, DXP reductoisomerase (DXR). Metabolic control analyses showed that DXS is the enzyme with the highest flux control coefficient of the MEP pathway and consequently the primary rate-limiting step of the pathway both in bacteria and plants (Volke et al., 2019; Wright et al., 2014).

Consistent with this central role in the regulation of pathway flux, DXS activity is regulated at multiple levels including transcriptional, post-transcriptional and post-translational (Pokhilko et al., 2015; Pulido et al., 2013, 2016; Rodriguez-Concepcion et al., 2019; Yu et al., 2021). Several reports have highlighted the importance of the metabolic regulation of the MEP pathway (Banerjee & Sharkey, 2014; Pokhilko et al., 2015; Rodríguez-Concepción & Boronat, 2015). Labeling experiments suggested a negative feedback regulation of plant DXS activity by MEP pathway products, IPP and DMAPP (Wolfertz et al., 2004), which was later confirmed both *in vitro* and *in vivo* (Aparajita Banerjee et al., 2013, 2016; Wright et al., 2014). Interestingly, MEP pathway flux has been observed to also modulate DXS protein levels in plants (Ghirardo et al., 2014; Han et al., 2013; Pokhilko et al., 2015). The feedback regulation of DXS levels and activity was shown to stabilize the plant pathway flux against changes in substrate supply and adjust it according to product demand under normal growth conditions (Pokhilko et al., 2015). More recently, *in vitro* assays have shown a similar feedback regulation for several bacterial DXS enzymes (Kudoh et al., 2017), but *in vivo* confirmation is still missing.

The initial crystallization of a truncated *Escherichia coli* DXS (Song et al., 2007) was recently followed by reported structures of DXS enzymes from another bacterium, *Deinococcus radiodurans* (Chen et al., 2019), and from the model plant *Arabidopsis thaliana* (Yu et al., 2021). From these studies it was concluded that the active enzyme is a dimer and that a highly hydrophobic surface is present in the interface of the two monomers. Computational analysis of the monomer structure suggested that these hydrophobic domains increase the aggregation propensity of the protein (Pulido et al., 2016). Arabidopsis DXS was actually found to easily aggregate, leading to subsequent degradation by the plastidial Clp protease complex. When stress conditions compromise the proteolytic capacity of the plastid, however, disaggregation rather than degradation is promoted for spontaneous refolding and reactivation of DXS

(Pulido et al., 2013, 2016). The high aggregation propensity of DXS was also confirmed in cyanobacteria (Kudoh et al., 2017) and *E. coli* (Kudoh et al., 2017), whereas the involvement of the Clp protease in DXS degradation has also been described in tobacco *(Nicotiana tabacum)* leaves (Moreno et al., 2018), tomato *(Solanum lycopersicum)* fruit (D'Andrea et al., 2018), the malaria parasite *Plasmodium falciparum* (Florentin et al., 2017) and *E. coli* (Ninnis et al., 2009).

Considering (a) that reduced IPP and DMAPP levels result in higher DXS activity and protein levels, (b) that DXS activity requires dimerization, (c) that monomers expose aggregation-prone hydrophobic domains, and (d) that aggregation normally leads to DXS degradation, we hypothesized that high IPP or DMAPP levels might displace the equilibrium towards the monomeric (i.e., inactive) conformation of the enzyme. If sustained, high IPP and DMAPP would lead to DXS monomer aggregation and eventual degradation. Here we tested this hypothesis using bacterial (*E. coli)* and plant (tomato) DXS enzymes.

2.2 Results

2.2.1 IPP/DMAPP inhibit EcDXS activity *in vivo*

Feedback regulation of DXS by MEP pathway products (IPP/DMAPP) has been described both *in vitro* and *in vivo* in plants (Banerjee et al., 2013; Wolfertz et al., 2004; Wright et al., 2014), but only *in vitro* in bacteria (Kudoh et al., 2017). To validate the effect of IPP and DMAPP on the activity of *E. coli* DXS (EcDXS) *in vivo,* we used engineered strains harboring a synthetic MVA operon that allows the production of IPP and DMAPP when MVA is supplied to the growth medium (Sauret-Güeto et al., 2006). To accurately detect changes in DXS activity, we used the EcAB4-10 strain, which lacks DXR and hence it is unable to convert DXP into MEP and downstream IPP and DMAPP (Sangari et al., 2010; Sauret-Güeto et al., 2006). Survival of EcAB4-10 cells was allowed by supplementing the growth medium with MVA, which was converted into IPP and DMAPP by the synthetic MVA operon (Sauret-Güeto et al., 2006). EcAB4-10 cells were grown at 37°C in LB medium in the presence of 0.5 or 10 mM MVA and collected during the exponential phase to ensure steady-state conditions. Intracellular metabolites were then extracted and measured by LC-MS. IPP and DMAPP were quantified together due to the technical difficulty of separating these double-bond isomers. As expected, increasing amounts of intracellular MVA were measured in MVA-supplemented cultures, demonstrating the uptake of the molecule from the medium (Fig. 2.1). Also as expected, IPP/DMAPP levels were much increased in cells growing with 10 mM MVA compared to those growing with 0.5 mM MVA (Fig. 2.1). By contrast, DXP levels were much lower with 10 mM MVA, which is consistent with the conclusion that enhanced IPP/DMAPP levels cause a reduction of EcDXS activity in living cells (Fig. 2.1). It is important to note that EcAB4-10 cells growing with 10 mM MVA did not show any growth impairment compared to those growing with 0.5 mM MVA (Fig. 2.1), suggesting that the amounts of MVA-derived IPP/DMAPP were not reaching the very high levels previously reported to trigger toxicity (Bongers et al., 2020; George et al., 2018). Taken together, these results confirm that the negative feedback mechanism reported to control the activity of DXS also operates *in vivo* to decrease the activity of the bacterial EcDXS enzyme when IPP/DMAPP levels are increased.

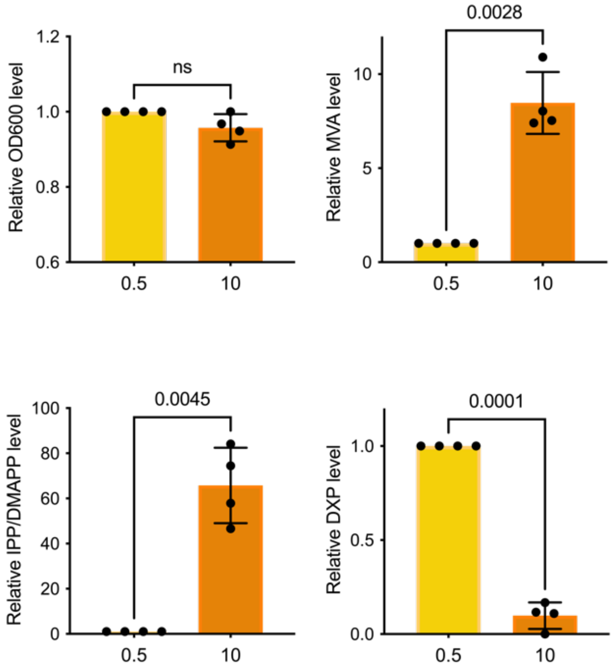

Figure 2. 1. Metabolite levels in MVA-supplemented EcAB4-10 cells. *E. coli* cells of the EcAB4-10 strain were grown in the presence of 0.5 or 10 mM MVA and collected during exponential phase (steady-state) for metabolite extraction and quantification by LC-MS. Bar plots represent relative values of cell growth measured by its optical density at 600 nm (OD600) and intracellular levels of MVA, DXP and IPP/DMAPP. Dots represent individual values. Mean and standard deviation of the n=4 replicates are shown. The p values of Student t-test analyses are also indicated.

2.2.2 IPP/DMAPP directly interact with DXS

Based on kinetic analyses and structural modeling, it was suggested that IPP and DMAPP might inhibit the activity of DXS by competing with its cofactor, thiamine diphosphate (TPP), for its binding site (Banerjee et al., 2013, 2016). However, direct evidence is missing. In order to experimentally confirm this model, the interaction of DXS with IPP and DMAPP was assessed by isothermal titration calorimetry (ITC). Specifically, we tested purified 6xHis tagged versions of the full-length EcDXS protein or a truncated version of the tomato SlDXS1 isoform lacking the N-terminal plastid targeting peptide. However, the purified EcDXS protein was found to rapidly aggregate in solution and therefore we decided to only use the plant SlDXS1 enzyme for the ITC experiments together with TPP, IPP or/and DMAPP as ligands. Based on the thermograms obtained and the corresponding binding isotherms using 20 µM SlDXS1 in the sample cell solution and 200 µM of the ligand in the syringe solution, we confirmed the interaction with TPP, as expected, with a dissociation constant (Kd) of 4.2 µM (Table 2.1 and Fig. 2. 2). IPP and DMAPP were also found to interact with the plant enzyme, showing similar dissociation constants (Kd of 2.2 µM for IPP and 1.7 µM for DMAPP). Similar Kd values were also found when the SlDXS1 protein was preincubated with 100 µM TPP in the sample cell (Kd of 1.2 µM for IPP and 4.3 µM for DMAPP), suggesting the absence of competition for the TPP binding (i.e. active) site and consequently the existence of an unrelated binding site for IPP/DMAPP (Table 2.1 and Fig. 2. 2).

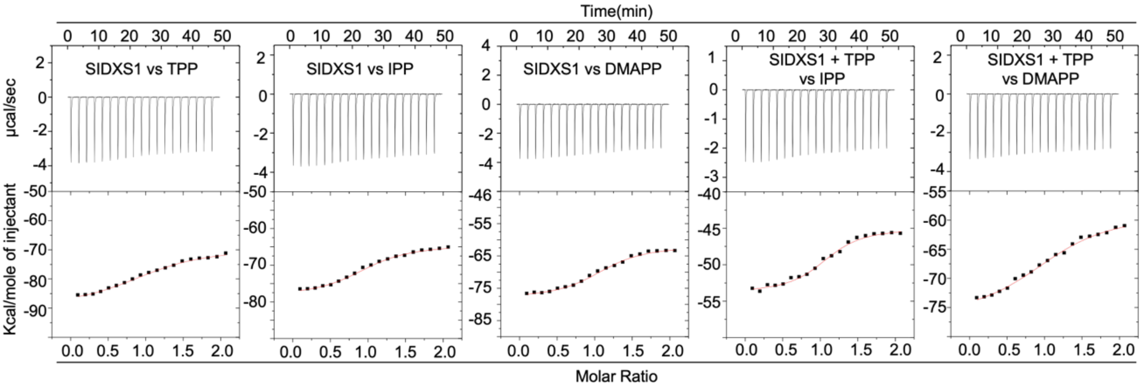

Figure 2. 2. Analysis of SlDXS1 interaction with metabolite ligands. ITC plots were obtained from the titration of 20 µM SlDXS1 (either alone or with 100 µM TPP) with 200 µM TPP, IPP or DMAPP as indicated. Assays were performed at 25 °C in 50 mM HEPES, 150 mM NaCl buffer. The plots in the upper panel show the thermogram (raw thermal power as a function of time), and the plots in the lower panel show the binding isotherm (heat released per injection normalized per mole of ligand injected as a function of the molar ratio, [ligand]/[protein], in the calorimetric cell). The solid lines represent the best fits of the experimental data after non-linear lest-squares analysis using a single-site binding model.

Table 2. 1 Dissociation constant of SlDXS1 interaction with metabolite ligands.

Protein	Ligands	K (M^{-1})	K_d (µM)
SlDXS1	TPP	$2.3 \cdot 10^5$	4.23
	IPP	$4.5 \cdot 10^5$	2.23
	DMAPP	$5.8 \cdot 10^5$	1.71
SlDXS1+TPP	IPP	$8.1 \cdot 10^5$	1.22
	DMAPP	$2.3 \cdot 10^5$	4.32

To explore whether binding of IPP and DMAPP had any effect on SlDXS1 structure, the protein was mixed with 50 µM TPP in the absence or presence of 50 µM IPP or DMAPP and then analyzed by dynamic light scattering (DLS). In the absence of IPP/DMAPP the estimated radius of the protein (5.8 nm) correlates with the dimeric form of the enzyme, of about 150 kDa. By contrast, in the presence of either IPP or DMAPP the peak shifted to a smaller radius while the range of particle sizes increased (Fig. 2.3). From these data we speculated that IPP and DMAPP binding might somehow interfere with the dimerization of SlDXS1, displacing the monomer-dimer equilibrium to the monomeric forms that might eventually aggregate forming the high molecular weight particles detected by DLS (Fig. 2.3).

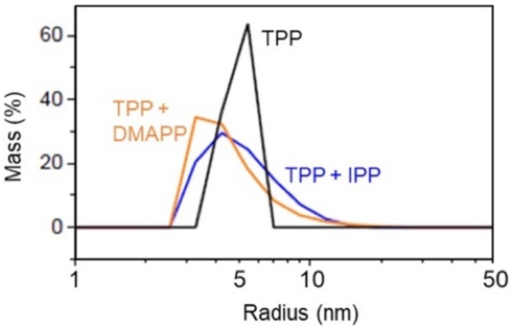

Figure 2. 3. DLS analysis of purified SlDXS1 with ligands. Recombinant SlDXS1 (20 µM) was premixed with 50 µM TPP and analyzed in the absence (black line) or presence of 50 µM IPP (blue line) or 50 µM DMAPP (orange line). For each measurement, 5 acquisitions of 5 seconds were taken.

2.2.3 IPP/DMAPP promote monomerization of bacterial and plant DXS enzymes

To further validate our interpretation of DLS experiments, we analyzed whether the oligomeric state of DXS enzymes from *E. coli* (EcDXS) and tomato (SlDXS1) changed in the presence of high levels of IPP/DMAPP *in vivo*. We initially expressed constructs encoding 6xHis-tagged versions of the full length EcDXS protein or the truncated SlDXS1 protein lacking the N-terminal plastid targeting peptide in the *E. coli* strain EcAM5, which harbors the MVA operon in a BL21(DE3) background (Rodríguez-Villalón et al., 2008). Transformed cells were grown at 37°C until exponential phase and then arabinose and IPTG were added to induce the expression of the MVA operon and the recombinant DXS enzyme, respectively. Cultures were also supplemented with 0, 1 or 10 mM MVA and then incubated for 3 additional hours at 26°C. The quaternary structure of recombinant DXS proteins in the cultures was analyzed by PAGE-SDS followed by immunoblot analysis with an anti-6xHis antibody after incubating total protein extracts with the crosslinker dimethyl suberimidate (DMS). In the presence of increasing concentrations of MVA, i.e., as IPP/DMAPP levels increased, the proportion of dimers decreased whereas monomeric forms of both EcDXS and SlDXS1 increased (Fig. 2.4). The structural change was even clearer when representing the dimer to monomer ratio (Fig. 2.4). These results demonstrate that high IPP and DMAPP levels decrease the proportion of active DXS dimers and increase the amounts of monomeric (inactive) forms of both bacterial and plant enzymes when expressed in *E. coli* cells.

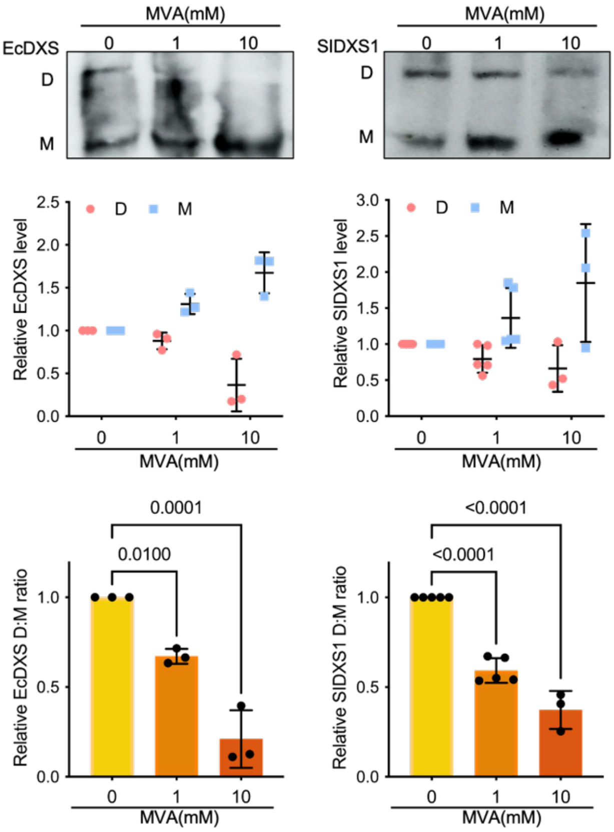

Figure 2. 4. Increasing IPP/DMAPP levels promote monomerization of bacteria and plant DXS enzymes in *E. coli*. EcAM5-1 cells were transformed with constructs to express 6xHis-tagged versions of bacterial (EcDXS) and plant (SlDXS1) enzymes and positive transformants were grown in media supplemented with the indicated amounts of MVA. Upper panels show representative images of immunoblot analyses with anti-6xHis antibodies. The position of dimers (D) and monomers (M) is indicated. Quantification of D and M abundance from immunoblot analyses of n≥3 independent experiments is represented in the plots. Individual data points as well as mean and standard deviation are represented. Numbers in the lower plots indicate p values (one-way ANOVA with Dunnett's multiple comparisons test).

In order to validate the results obtained with the tomato SlDXS1 protein using a plant system, we agroinfiltrated constructs encoding the full-length protein in tobacco (*Nicotiana benthamiana*) leaves. The amount of IPP/DMAPP in leaf cells was modulated using the MEP pathway inhibitor fosmidomycin (FSM) and the carotenoid biosynthesis inhibitor norflurazon (NF). While both inhibitors cause a bleaching phenotype due to the photooxidative damage associated to the loss of carotenoids (Fig. 2.5a), FSM causes a drop in IPP/DMAPP levels whereas NF is expected to increase the amount of these isoprenoid precursors by preventing their consumption by the carotenoid pathway. Leaf bleaching associated to the activity of these inhibitors, which were added to the agroinfiltration mixture, was clearly observed at 5 dpi (days post-inoculation) (Fig. 2.5a). This time point was selected to take samples for LC-MS analysis of MEP pathway intermediates as well as for protein extraction and immunoblot analysis using an anti-DXS serum (Pulido et al., 2013). Our LC-MS method was not sensitive enough to detect IPP/DMAPP in agroinfiltrated tobacco leaves but we could successfully quantify the levels of DXP, MEP and the downstream intermediate methylerythritol-cyclodiphosphate (ME-cPP). FSM treatment caused an accumulation of DXP and a drop in MEP and MEcPP levels compared to untreated controls. By contrast, NF treatment reduced the levels of DXP, MEP and MEcPP, as expected considering that increased IPP/DMAPP levels should result in lower DXS activity. Consistent with our interpretation, the dimer to monomer ratio of SlDXS1 decreased in plant leaves treated with NF (Fig. 2.5b). Also, in agreement with the results previously obtained in E. coli, reduced IPP/DMAPP levels in FSM-treated samples led to an increased dimer to monomer ratio compared to mock-treated controls (Fig. 2.5b). This negative correlation between dimer to monomer ratio and IPP/DMAPP levels strongly supports our conclusion that IPP and DMAPP are able to modulate DXS activity in vivo by interfering with the formation of the active dimeric form of the enzyme in both bacterial and plant systems.

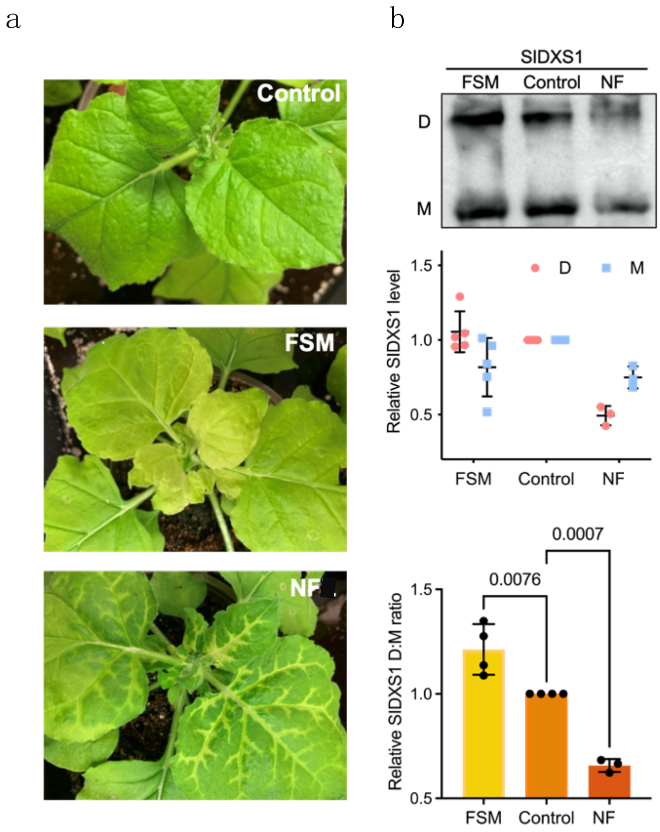

Figure 2. 5. Changing IPP/DMAPP levels modulate monomerization of SlDXS1 in *N. benthamiana*. Leaves from *N. benthamiana* plants were infiltrated with constructs to produce SlDXS1 together with either water (control) or inhibitors, and samples were collected 5 days later. (A) Representative images of the leaves. (B) Immunoblot analyses of protein extracts with anti-DXS antibodies. The position of dimers (D) and monomers (M) is indicated in the upper panel, which shows the result of a representative experiment. Quantification of D and M abundance from immunoblot analyses of n≥3 independent experiments is represented in the plots. Individual data points as well as mean and standard deviation are represented. Numbers in the lower plots indicate p values (one-way ANOVA with Dunnett's multiple comparisons test).

2.2.4 IPP/DMAPP promote DXS aggregation in bacteria and plant cells

DXS monomers expose highly hydrophobic domains that make them prone to aggregation (Pulido et al., 2016). To test whether IPP/DMAPP-induced monomerization of EcDXS and SlDXS1 proteins favored their aggregation, we expressed 6xHis-tagged versions of both proteins in EcAM5 cells, induced IPP/DMAPP accumulation *in vivo* by supplementing the growth medium with 1 or 10 mM MVA, and then isolated proteins from soluble and insoluble fractions for immunoblot analysis with anti-6xHis serum (Fig. 2.6). Most of the recombinant EcDXS and SlDXS1 proteins were found in the soluble fractions, with no major differences in the presence or absence of MVA (Fig. 2.6). In the insoluble fraction, however, the amounts of EcDXS and SlDXS1 increased in MVA-supplemented cells (i.e., when IPP/DMAPP levels increased), resulting in a higher ratio of insoluble (aggregated) vs. soluble (disaggregated) protein (Fig. 2.6).

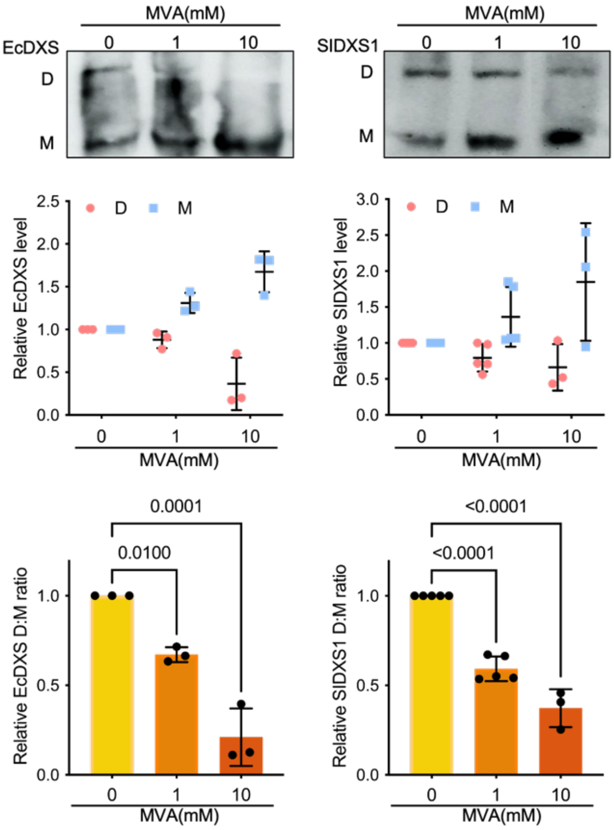

Figure 2. 6. Increasing IPP/DMAPP levels promote aggregation of bacteria and plant DXS enzymes in *E. coli.* EcAM5 cells were transformed with constructs to express 6xHis-tagged versions of bacterial (EcDXS) and plant (SlDXS1) enzymes and positive transformants were grown in media supplemented with the indicated amounts of MVA. Upper panels show representative images of immunoblot analyses with anti-6xHis antibodies. Extracts corresponding to soluble (S) and insoluble/aggregated (I) proteins are indicated. Quantification of S and I abundance from immunoblot analyses of n≥3 independent experiments is represented in the plots. Individual data points as well as mean and standard deviation are represented.

Numbers in the lower plots indicate p values (one-way ANOVA with Dunnett's multiple comparisons test).

The aggregation status of the SlDXS1 protein was confirmed in plant cells by observing the accumulation of a GFP-tagged fusion, SlDXS1-GFP. Arabidopsis DXS enzymes fused to GFP are localized in the plastid stroma but they can also be observed forming a spotted distribution corresponding to aggregates when overexpressed (Perello et al., 2016; Pulido et al., 2013, 2016). As expected, transient overexpression of SlDXS1-GFP in N. benthamiana leaves also led to the formation of fluorescent speckles identified by confocal laser scanning microscopy (Fig. 2.7). Quantification of the number of fluorescent spots (i.e. SlDXS1-GFP aggregates) per chloroplast in leaves treated with inhibitors altering IPP/DMAPP levels showed higher amounts with FSM and lower with NF compared to untreated controls (Fig. 2.7). The size of the spots, however, was higher in NF samples, in agreement with the conclusion that SlDXS1 aggregation increases when IPP/DMAPP levels increase.

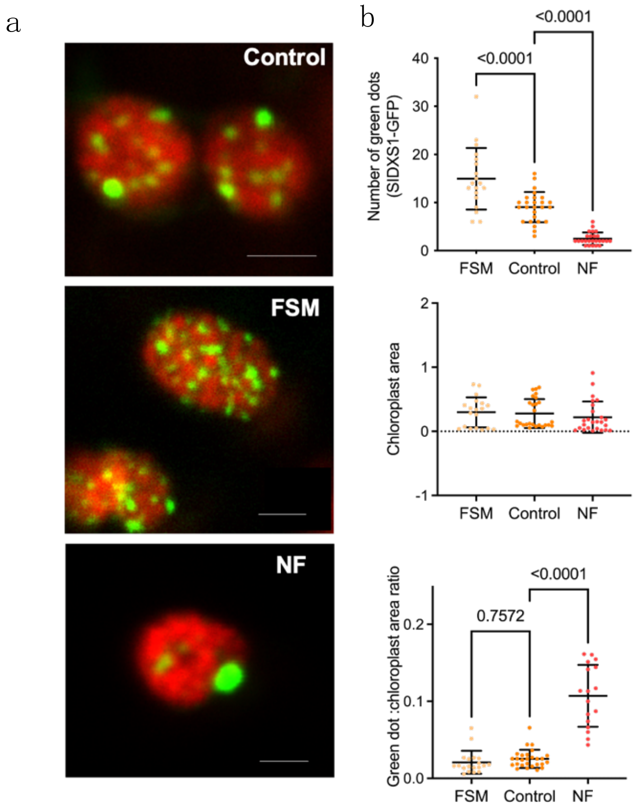

Figure 2. 7. Changing IPP/DMAPP levels modulate monomerization of SlDXS1 in *N. benthamiana*. Leaves from *N. benthamiana* plants were infiltrated with constructs to produce SlDXS1 together with either water (control) or inhibitors, and samples were collected 5 days later. (a) Representative images of the leaves. (b) Immunoblot analyses of protein extracts with anti-DXS antibodies. The position of dimers (D) and monomers (M) is indicated in the upper panel, which shows the result of a representative experiment. Quantification of D and M abundance from immunoblot analyses of n≥3 independent experiments is represented in the plots. Individual data points as well as mean and standard deviation are represented. Numbers in the lower plots indicate p values (one-way ANOVA with Dunnett's multiple comparisons test).

2.3 Discussion

The regulation of the MEP pathway to produce the building blocks required for the biosynthesis of isoprenoids (IPP and DMAPP) has revealed to be rather complex, encompassing transcriptional, post-transcriptional, post-translational and metabolic levels (Banerjee & Sharkey, 2014; Hemmerlin, 2013; Rodríguez-Concepción & Boronat, 2015; Vranová et al., 2013). This is especially relevant for the first enzyme of the pathway (DXS), which catalyzes the TPP-dependent conversion of pyruvate and GAP into DXP in the main rate-limiting step of the pathway (Volke et al., 2019; Wright et al., 2014). Here we show that the mechanism by which accumulation of intracellular IPP and DMAPP inhibits the activity of DXS is conserved in bacteria and plant plastids and it involves (i) direct binding of IPP/DMAPP to DXS in a location different from the TPP-binding active site, followed by (ii) enhanced monomerization of the enzyme, and eventually (iii) aggregation of the inactive monomers. This mechanism allows a short-term response to a sudden increase or decrease in IPP/DMAPP supply (by rapidly shifting the dimer-monomer equilibrium accordingly) but also a long-term response if IPP/DMAPP abundance persists (as monomers aggregate and become unavailable to form active DXS dimers).

DXS, in its dimeric active form, has been described as a three-domain polypeptide with domains I and II from the same chain involved in the formation of the active site and the binding of TPP. Domain III is involved in the formation of the dimer interface that includes a highly hydrophobic surface that remains unexposed to the solvent in the dimer (Chen et al., 2019; Song et al., 2007). The reaction mechanism involves the formation of a covalent intermediate between enzyme-bound TPP and pyruvate, followed by the GAP-stimulated decarboxylation of the bound pyruvate, and incorporation of GAP to the remaining fragment to generate DXP (Chen et al., 2019; Song et al., 2007; Vranová et al., 2013). Using a recombinant DXS protein from the tree *Populus trichocarpa* (PtDXS), it was proposed that IPP and DMAPP might compete with TPP for its pocket in the active site formed in each of the two subunits of the homodimer, with Ki values of ca. 65 µM for IPP and 81 µM for DMAPP (Banerjee et al., 2013). This competition is striking, however, considering that TPP is generally thought to be tightly bound as an integral part of DXS but also that it functions as a cofactor of many enzymes unrelated to isoprenoid synthesis for whom a regulation by IPP/DMAPP might not be physiologically meaningful. Experimental data from enzyme activity assays could not be fit to standard competitive inhibition kinetics and instead it was proposed a negative cooperative model in which binding of IPP/DMAPP to one of the DXS monomers would somehow hamper the binding of a second molecule to the other subunit of the homodimer (Banerjee et al., 2013). Furthermore, mutation of residues at the PtDXS active site that were predicted to be critical for binding the IPP/DMAPP carbon chain were found to have opposite effects on IPP-mediated inhibition of enzyme activity (Banerjee et al., 2016). Our ITC experiments using SlDXS1 in the presence of an excess of TPP, IPP and DMAPP confirmed that these three small diphosphate metabolites could indeed interact with the enzyme but showed Kd values for IPP and DMAPP that were very similar in the absence or presence of TPP (Table 2.1 and Fig. 2.2). These results argue against a direct competition of IPP/DMAPP for the TPP binding site in DXS and instead suggest the existence of an alternative binding site for the MEP pathway products. The inconsistencies between these two models of DXS inhibition by IPP/DMAPP might derive from the underlying methodology, i.e., enzymatic vs. calorimetric assays. ITC is a powerful tool to study protein/ligand binding interactions that involves directly measuring the heat that is released or absorbed in real time when one solution is titrated into another (Wang et al., 2020) ITC is capable of providing information on enzymatic reactions that is difficult to obtain using traditional (indirect) biochemical assays. In particular, the use of DXS substrates (pyruvate) together with additional metabolites (dihydroxyacetone phosphate) and enzymes (triose-phosphate isomerase from rabbit muscle) in the reaction mixtures previously used to determine the inhibition dynamics of IPP/DMAPP (Banerjee et al., 2013) might have resulted in interferences influencing the final interpretation of the data.

Allosteric regulation of protein function occurs when the binding of a molecule either activating or inhibiting the activity of the protein takes place away from the active site. This differs from competitive inhibition wherein the inhibitor binds to the active site and prevents the protein's natural substrate gaining access. In contrast with the previously proposed model of negative cooperative inhibition of TPP binding by IPP/DMAPP (Banerjee et al., 2013, 2016), our data supports the conclusion that IPP/DMAPP-mediated inhibition of DXS is truly allosteric as the modulation of enzyme activity involves binding outside the active site. Many proteins are allosterically regulated with a variety of mechanisms (Laskowski et al., 2009). Our model of IPP/DMAPP inhibition of DXS activity is a typical example of allosteric regulation in which the downstream products of a biosynthetic pathway down-regulate the activity of the enzyme catalyzing the first committed step through feedback inhibition, hence ensuring that pathway flux is adjusted to end-product usage (Gerhart, 2014; Ruszkowski, 2018; Wang et al., 2020). In most cases, binding of the ligand inhibits enzyme activity by promoting a conformational change (Laskowski et al., 2009). Because prediction of the energetics and mechanisms of protein conformational changes by ITC remains very challenging, we used DLS to initially address this possibility. Indeed, our results showed that IPP/DMAPP binding disrupts the dimeric structure of the active SlDXS1 enzyme, causing a peak shift towards smaller (monomeric) forms (Fig. 2.3). Further analysis of EcDXS and SlDXS1 dimeric and monomeric forms by immunoblot experiments confirmed a decreased dimer to monomer ratio when levels of IPP and DMAPP increased in living bacteria (Fig. 2.4) and plant cells (Fig. 2.5). Changes in the dimer:monomer ratio triggered by allosteric regulation has been observed in different proteins, including receptors, membrane translocators (Jaipuria et al., 2017) and enzymes (Seetoh & Abell, 2016). In the case of DXS, however, IPP/DMAPP-mediated monomerization was found to be linked to increased aggregation both in bacteria (Fig. 2.6) and plastids (Fig. 2.7). DXS exists in a bimodal distribution of open and closed conformations. In the catalytic reaction mechanism described for DXS, the dimeric enzyme adopts a closed conformation when pyruvate binds the TPP-containing active site, and then changes to an open state upon GAP binding (Chen et al., 2019; Decolli et al., 2019b; Zhou et al., 2017). In the open conformation, solvent-accessible areas are increased in different segments near the active site, exposing hydrophobic domains in the interface of the two monomers which are normally buried in the quaternary structure of closed dimer. This might partially explain the previously observed propensity to aggregation of this protein in plants (Pulido et al., 2013, 2016) and microbes (Kudoh et al., 2017; Kudoh et al., 2017). Most interestingly, DXS monomers fully expose the aggregation-prone domains (Pulido et al., 2016). We therefore conclude that high IPP/DMAPP levels shift the dimer:monomer equilibrium of DXS to monomeric forms for rapid down-regulation of enzyme activity. Monomers would be expectedly available for immediate dimerization and hence enzyme reactivation upon return of IPP/DMAPP to steady state levels. However, if IPP/DMAPP levels remain high (e.g., by a blockage in their consumption by downstream isoprenoid biosynthetic pathways), monomers would remain available for aggregation to drastically reduce DXS activity.

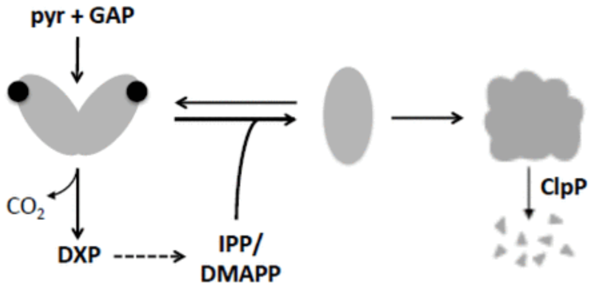

Figure 2. 8. Model of the molecular mechanism involved in the allosteric feedback inhibition of DXS. The first step of the MEP pathway is the production of DXP from pyruvate and GAP, a reaction catalyzed by DXS. The active DXS enzyme is a dimer and requires binding of the cofactor TPP (represented as black circles) to the active site present in each of the subunits. Allosteric changes induced by binding of IPP/DMAPP shifts the dimer:monomer equilibrium towards the monomeric (inactive) forms, causing an immediate down-regulation of enzyme activity. If monomers are not used to make new dimers (e.g., due to the presence of high IPP/DMAPP levels), exposed hydrophobic domains can lead to monomer aggregation and eventual degradation by Clp protease complex (ClpP), resulting in a sustained removal of DXS activity.

Our model, summarized in Fig. 2.8, not only provides a mechanistic explanation of how MEP-derived IPP and DMAPP supply can be adapted to changes in their demand but it can also explain the changes in DXS protein levels observed after long-term interference of the MEP pathway flux (Ghirardo et al., 2014; Han et al., 2013; Pokhilko et al., 2015). In particular, genetic or pharmacological reduction of IPP/DMAPP levels cause an up-regulation of DXS protein levels without concomitant changes in gene expression (Ghirardo et al., 2014; Han et al., 2013; Pokhilko et al., 2015), whereas mutants unable to produce carotenoids and hence expectedly accumulating higher IPP/DMAPP levels (similar to NF treatments) showed an opposite phenotype of reduced DXS protein levels (Pokhilko et al., 2015). Under non-stressed conditions, DXS aggregates are degraded by the Clp protease complex in different organisms, including plants but also microorganisms (Andrea et al., 2018; Florentin et al., 2017; Moreno et al., 2018; Ninnis et al., 2009; Pulido et al., 2013, 2016). We therefore propose that a prolonged shortage of IPP/DMAPP might lead to mostly dimeric (i.e., soluble and enzymatically active) DXS whereas sustained IPP/DMAPP abundance would cause an enhanced monomerization and aggregation of the protein, eventually resulting in their efficient removal by the Clp protease (Fig. 2.8).

In summary, we discovered an evolutionary conserved and mechanistically simple system in which the MEP pathway products IPP and DMAPP (the universal precursors of all isoprenoids) allosterically control the activity of DXS (the main rate-limiting enzyme of the pathway) by modulating the equilibrium between dimeric (active) and monomeric/aggregated (inactive) forms of the enzyme. Peak demands of MEP pathway products (e.g., to produce monoterpenes in response to a pathogen attack or carotenoids upon a high light stress) would decrease the amount of IPP/DMAPP, promoting DXS dimerization and hence maximizing activity to increase pathway flux. By contrast, reduced IPP/DMAPP consumption causing the build-up of these metabolites would promote DXS monomerization, which could eventually result in aggregation and subsequent proteolytic removal of the protein(Fig. 2.8). Overall, these results improve our surprisingly scarce knowledge of how the MEP pathway is regulated (Banerjee & Sharkey, 2014; Kipchirchir Bitok & Meyers, 2012; Pokhilko et al., 2015). The MEP pathway, probably the main metabolic pathway elucidated in this century (Rodríguez-Concepción & Boronat, 2002), provides the precursors for a large diversity of isoprenoids with high added value from the industrial and nutritional points of view. Understanding its regulation, in particular that of its main enzyme, DXS, is therefore a must for the rational design of biotechnological endeavors aimed at increasing isoprenoid contents in microbial and plant systems.

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Chapter 3

Chapter 3. Feedback inhibition of DXR (but not DRL) by IPP/DMAPP

3.1 Introduction

The methylerythritol 4-phosphate (MEP) pathway synthesizes isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP), the universal precursors for the synthesis of isoprenoids in most bacteria and in plant plastids. Isoprenoids include metabolites with high added value as pigments, nutrients, chemicals or drugs, and increasing the metabolic flux through the MEP pathway by genetic engineering is one of the key factors contributing to improve isoprenoid production in bacterial and plant biofactories (Rodríguez-Concepción & Boronat, 2002). The first two pathway enzymes, deoxyxylulose 5-phosphate (DXP) synthase (DXS) and DXP reductoisomerase (DXR) are often targets to overexpress in order to promote the metabolic flux of the MEP pathway. Because DXS-produced DXP is also required for the production of non-isoprenoid metabolites such as thiamin diphosphate (vitamin B1) and pyridoxal phosphate (vitamin B6) in bacteria, DXR can be considered as the first enzyme completely exclusive of the MEP pathway (Rodríguez-Concepción & Boronat, 2002).

DXR is a NADPH-dependent enzyme that converts DXP into MEP in the first committed step of the pathway (Koppisch et al., 2001; Kuzuyama et al., 2000; Takahashi et al., 1998). DXR enzymes contain three domains: a large N-terminal NADPH-binding domain, a connective or linker domain, and a smaller C-terminal alpha-helical domain. The active enzyme is a dimer with crystal symmetry (Sweeney et al., 2005). Genes encoding DXR are present in virtually all organisms that have a functional MEP pathway.

In a few groups of bacteria such as *Brucella* and *Bartonella*, however, DXR is missing and a DXR-like (DRL also called DXR class 2) enzyme was found to be present to catalyze the exact same reaction of DXP to MEP conversion (Sangari et al., 2010). The DRL enzyme is also a dimer with three distinct domains: a N-terminal NADPH-binding domain, a central or catalytic domain, and a smaller C-terminal domain (Pérez-Gil et al., 2012). The central/catalytic domain of DRL presents low structural relationship with DXR (Pérez-Gil et al., 2012). DRL was found to resist inhibition with the DXR inhibitor fosmidomycin (FSM) likely because of the central domain differences between the two enzymes, especially in the so-called "lid" regions (Pérez-Gil et al., 2012; Sangari et al., 2010).

Metabolic flux analysis identified DXS as the main regulator of the MEP pathway flux (Volke et al., 2019; Wright et al., 2014). However, DXR might become important when the primary rate-limiting step is released. In the case of DXS, protein levels and enzyme activity are feedback regulated by IPP and DMAPP to stabilize the MEP pathway flux against changes in substrate supply and adjust it according to product demand under normal growth conditions (Banerjee et al., 2013; Pokhilko et al., 2015). More recently, our work has shown that direct binding of IPP and DMAPP to DXS promote monomerization and hence deactivation of the enzyme, which might eventually lead to aggregation and degradation of the monomers (Di et al., 2022). Here we tested whether a similar mechanism might also regulate DXR and/or DRL.

3.2 Results

3.2.1 IPP and DMAPP inhibit the activity of EcDXR but not BaDRL

To explore a possible feedback regulation of DXR and/or DRL by IPP and DMAPP, the activity of recombinant enzymes from *Escherichia coli* (EcDXR) and *Brucella abortus* (BaDRL) was assessed *in vitro* with and without IPP or DMAPP added to the reaction media. DXR and DRL convert DXP to MEP by using NADPH as a cofactor for intramolecular rearrangement and reduction (Yajima et al., 2007). So, the activity of DXR or DRL can be analyzed by a spectrophotometric assay that measures the consumption of NADPH detected at 340 nm. After cloning 6xHis-tagged versions of EcDXR and BaDRL for expression in *E. coli* cells, the recombinant proteins were purified by IMAC and used for activity assays in media supplemented with water (as a control), 1mM IPP, 1mM DMAPP, or a mix of 0.5mM IPP and 0.5mM DMAPP. NADPH was consumed rapidly by EcDXR when supplemented only with water but much more slowly when IPP and/or DMAPP were added (Fig. 3.1a). Interestingly, the consumption of NADPH by BaDRL kept a similar level with or without IPP and/or DMAPP (Fig. 3.1a). To provide quantitative data, the IC50 value was calculated based on the NADPH absorbance drop. EcDXR enzyme activity was similarly inhibited by 1 mM IPP, 1 mM DMAPP, or the mixture of 0.5mM IPP and 0.5mM DMAPP, but there was no obvious change for BaDRL activity (Fig. 3.1b). The described data indicate that IPP and DMAPP inhibit the activity of DXR (similarly to that described for DXS) but not the activity of DRL.

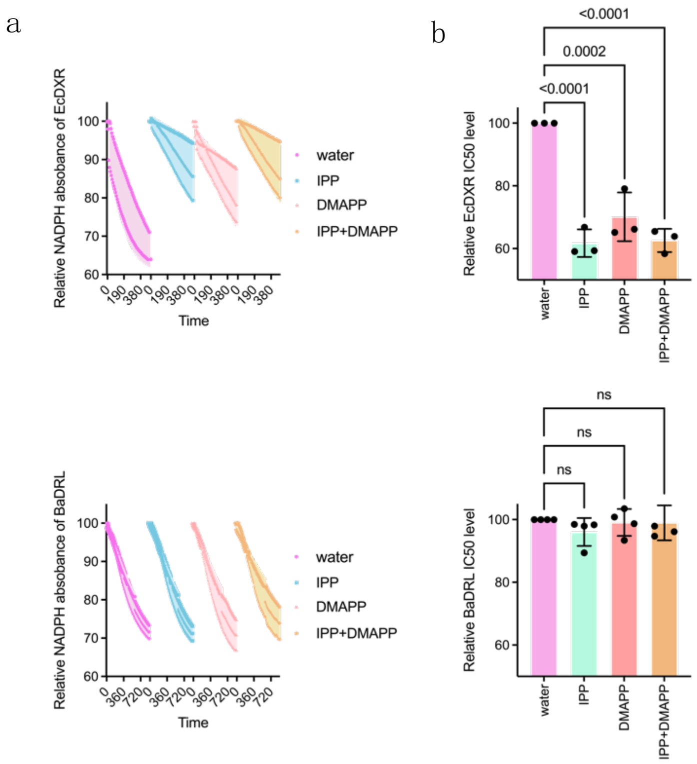

Figure 3. 1. DXR enzyme activity assays. NADPH absorbance was measured as an estimate of DXR enzyme activity *in vitro*. (a) NADPH consumption with time in reaction mixtures containing purified EcDXR and BaDRL and supplemented with water, 1mM IPP, 1mM DMAPP, or a combination of 0.5mM IPP and 0.5mM DMAPP. (b) The IC50 of EcDXR and BaDRL under the treatments described in (a). Individual data points as well as mean and standard deviation of n≥3 independent experiments are represented. Numbers in the lower bar plots indicate *p* values (one-way ANOVA with Dunnett's multiple comparisons test).

3.2.2 IPP/DMAPP directly interact with EcDXR and BaDRL.

IPP and DMAPP inhibit DXS activity by direct interaction with the protein (Di et al., 2022). In order to experimentally explore if IPP and DMAPP also inhibit DXR activity by binding to the protein we used isothermal titration calorimetry (ITC). Specifically, we tested purified 6xHis-tagged versions of the full-length EcDXR and BaDRL proteins (Fig. 3.2). Based on the thermograms obtained and the corresponding binding isotherms using 20 μM EcDXR in the sample cell solution and 200 μM of the ligands in the syringe solution, IPP and DMAPP were found to interact with the EcDXR enzyme, with dissociation constants K_d of 0.20 μM for IPP and 0.04 μM for DMAPP (Table 3.1), indicating a higher affinity of DMAPP. Surprisingly, BaDRL was also detected to interact with IPP and DMAPP despite the lack of effect of these metabolites on BaDRL activity (Fig. 3.1). In this case, the dissociation constants were of 0.98 μM for IPP and 0.18 μM for DMAPP (Table 3.1). A closer analysis of the data indicated that the heat power of BaDRL is high at the beginning of the experiment (when the protein and the ligands are put together) and then decrease as the standard ITC result (Fig. 3.2). Compared with BaDRL, the heat power of EcDXR was lower at the beginning and it reached a peak at 20 min with both IPP and DMAPP treatments (Fig. 3.2).

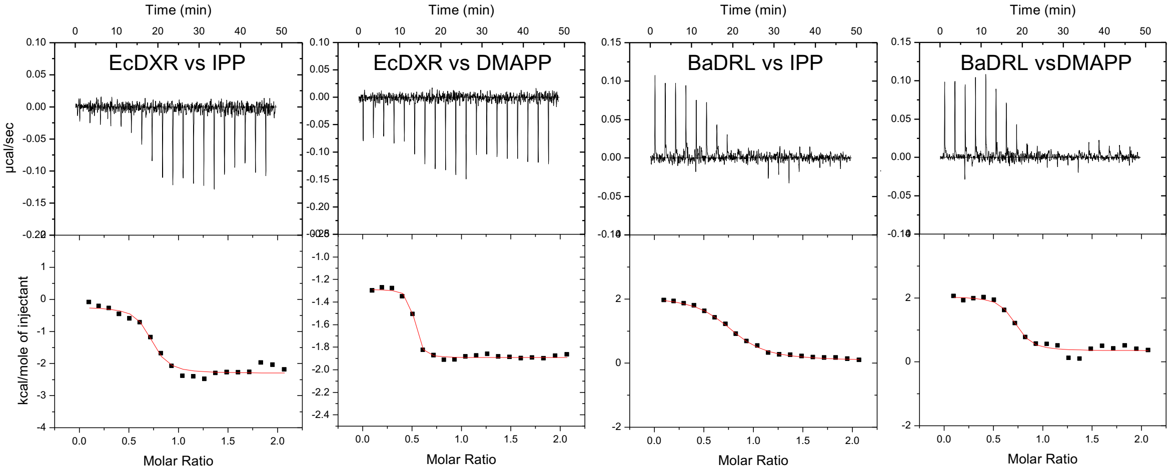

Figure 3. 2. Analysis of EcDXR or BaDRL interaction with IPP and DMAPP ligands. ITC plots were obtained from the titration of 20 μM EcDXR with 200 μM IPP or DMAPP as indicated, and from 20 μM BaDRL with 200 μM IPP or DMAPP as indicated. Assays were performed at 25 °C in 50 mM HEPES, 150 mM NaCl buffer. The plots in the upper panel show the thermogram (raw thermal power as a function of time), and the plots in the lower panel show the binding isotherm (heat released per injection normalized per mole of ligand injected as a function of the molar ratio, [ligand]/[protein], in the calorimetric cell). The solid lines represent the best fits of the experimental data after non-linear lest-squares analysis using a single-site binding model.

Table 3. 1 Dissociation constant of EcDXS and BaDRL interaction with IPP/DMAPP

Protein	Ligands	K (M^{-1})	K_d (μM)
EcDXR	IPP	$5.08 \cdot 10^6$	0.20
	DMAPP	$2.6 \cdot 10^7$	0.04
BaDRL	IPP	$1.08 \cdot 10^6$	0.93
	DMAPP	$5.32 \cdot 10^6$	0.18

3.2.3 Prediction of the IPP/DMAPP interaction sites

To next investigate the possible binding sites of IPP and DMAPP in the DXR enzyme, the crystal structure of EcDXR (Mac Sweeney et al., 2005) was used to dock with IPP and DMAPP using Autodock software (Fig. 3.3), a molecular modeling and docking simulation software that can predict the preferred interaction site when ligand and target protein bind to each other (Lengauer & Rarey, 1996). Analysis of the crystal structure found that the active site of EcDXR is formed by the highly conserved residues Asp150, Glu152, His 209, Glu231, Glu234 and His257 (Sweeney et al., 2005). The substrate (DXP) was found to bind to the C-terminal and connective domains, and then induce a conformational change. The residues 206-216 from EcDXR form a flexible loop that connects both domains with an induced fit adaptation and functions as a ‘lid’ over the active site (Sweeney et al., 2005). Without NADPH or DXP, the flexible ‘lid’ is open, but it closes upon DXP and NADPH binding to shield the active site from the solvent (Sweeney et al., 2005). Docking showed that both IPP and DMAPP might bind to the active site of the EcDXR protein covered by the flexible loop (Fig. 3.3a). The phosphonate moiety of IPP was predicted to interact with Thr10, Asn38, Lys37 and Gly36, and the phosphonate moiety of DMAPP was predicted to interact with Gly8, Gly11, Ser121, Ala100 and Val102 (Fig. 3.3c). Interestingly, these interactions were predicted to compete for the interaction site with the phosphonate moiety of NADPH, suggesting that both IPP and DMAPP might inhibit DXR activity by competing with NADPH for the active site. In addition, DMAPP was also predicted to bind at the interaction surface of monomer DXR with residues Gln165, His282, Trp286, Pro278 and Arg289 (Fig. 3.3b). This interaction may interfere with the formation of DXR dimers.

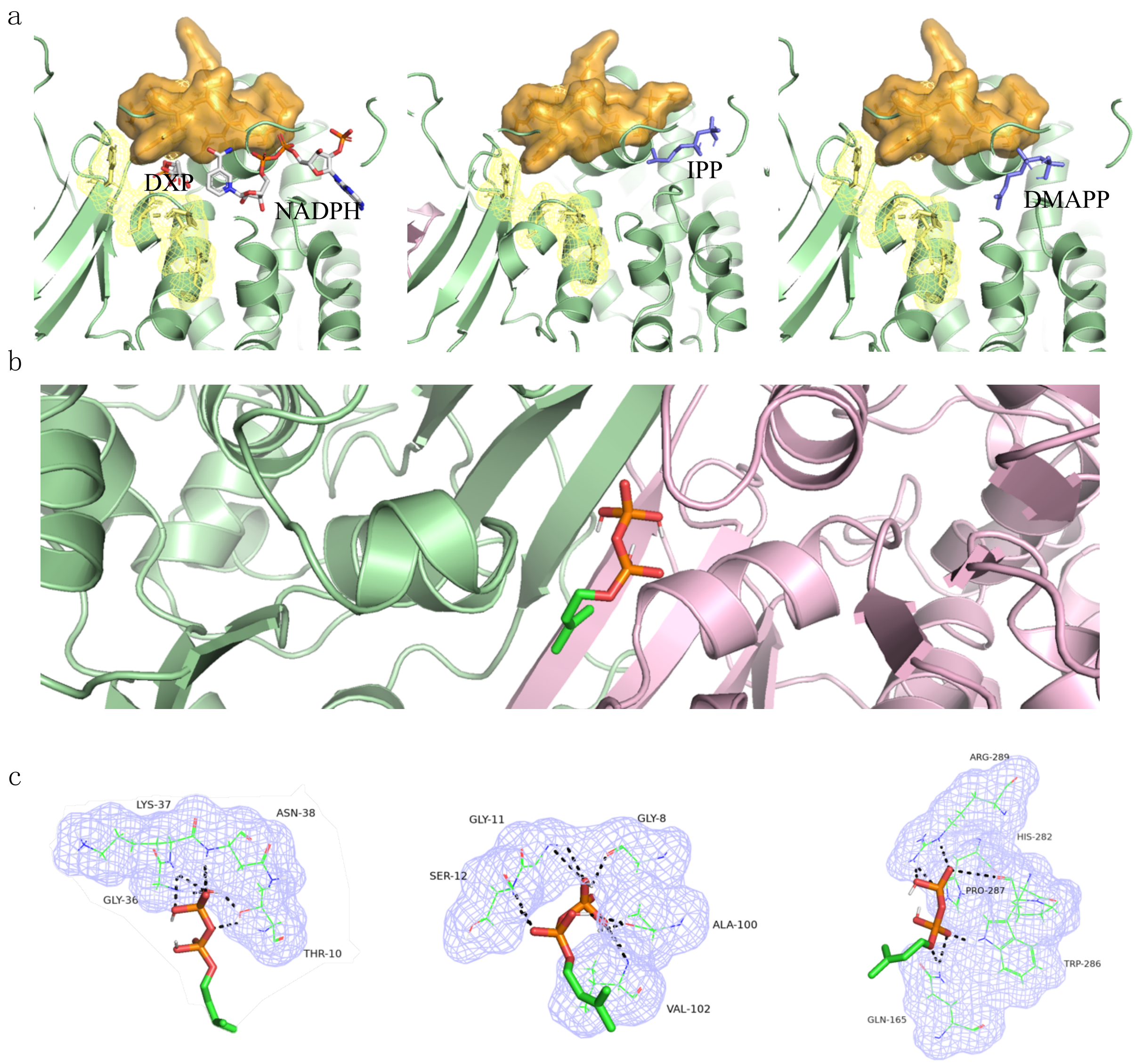

Figure 3.3.Computational modeling of ligand binding sites of EcDXR. The crystal structure of EcDXR (PDB: 1jvs) is used to dock with IPP and DMAPP, respectively by Autodock software. The flexible 'lid' (residues 206-216) is shown in orange, and the active site (conserved residues Asp150, Glu152, His 209, Glu231, Glu234 and His257) is in yellow. (a) From left to right, the DXP and NADPH binding domain, IPP binding domain and DMAPP binding domain. (b) The second binding site of DMAPP (the two monomers in the DXR dimer are shown in green and pink). (c) From left to right, the interaction residues of IPP, DMAPP at the active site and DMAPP at the interaction surface of the monomer.

We also modelled the possible interaction of IPP and DMAPP with DRL using the crystal structure of BaDRL (Pérez-Gil et al., 2012) and the Autodock software (Fig. 3.4). Both IPP and DMAPP might bind to the active site of the BaDRL protein but not fully covered by the flexible lid. Also, the residues predicted to be involved in their binding are very different in DXR and DRL (Fig. 3.4b). The phosphonate moiety of IPP was predicted to interact with Ans194 and Lys309, and the phosphonate moiety of DMAPP was predicted to interact with Lys193,Ans194 and Ser287. Interestingly, DMAPP was predicted to interact with Lys193, a residue that was previously found to interact with FSM (Pérez-Gil et al., 2012).

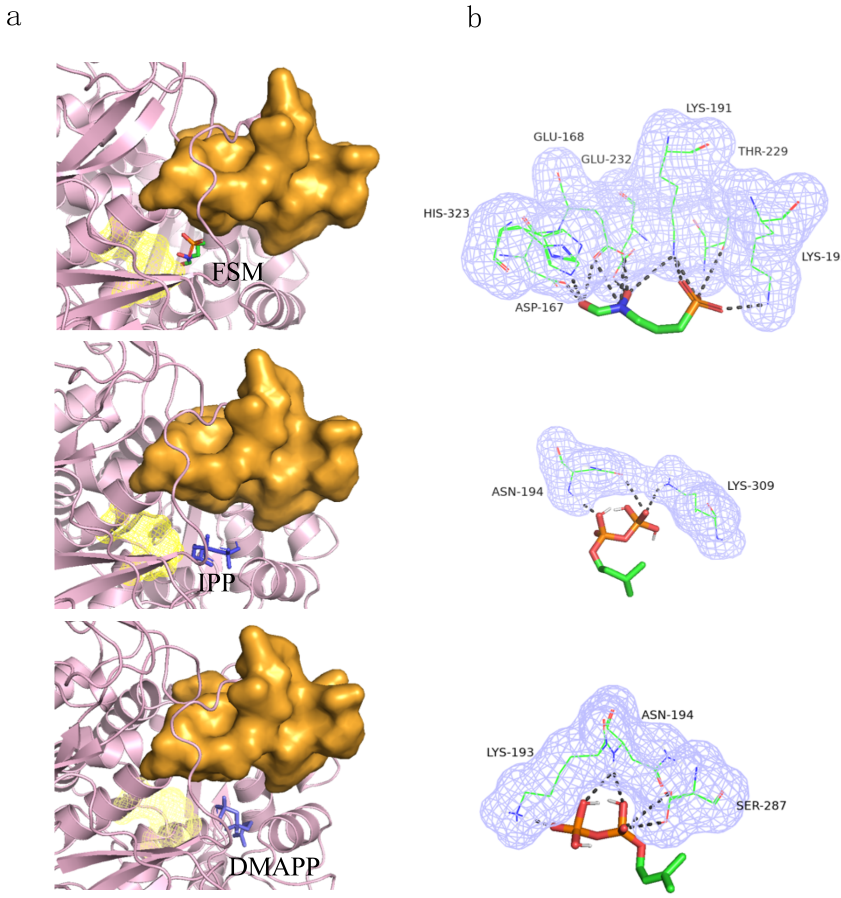

Figure 3. 4. Computational modeling of ligand binding sites of BaDRL. The crystal structure of BaDRL (PDB: 3upy) is used to dock with IPP and DMAPP, respectively by Autodock software. The flexible 'lid' is shown in orange, and the active site is in yellow. (a) From up to down, the FSM binding domain, IPP binding domain and DMAPP binding domain. (b) From up to down, the interaction residues of FSM, IPP and DMAPP at the active site.

3.2.4 IPP and particularly DMAPP binding might change EcDXR conformation.

To next explore whether binding of IPP and DMAPP had any effect on EcDXR and BaDRL structure, the proteins were mixed with increasing concentrations of IPP or DMAPP and then analyzed by dynamic light scattering (DLS). In the absence of IPP and DMAPP, the estimated radius of the EcDXR protein is consistent with the dimeric form of the enzyme, of about 79 kDa. As the concentration of IPP increases, the peak moves toward smaller radius, although the change is not substantial (Fig. 3.5a). The peak shifted to smaller radius with 10μM DMAPP treatment but it progressively returned to the dimeric form as the DMAPP concentration increased (Fig. 3.5a). BaDRL showed an estimated radius of about 133 kDa in the absence of IPP and DMAPP, again consistent with the dimeric form of the enzyme. Compared with the EcDXR sample, however, the range of BaDRL particle sizes was wider (Fig. 3.5b). No major changes were observed in the presence of different concentration of IPP and DMAPP (Fig. 3.5b). From these data we speculate that DMAPP binding may somehow interfere with the dimerization of EcDXR at low concentration. Despite IPP and DMAPP can directly interact with BaDRL, this interaction does not change the quaternary structure of the enzyme.

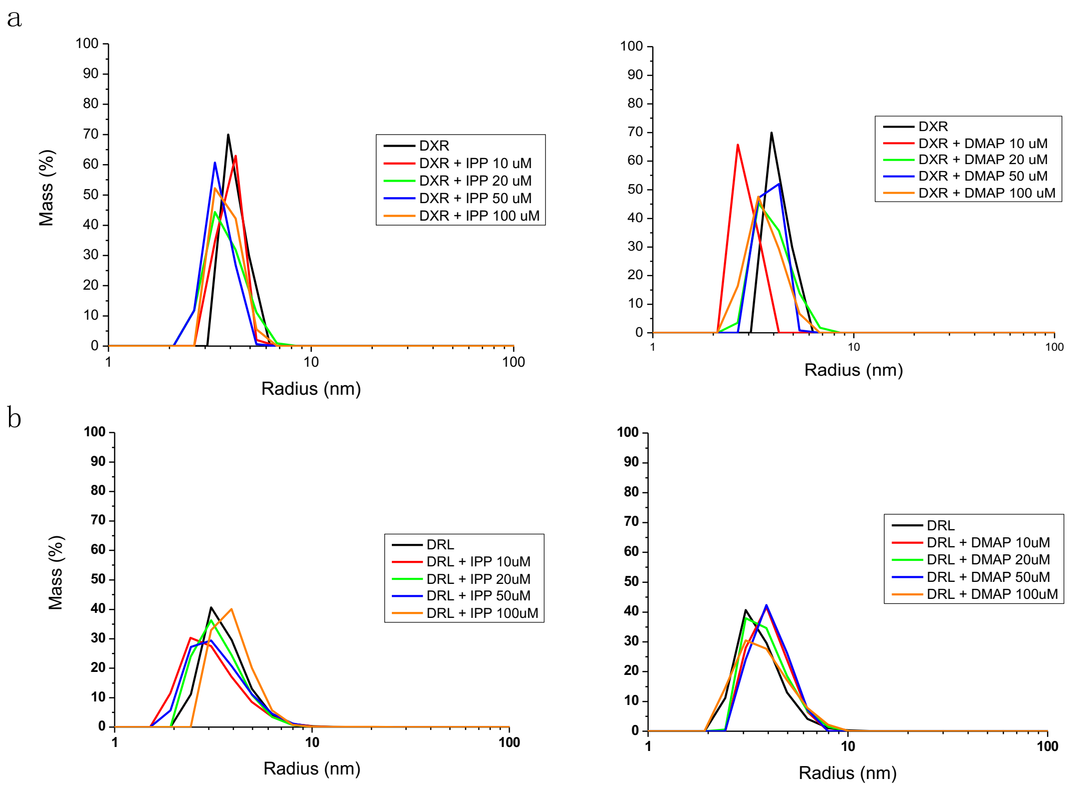

Figure 3. 5. DLS analysis of purified EcDXR or BaDRL protein with ligands. (a) Recombinant EcDXR (20 μM) was analyzed in the absence (black line) or presence of 10μM(red), 20μM(green), 50μM(blue) and 100μM(orange) IPP (right) or DMAPP (left). (b) Recombinant BaDRL (20 μM) was analyzed in the absence (black line) or presence of 10μM(red), 20μM(green), 50μM(blue) and

100µM(orange) IPP (right) or DMAPP (left).For each measurement, 5 acquisitions of 5 seconds were taken.

3.2.5 IPP/DMAPP promotes monomerization of EcDXR but not BaDRL in bacteria

To further investigate the molecular effects of IPP and DMAPP on DXR and DRL structure, we analyzed whether the oligomeric state of EcDXR and BaDRL changed in the presence of high levels of IPP/DMAPP *in vivo*. Recombinant EcDXR and BaDRL proteins with a 6xHis tag were expressed in *E. coli* cells of the EcAM5 strain, which harbors a synthetic arabinose-induced MVA operon allowing the production of IPP and DMAPP when MVA is supplied to the growth medium in a BL21(DE3) background (Rodriguez-Villalon et al., 2008). When *t*ransformed cells reached the exponential phase at 37 ºC, arabinose and IPTG were added to the culture to induce the expression of the MVA operon and the expression of the recombinant (EcDXR or BaDRL) enzymes, respectively. Cultures were also supplemented with 0,1 or 10 mM MVA and then they were incubated for 3 h or overnight at 26 ºC. At these time points, samples were taken for protein extraction, PAGE-SDS and immunoblot analyses with an anti-6xHis antibody. Protein extracts were incubated with the crosslinker dimethyl suberimidate (DMS) to preserve the quaternary structure of the recombinant proteins. At 3 h, the proportion of EcDXR dimers to monomers was similar at different concentrations of MVA (*i.e.*, in the presence of increasing IPP/DMAPP levels). However, in overnight cultures exposed for longer to increasing concentrations of MV*A* and hence higher IPP/DMAPP production*,* the proportion of EcDXR dimers decreased and monomeric forms increased as IPP/DMAPP levels increased (Fig. 3.6). The calculation of the dimer to monomer ratio confirmed that the presence of increasing concentrations of MVA promoted the change of the DXR quaternary structure from dimer to monomer. On the contrary, the quaternary structure of BaDRL was not affected by the increasing concentrations of MVA under the same experimental conditions used for EcDXR (Fig. 3.6).

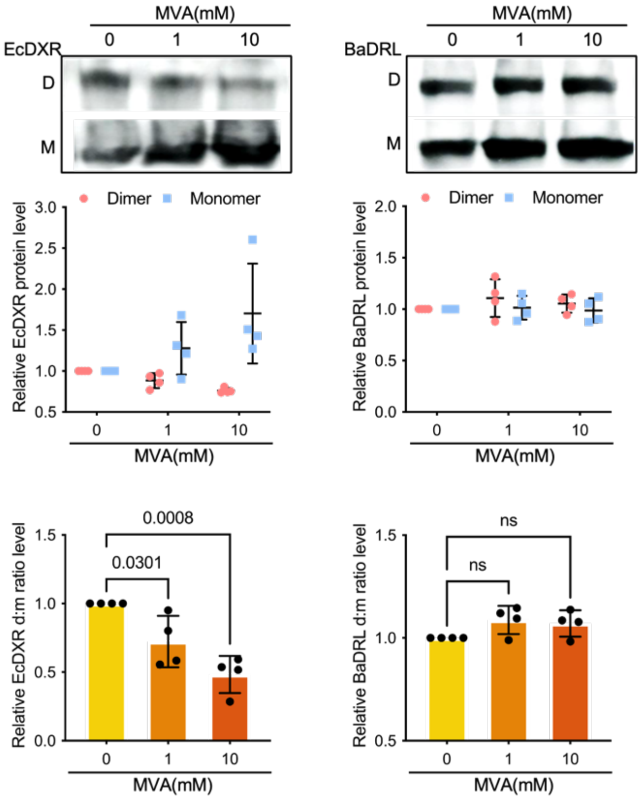

Figure 3. 6. Increasing IPP/DMAPP levels promote monomerization of EcDXR but not BaDRL enzymes in *E. coli.* EcAM5-1 cells were transformed with constructs to express 6xHis-tagged versions of EcDXR and BaDRL enzymes and positive transformants were grown in media supplemented with the indicated amounts of MVA. Upper panels show representative images of immunoblot analyses with anti-6xHis antibodies. The position of dimers (D) and monomers (M) is indicated. Quantification of D and M abundance and ratios from immunoblot analyses is represented in the plots. Individual data points as well as mean and standard deviation of n≥3 independent experiments are represented. Numbers in the lower bar plots indicate *p* values (one-way ANOVA with Dunnett's multiple comparisons test).

3.2.6 IPP/DMAPP promotes monomerization of tomato DXR in plant cells

In order to validate the results in a plant system, the full-length tomato DXR protein (SlDXR) was cloned and then transiently expressed *in tobacco (Nicotiana* benthamiana) leaves by agroinfiltration (Fig. 3.7). The amount of IPP/DMAPP in leaf cells was modulated using specific inhibitors. The DXS inhibitor clomazone (CLZ) was used to decrease IPP/DMAPP production in plastids, and the carotenoid biosynthesis inhibitor norflurazon (NF) was used to increase the amount of these isoprenoid precursors by preventing their consumption by the carotenoid pathway (Di et al., 2022). A leaf bleaching phenotype associated with the activity of these inhibitors, which were added to the agroinfiltration mixture, was clearly observed (Fig. 3.7a). The agroinfiltrated leaf tissues were collected at 5 dpi (days post-inoculation) for protein extraction and immunoblot analysis using an anti-DXR serum (Rodríguez-Concepción et al., 2001). Compared to mock controls treated with water, CLZ treatment led to a rise in the proportion of dimers and a reduction in monomeric forms of SIDXR (Fig. 3.7b). Consistently, the NF treatment caused a decreased proportion of SlDXR dimers (Fig. 3.6b). This negative correlation between the dimer to monomer ratio and the IPP/DMAPP levels in both bacteria (Fig. 3.6) and plant cells (Fig. 3.7) strongly supports our conclusion that IPP and DMAPP can modulate DXR activity in vivo by promoting the dissociation of the active dimeric form of the enzyme. Excess IPP and DMAPP favor the monomeric (i.e. inactive) forms whereas low levels of these isoprenoid precursors promote dimerization and hence increase DXR activity, similar to that described for DXS (Di et al., 2022).

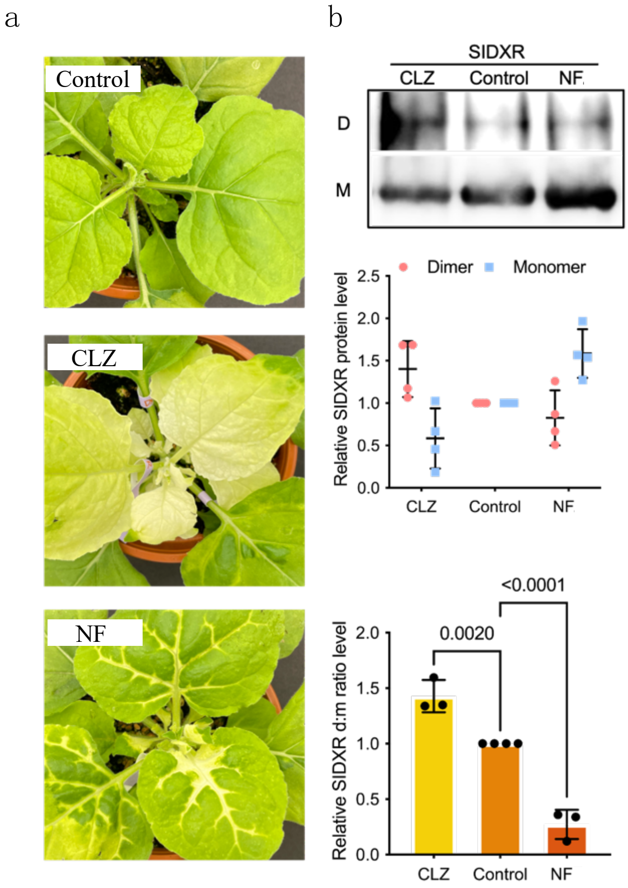

Figure 3. 7. Changing IPP/ DMAPP levels modulate monomerization of SlDXR in plants. Leaves from *N. benthamiana* plants were infiltrated with constructs to produce SlDXR together with either water (control) or the indicated inhibitors, and samples were collected 5 days later. (a) Representative images of the leaves. (b) Immunoblot analyses of protein extracts with anti-DXR antibodies. The position of dimers (D) and monomers (M) is indicated in the upper panel, which shows the result of a representative experiment. Quantification of D and M abundance from immunoblot analyses of n≥3 independent experiments is represented in the plots. Individual data points as well as mean and standard deviation are represented. Numbers in the lower plots indicate p values (one-way ANOVA with Dunnett's multiple comparisons test).

3.3 Discussion

The MEP pathway produces IPP and DMAPP, the universal precursors of isoprenoids in bacteria and the plastids of plants. Compared to the alternative MVA pathway that produces IPP and DMAPP in archaea and the cytosol of eukaryotic cells, the MEP pathway has a higher theoretical capacity to produce isoprenoid precursors (Rude & Schirmer, 2009; Vickers et al., 2015). However, biotechnological approaches directed towards increasing the production of isoprenoids of interest by up-regulating the MEP pathway to supply more IPP and DMAPP precursors have obtained results that are still far from the predicted maximum yields of the pathway. In part, this might be due to the feedback regulation of the pathway flux by IPP and DMAPP, which slows down the pathway flux when IPP and DMAPP are not consumed and accumulate. Therefore, understanding this feedback regulation can provide useful tools for promoting the production of MEP-derived precursors. Because DXP is an intermediate of non-isoprenoid side pathways (Julliard & Douce, 1991), DXR is actually considered as the first committed enzyme in the MEP pathway. Why DXR controls the metabolic flux of the pathway is some species and tissues but not in others is still unclear (Li et al., 2020).

Research on DXS, the main rate-limiting enzyme of the MEP pathway, showed that IPP and DMAPP inhibit DXS activity (Banerjee et al., 2013a; Ghirardo et al., 2014a). Our results indicate that the underlying mechanism involves binding of IPP and DMAPP, which rapidly causes a decreased activity by promoting monomerization (Di et al., 2022) (Chapter 2). Here we report for the first time that IPP and DMAPP also inhibit the activity of DXR *in vitro* (Fig. 3.1). On the contrary, the activity of the DXR-like enzyme DRL was not influenced by the presence of changing IPP and DMAPP levels in the reaction media. Similarly, DRL was found to resist to FSM, a competitive inhibitor of DXR, because of the unique arrangement of the DRL active site (Sangari et al., 2010; Steinbacher et al., 2003). Strikingly, ITC experiments demonstrated that IPP and DMAPP can directly interact with both DXR and DRL (Fig. 3.2). This binding only resulted in structural changes detected by DLS in the case of DXR when incubated with 10μM DMAPP, but not with higher concentrations of DMAPP (Fig. 3.5). Binding of IPP and DMAPP to DRL did not result in structural changes (Fig. 3.5), perhaps explaining the lack of effect observed in enzyme activity (Fig. 3.1). Further experiments in bacteria (Fig. 3.6) and plant systems (Fig. 3.7) confirmed that increasing IPP/DMAPP levels promoted monomerization of DXR *in vivo*. The DXR monomerization process was slow compared with DXS (as it was observed at 3 h for DXS but at least 15 h for DXR after increasing IPP/DMAPP levels in bacteria). Moreover, according to ITC results, the binding affinities based on the calculated association constants (Ka) were 4.5 $x10^5$ (IPP) and 5.8 $x10^5$ (DMAPP) for DXS but an order of magnitude higher for DXR, 5.08 $x10^6$ (IPP) and 2.6 $x10^7$ (DMAPP) (Supplemental Table 3.S1). This means that IPP/DMAPP bind to DXS with less affinity than to DXR, and that DMAPP binds to DXR with more affinity than IPP. The differences in affinities strongly suggest unique binding mechanisms. In the case of DXR, computational modeling suggests that IPP and DMAPP might compete with NADPH to inhibit the activity of DXR (Fig. 3.3). We speculate that this mechanism might be the main responsible for the drop in DXR activity detected in the *in vitro* activity assays when supplemented with IPP/DMAPP (Fig. 3.1). Moreover, DMAPP can potentially bind to the DXR monomer-monomer interaction surface. Perhaps this binding is what eventually destabilizes the DXR dimer, resulting in the monomerization observed *in vivo* (Fig. 3.6 and 3.7). The crystal structure analysis of DXR-IPP and DXR-DMAPP would be necessary to ascertain the binding mechanism and to explain the observed effects on DXR activity and dimerization status. The same is true for DRL, an enzyme whose unique active site arrangement leads to resist inhibition by FSM (Pérez-Gil et al., 2012) and repression by IPP/DMAPP (Fig. 3.1 and 3.4). It is remarkable that, despite the overall similarity of the binding of FSM, IPP and DMAPP to the active site of DRL and DXR, none of the residues participating directly in their binding are structurally equivalent. It is therefore likely that the similar mechanism prevent inhibition of DRL enzyme activity by FSM and IPP/DMAPP. This is a very interesting feature that makes DRL a super-enzyme insensitive to feedback regulation by the MEP pathway products and a promising new tool for new biotechnological approaches for the metabolic engineering of isoprenoids in plant and bacteria bio factories.

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General discussion

General discussion

In most bacteria and the plastids of plants, IPP and DMAPP, the universal precursors of all isoprenoids, are simultaneously produced by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Rodríguez-Concepción & Boronat, 2002; Rohdich et al., 2003). Compared to the mevanolic acid (MVA) pathway present in archaea, some bacteria, and the cytosol of eukaryotic cells, the MEP pathway has a higher theoretical capacity for the production of isoprenoid precursors, which has stimulated extensive research on further improving MEP pathway flux in microbial and plant systems (Rude & Schirmer, 2009; Vickers et al., 2015). However, biotechnological approaches directed towards increasing the production of isoprenoids of interest by up-regulating the MEP pathway to supply more IPP and DMAPP precursors have obtained results that are still far from the predicted maximum yields of the pathway. In part, this might be due to the feedback regulation of the pathway flux by IPP and DMAPP, which slows down the pathway flux when IPP and DMAPP are not consumed and hence accumulate. Therefore, understanding this feedback regulation can provide useful biotechnological tools for promoting the production of MEP-derived precursors.

In the MEP pathway, DXS catalyzes the production of DXP and controls the flux of the whole pathway as the main rate-limiting enzyme (Volke et al., 2019; Wright & Phillips, 2014). Corresponding with the primary regulatory role of DXS, its enzymatic activity is tightly regulated at many different levels, including post-translational levels (Banerjee & Sharkey, 2014; Hemmerlin, 2013; Rodríguez-Concepción & Boronat, 2015). Among them, DXS was the only MEP pathway enzyme identified to be feedback regulated by IPP/DMAPP at the beginning of this PhD thesis. The specific molecular mechanism behind this feedback regulation was unknown. On the other hand, DXP is an intermediate of non-isoprenoid side pathways (Julliard & Douce, 1991) and therefore the first committed step of the MEP pathway is the conversion of DXP into MEP catalyzed by DXR in most organisms and DRL in just a few groups of bacteria (Pérez-Gil & Rodríguez-Concepcíon, 2013). Why DXR controls the metabolic flux of the pathway is some species and tissues but not in others is still unclear (Li et al., 2020). In this PhD work we show for the first time that DXR but not DRL is also feedback regulated by IPP/DMAPP, even though the mechanism might differ from that regulating DXS. Besides unveiling these mechanisms, in this work we also characterized the tomato DXS family, formed by three paralogs of which only two (SlDXS1 and SlDXS2) are true DXS enzymes. DXR is encoded by a single gene in tomato and many other plants.

4.1 The tomato DXS family

In tomato, DXS is encoded by 3 different genes encoding proteins which belong to DXS class 1 (SlDXS1). class 2 (SlDXS2) and class 3 (SlDXS3), respectively. Besides this phylogenetic classification and gene expression analyses, information on these three isoforms is scattered and incomplete in the literature. *SIDXS1* is expressed at high levels during plant development, consistent with a role of housekeeping gene which contributes to phytohormone and photosynthetic pigment synthesis but it is also required to support carotenoid synthesis during fruit ripening (Kim et al., 2005; Lois et al., 2000). *SIDXS2* is expressed at tissues (e.g., leaf trichomes) and conditions associated to the synthesis of specific isoprenoids involved in defense responses and signaling (Paetzold et al., 2010; Walter et al., 2002). And *SIDXS3* is expressed at very low levels and encodes a protein considered to lack DXS activity (Luna-Valdez et al., 2021). Using our DXS-deficient *E. coli* strain we confirmed that both SIDXS1 and SIDXS2 are true DXS enzymes, confirming previous research (Lois et al., 2000; Paetzold et al., 2010). We also confirmed the prediction that SIDXS3 lacks DXS activity (Luna-Valdez et al., 2021).

Computational modeling showed that SlDXS1, SlDXS2 and SlDXS3 had similar protein structures. Two monomers were arranged side by side to form a dimer protein, with each monomer containing three domains. The residues in the active site are strictly conserved in *E. coli*, *D. radiodurans* and *A. thaliana* DXS sequences (His80/82/146, Asp152/154/208, Ile368/371/448, Glu370/373/450/418, and Phe395/398/475, respectively). We found that loss of cofactor (TPP) binding might explain the lack of DXS activity in SlDXS3, as previously suggested (Luna-Valdez et al., 2021).

Using fusions to fluorescent proteins, SlDXS1 and SlDXS2 were confirmed to be located in plastids, where the MEP pathway takes place. Moreover, the subplastidial localization of SlDXS1 and SlDXS2 was found to be overlapping. Co-immunoprecipitation assays further showed that SlDXS1 and SlDXS2 can interact in vivo, whereas structure modeling confirmed that SlDXS1 and SlDXS2 monomers could form catalytically active heterodimers very similar to DXS homodimers. The potential biological function of such heterodimers is difficult to predict. In the tomato plant, SlDXS1 and SlDXS2 transcripts are found in many tissues, but sometimes they show opposite accumulation profiles (e.g. during fruit ripening and seed development). Interestingly, they are expressed at similar levels in young (1-2cm) fruit, suggesting that SlDXS1-SlDXS2 heterodimers might be preferentially formed at phases of rapid growth. It is also possible that SlDXS3 may interact with SlDXS1 or SlDXS2 to form SlDXS1-SlDXS3 or SlDXS2-SlDXS3 heterodimers that might contribute to decrease the DXS activity of SlDXS1 and SlDXS2 isoforms. However, the virtually constitutive profile and low expression level of SlDXS3 argues against a major regulatory role for this protein.

4.2 Feedback-regulation of the MEP pathway: not one but multiple checkpoints to control metabolic flux

SIDXS1 was selected to investigate the feedback regulation mechanism together with the *E.coli* DXS (EcDXS) enzyme. Our ITC experiments using SlDXS1 in the presence of an excess of TPP, IPP and DMAPP confirmed that these three small diphosphate metabolites could indeed interact with the enzyme but showed Kd values for IPP and DMAPP that were very similar in the absence or presence of the TPP cofactor. These results argue against a direct competition of IPP/DMAPP for the TPP binding site in DXS as originally proposed (Banerjee et al., 2013b). Instead. Our results suggest the existence of a truly allosteric mechanism behind the inhibition of DXS activity by IPP/DMAPP. Allosteric regulation of protein function occurs when the binding of a molecule either activating or inhibiting the activity of the protein takes place away from the active site.

Further analysis of the effect of IPP/DMAPP on EcDXS and SlDXS1 structure confirmed a decreased dimer to monomer ratio when levels of IPP and DMAPP increased in living bacteria and plant cells. Changes in the dimer:monomer ratio triggered by allosteric regulation has been observed in different proteins, including receptors (Petersen et al., 2017), membrane translocators (Jaipuria et al., 2017) and enzymes (Seetoh & Abell, 2016). In the case of DXS, however, IPP/DMAPP-mediated monomerization was found to be linked to increased aggregation both in bacteria and plastids, likely because isolated monomers expose hydrophobic stretches that are otherwise hidden in the dimer. We propose that DXS monomerization might be a relatively fast and reversible response to high IPP/DMAPP levels. If excess IPP/DMAPP persist, monomers aggregate and they are eventually degraded (Fig. 4.1).

Here we found for the first time that IPP and DMAPP inhibit the activity of EcDXR but not BaDRL despite ITC experiments demonstrated that IPP and DMAPP can directly interact with both enzymes. This binding only resulted in structural changes detected by DLS in the case of DXR when incubated with 10µM DMAPP, but not with IPP or with higher concentrations of DMAPP. Further experiments in bacteria and plant systems confirmed that increasing IPP/DMAPP levels promoted DXR monomerization *in vivo*. The DXR monomerization process was slow compared with that observed for DXS (as it only took 3h for DXS but at least 15h for DXR after increasing IPP/DMAPP levels in bacteria). Moreover, according to ITC results, the binding affinities based on the calculated association constants (Ka) were one (IPP) or two (DMAPP) orders of magnitude higher for DXR compared to DXS. This means that IPP/DMAPP bind to DXS with less affinity than to DXR, and that DMAPP binds to DXR with more affinity than IPP. These differences in affinities strongly suggest unique binding mechanisms.

Unlike DXS, DXR is an NADPH-dependent enzyme (Koppisch et al., 2001; Kuzuyama et al., 2000; Takahashi et al., 1998). A flexible 'lid' exists in an open conformation on the active site when the NADPH cofactor and the DXP substrate are absent, and it closes upon NADPH and DXP binding to shield the active site from the solvent (Sweeney et al., 2005). Computational modeling suggests that the phosphonate moiety of IPP and DMAPP compete for the active site with the phosphonate moiety of NADPH. Therefore, excess IPP/DMAPP might rapidly inhibit the activity of DXR by preventing the binding of the NADPH cofactor. We speculate that this mechanism might be the main responsible for the drop in DXR activity detected in the *in vitro* activity assays when supplemented with IPP/DMAPP. Moreover, DMAPP can potentially bind to the DXR monomer-monomer interaction surface. Perhaps this binding is what eventually destabilizes the DXR dimer, resulting in the monomerization observed *in vivo*. We therefore propose that, similar to that described for DXS, regulation of DXR activity by IPP/DMAPP might have two phases even though the mechanisms appear to be different for these two enzymes (Fig. 4.1). In the case of DXR, completion between IPP/DMAPP and NADPH for active site would define the first (fast and reversible) phase, whereas DMAPP-triggered monomerization would be a second (slow and non-reversible) phase (Fig. 4.1).

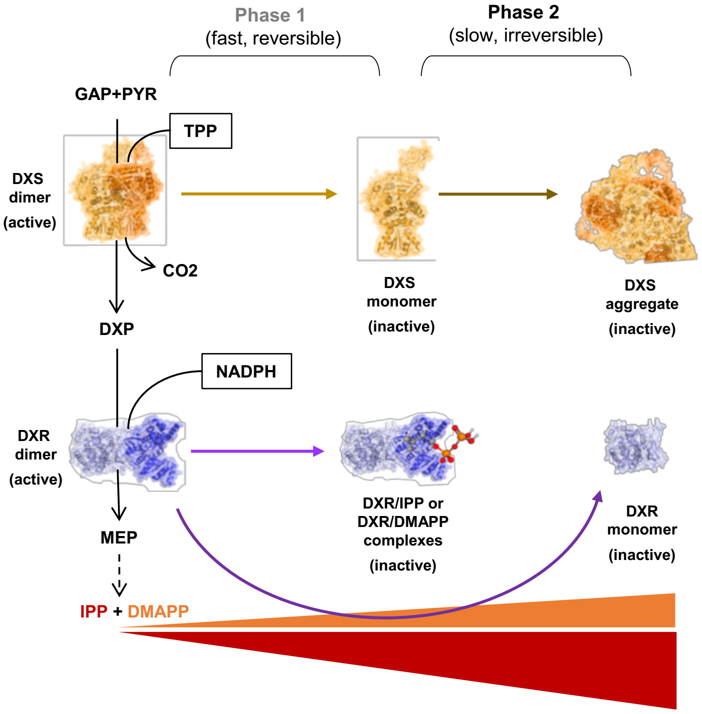

Figure 4. 1. Multiple checkpoints for the feedback control of the MEP pathway flux. DXS and DXR are dimeric enzymes that catalyze the first two steps of the MEP pathway for the production of a 5:1 ratio of IPP and DMAPP (represented by triangles of different size). Accumulation of IPP/DMAPP can be toxic for the cell. To prevent deleterious effects and, at the same time, adjust IPP/DMAPP supply to cell needs, different feedback mechanisms regulate DXS and DXR activities as represented.

4.3 DRL is immune to IPP/DMAPP feedback regulation

DRL catalyzes the same reaction as DXR, but they have low structural relationship especially at the active domain (Pérez-Gil et al., 2012; Sangari et al., 2010). This structural difference allows DRL to resist inhibition with FSM, a specific inhibitor of DXR, even though FSM binding to DXR and DRL is overall similar (Pérez-Gil et al., 2012; Sangari et al., 2010). According to enzyme activity assays, the activity of DRL is not inhibited by IPP/DMAPP even though ITC experiments demonstrated that IPP and DMAPP can directly interact with DRL. Computational modeling suggests that both IPP and DMAPP might bind to the active site of the BaDRL protein but to residues different from those in DXR (Fig. 3.4b). It is therefore likely that a similar mechanism prevents inhibition of DRL enzyme activity by FSM and IPP/DMAPP.

The insensitivity to feedback inhibition by IPP/DMAPP is a very interesting feature that makes DRL a super-enzyme insensitive to feedback regulation by the MEP pathway products and a promising new tool for new biotechnological approaches for the metabolic engineering of isoprenoids in plant and bacteria bio-factories.

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Conclusions

Conclusions

1. Tomato has three DXS-like sequences, but the DXS3 isoform does not have DXS activity.
2. The absence of DXS activity of DXS3 is likely due to its lacking a conserved TPP binding domain.
3. Tomato DXS1 and DXS2 isoforms can interact and form heterodimers.
4. IPP and DMAPP allosterically inhibit the activity of bacterial and plant (tomato) DXS by directly binding away from the active site of the enzyme.
5. IPP and DMAPP binding promotes monomerization of the active DXS dimer.
6. DXS monomers can form aggregates that are eventually degraded.
7. IPP and DMAPP also feedback inhibit DXR activity likely by competing with the NADPH cofactor for the active site.
8. DMAPP may also allosterically inhibit DXR by monomerization of the active dimer.
9. The activity of DRL is not affected by IPP and DMAPP.

Appendices

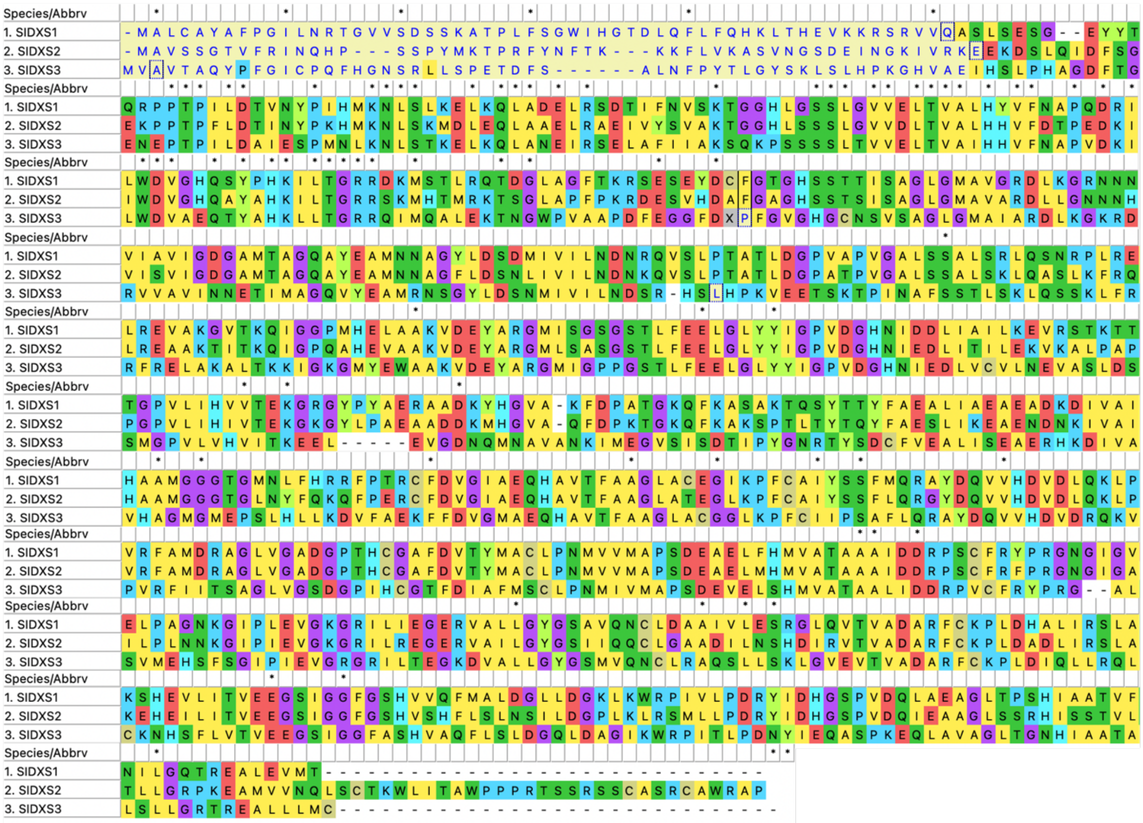
Appendices

List of primers used in this work

Primer Name	Sequence (5' to 3')	Gene ID	Use
SlDXS1-F	ATCATGGCTTTGTGTGCTTATGCATTTCCTG	Solyc01g067890	Cloning
Nhel-Myc-SlDXS1-F	ATGGCTAGCGAACAAAAACTCATCTCAGAAGAGGATCTGGCTTCCTTATCAGAATCTGG	Solyc01g067890	Cloning
SlDXS1-XhoL-R	CTTACTCGAGTGTCATGACCTCTAGAGCCTCTC	Solyc01g067890	Cloning
SlDXS2-F	CAGATGGCAGTTTCTTCAGGCACTGTATTTAG	Solyc11g010850	Cloning
Ncol-HA-SlDXS2-F	ATACCATGGACTACCCATACGATGTTCCAGATTACGCTGAAAAAGATAGTTTGCAGATTG	Solyc11g010850	Cloning
SlDXS2-SalI-R	CTTAGTCGACGTTTACTACCATAGCTTCTTTTG	Solyc11g010850	Cloning
SlDXS3-F	TGATGGTTGCTGTTACTGCTCAGTACCCATTTG	Solyc08g066950	Cloning
Nhel-FLAG-SlDXS3-F	ATGGCTAGCGACTACAAAGACGATGACGACAAGCATTCTTTGCCACATGCTGGTG	Solyc08g066950	Cloning
SlDXS3-SalL-R	GTTAGTCGACGCACATCAAAAGAAGAGCTTCTC	Solyc08g066950	Cloning
GateWaySIDXS1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGCTTTGTGTGCTTATGCAT	Solyc01g067890	Gateway cloning
GateWaySIDXS1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTCATGACCTCTAGAGC	Solyc01g067890	Gateway cloning
GateWaySIDXS2-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGCAGTTTCTTCAGGCACT	Solyc11g010850	Gateway cloning
GateWaySIDXS2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTTACTACCATAGCTTC	Solyc11g010850	Gateway cloning
GateWaySIDXS3-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGTTGCTGTTACTGCTCAG	Solyc08g066950	Gateway cloning
GateWaySIDXS3-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCACATCAAAAGAAGAGC	Solyc08g066950	Gateway cloning
SIDXR-F	ATCATGGCCCTCAATTTGCTTTCTCCTGCTG	Solyc03g114340	Cloning
NdeI-Myc-SIDXR-F	ACTCATATGGAACAAAAACTCATCTCAGAAGAGGATCTGGCCTGGCCTGGAAGGGCTGT	Solyc03g114340	Cloning
SIDXR-NotI-R	ACAGCGGCCGCTACAAGAGCTGGACTCAAAC	Solyc03g114340	Cloning
GateWaySIDXR-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGCCCTCAATTTGCTTTCTC	Solyc03g114340	Gateway cloning
GateWaySIDXR-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACAAGAGCTGGACTCAA	Solyc03g114340	Gateway cloning

List of constructs used in this work

Plasmids	**Backbone**	**Antibiotic**	**Cloning method**	**Tag**
pBSK-SIDXS1	pBSK	Ampicillin	Ligation	-
pBSK-SIDXS2	pBSK	Ampicillin	Ligation	-
pBSK-SIDXS2	pBSK	Ampicillin	Ligation	-
pET23a-SIDXS1-	pET23	Ampicillin	Ligation	6His(C-terminal)
pET23d-SIDXS2	pET23	Ampicillin	Ligation	6His(C-terminal)
pET23a-SIDXS3	pET23	Ampicillin	Ligation	6His(C-terminal)
pET23a-EcDXR	pET23	Ampicillin	Ligation	6His(C-terminal)
pET23a-BaDRL	pET23	Ampicillin	Ligation	6His(C-terminal)
pGWB405-SIDXS1	pGWB405	Spectinomycin	Gateway	GFP(C-terminal)
pGWB420-SIDXS1	pGWB420	Spectinomycin	Gateway	Myc(C-terminal)
pGWB454-SIDXS1	pGWB454	Spectinomycin	Gateway	RFP(C-terminal)
pGWB405-SIDXS2	pGWB405	Spectinomycin	Gateway	GFP(C-terminal)
pGWB414-SIDXS2	pGWB414	Spectinomycin	Gateway	HA(C-terminal)
pGWB420-SIDXR	pGWB420	Spectinomycin	Gateway	Myc(C-terminal)
pGWB420-RPK	pGWB420	Spectinomycin	Gateway	Myc(C-terminal)



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1. SIDXS1  -MALCAYAFPGILNRTGVVSDSSKATPLFSGWIHGTDLQFLFQHKLTHEVKKRSRVVQASLSESG--EYYT
2. SIDXS2  -MAVSSGTVFRINQHP---SSPYMKTPRFYNFTK---KKFLVKASVNGSDEINGKIVRKEEKDSLQIDFSG
3. SIDXS3  MVAVTAQYPFGICPQFHGNSRLLSPETDFS------ALNFPYTLGYSKLSLHPKGHVAEIHSLPHAGDFTG

1. SIDXS1  QRPPTPILDTVNYPIHMKNLSLKELKQLADELRSDTIFNVSKTGGHLGSSLGVVELTVALHYVFNAPQDRI
2. SIDXS2  EKPPTPFLDTINYPKHMKNLSKMDLEQLAAELRAEIVYSVAKTGGHLSSSLGVVDLTVALHHVFDTPEDKI
3. SIDXS3  ENEPTPILDAIESPMNLKNLSTKELKQLANEIRSELAFIIAKSQKPSSSSLTVVELTVAIHHVFNAPVDKI

1. SIDXS1  LWDVGHQSYPHKILTGRRDKMSTLRQTDGLAGFTKRSESEYDCFGTGHSSTTISAGLGMAVGRDLKGRNNN
2. SIDXS2  IWDVGHQAYAHKILTGRRSKMHTMRKTSGLAPFPKRDESVHDAFGAGHSSTSISAGLGMAVARDLLGNNNH
3. SIDXS3  LWDVAEQTYAHKLLTGRRQIMQALEKTNGWPVAAPDFEGGFDXPFGVGHGCNSVSAGLGMAIARDLKGKRD

1. SIDXS1  VIAVIGDGAMTAGQAYEAMNNAGYLDSDMIVILNDNRQVSLPTATLDGPVAPVGALSSALSRLQSNRPLRE
2. SIDXS2  VISVIGDGAMTAGQAYEAMNNAGFLDSNLIVILNDNKQVSLPTATLDGPATPVGALSSALSKLQASLKFRQ
3. SIDXS3  RVVAVINNETIMAGQVYEAMRNSGYLDSNMIVILNDSR-HSLHPKVEETSKTPINAFSSTLSKLQSSKLFR

1. SIDXS1  LREVAKGVTKQIGGPMHELAAKVDEYARGMISGSGSTLFEELGLYYIGPVDGHNIDDLIAILKEVRSTKTT
2. SIDXS2  LREAAKTITKQIGPQAHEVAAKVDEYARGMLSASGSTLFEELGLYYIGPVDGHNIEDLITILEKVKALPAP
3. SIDXS3  RFRELAKALTKKIGKGMYEWAAKVDEYARGMIGPPGSTLFEELGLYYIGPVDGHNIEDLVCVLNEVASLDS

1. SIDXS1  TGPVLIHVVTEKGRGYPYAERAADKYHGVA-KFDPATGKQFKASAKTQSYTTYFAEALIAEAEADKDIVAI
2. SIDXS2  PGPVLIHIVTEKGKGYLPAEAADDKMHGVA-QFDPKTGKQFKAKSPTLTYTQYFAESLIKEAENDNKIVAI
3. SIDXS3  SMGPVLVHVITKEEL-----EVGDNQMNAVANKIMEGVSISDTIPYGNRTYSDCFVEALISEAERHKDIVA

1. SIDXS1  HAAMGGGTGMNLFHRRFPTRCFDVGIAEQHAVTFAAGLACEGIKPFCAIYSSFMQRAYDQVVHDVDLQKLP
2. SIDXS2  HAAMGGGTGLNYFQKQFPERCFDVGIAEQHAVTFAAGLATEGLKPFCAIYSSFLQRGYDQVVHDVDLQKLP
3. SIDXS3  VHAGMGMEPSLHLLKDVFAEKFFDVGMAEQHAVTFAAGLACGGLKPFCIIPSAFLQRAYDQVVHDVDRQKV

1. SIDXS1  VRFAMDRAGLVGADGPTHCGAFDVTYMACLPNMVVMAPSDEAELFHMVATAAAIDDRPSCFRYPRGNGIGV
2. SIDXS2  VRFAMDRAGLVGADGPTHCGAFDVTYMACLPNMVVMAPSDEAELMHMVATAAAIDDRPSCFRFPRGNGIGA
3. SIDXS3  PVRFIITSAGLVGSDGPIHCGTFDIAFMSCLPNMIVMAPSDEVELSHMVATAALIDDRPVCFRYPRG--AL

1. SIDXS1  ELPAGNKGIPLEVGKGRILIEGERVALLGYGSAVQNCLDAAIVLESRGLQVTVADARFCKPLDHALIRSLA
2. SIDXS2  ILPLNNKGIPIEVGKGRILREGERVAILGYGSIIQQCLGAADILNSHDIRVTVADARFCKPLDADLIRSLA
3. SIDXS3  SVMEHSFSGIPIEVGRGRILTEGKDVALLGYGSMVQNCLRAQSLLSKLGVEVTVADARFCKPLDIQLLRQL

1. SIDXS1  KSHEVLITVEEGSIGGFGSHVVQFMALDGLLDGKLKWRPIVLPDRYIDHGSPVDQLAEAGLTPSHIAATVF
2. SIDXS2  KEHEILITVEEGSIGGFGSHVSHFLSLNSILDGPLKLRSMLLPDRYIDHGSPVDQIEAAGLSSRHISSTVL
3. SIDXS3  CKNHSFLVTVEEGSIGGFASHVAQFLSLDGQLDAGIKWRPITLPDNYIEQASPKEQLAVAGLTGNHIAATA

1. SIDXS1  NILGQTREALEVMT-------------------------------
2. SIDXS2  TLLGRPKEAMVVNQLSCTKWLITAWPPPRTSSRSSCASRCAWRAP
3. SIDXS3  LSLLGRTREALLLMC-------------------------------
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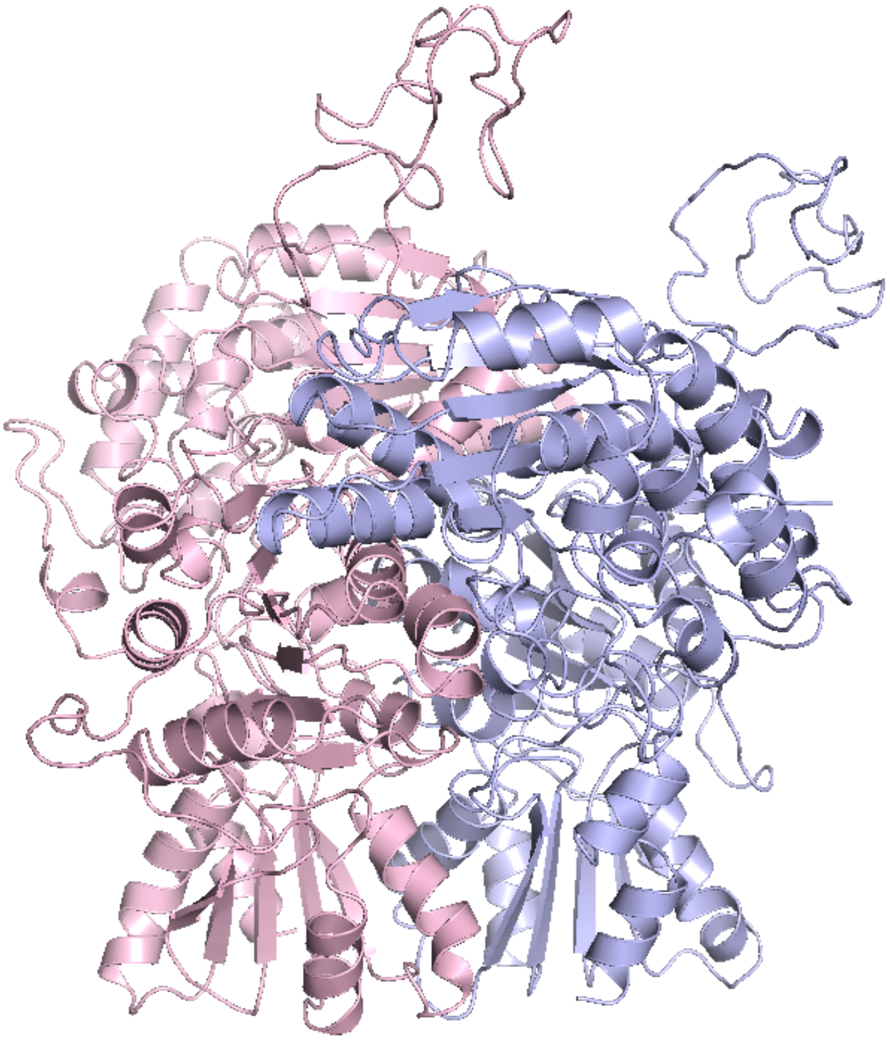
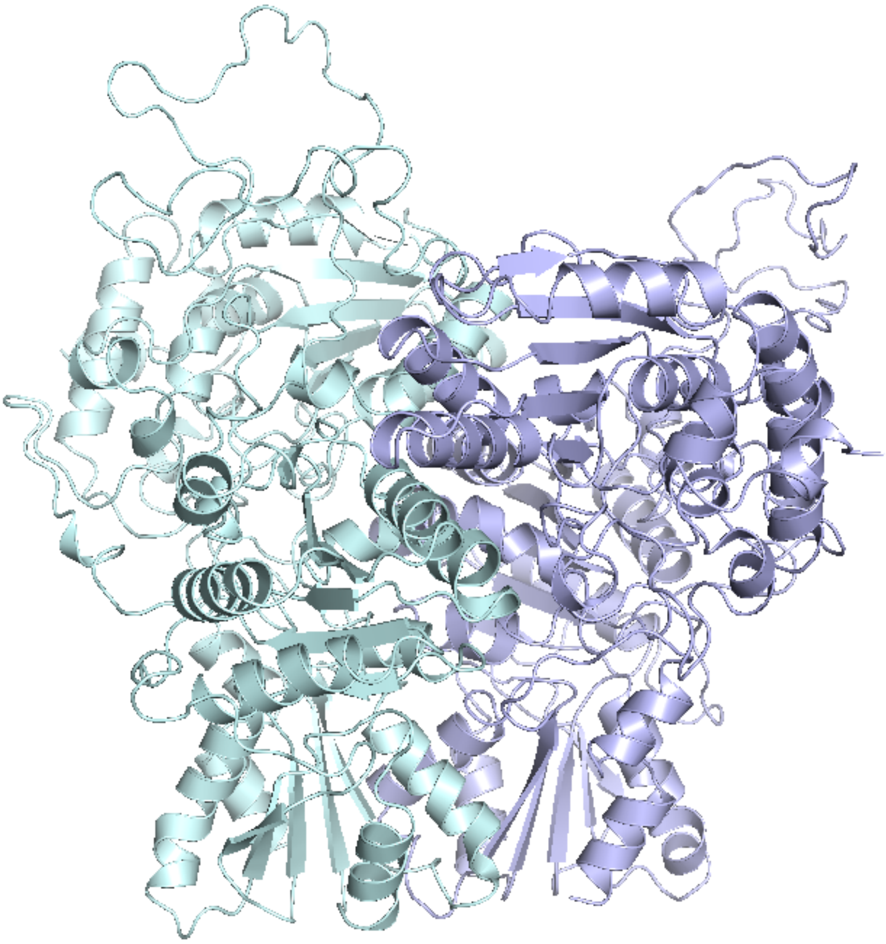
Supplemental Figure 1. Alignment of SIDXS1, SIDXS2 and SIDXS2 sequences. The predicted plastid-targeting sequences of three SIDXS isoforms highlight in yellow.

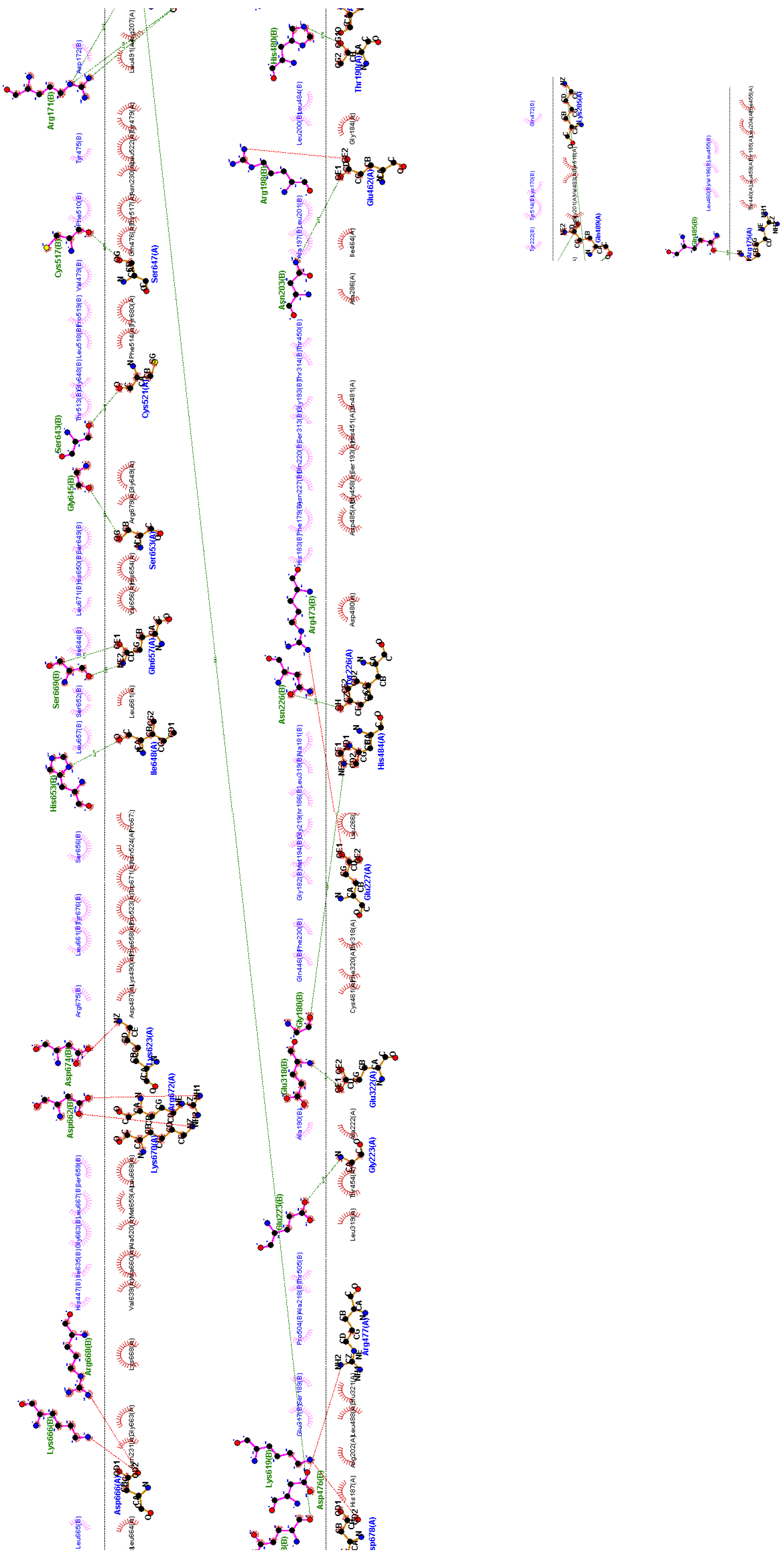


Supplemental Figure 2. The computational modeling of SlDXS1 and SlDXS3 heterodimer. SlDXS1 is colored in pink and SlDXS3 is colored in purple

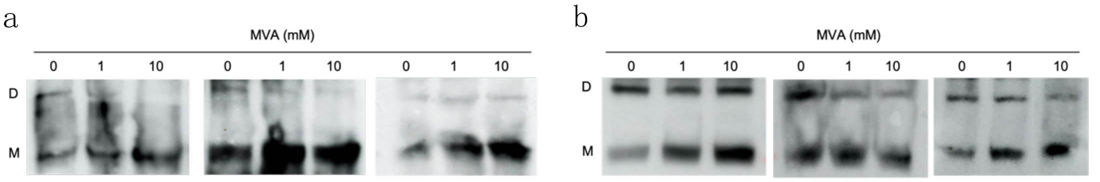


Supplemental Figure 3. The computational modeling of SlDXS2 and SlDXS3 heterodimer.

SlDXS2 is colored in blue and SlDXS3 is colored in purple



Supplemental Figure 4. The interaction surface of SIDXS1 and SIDXS2



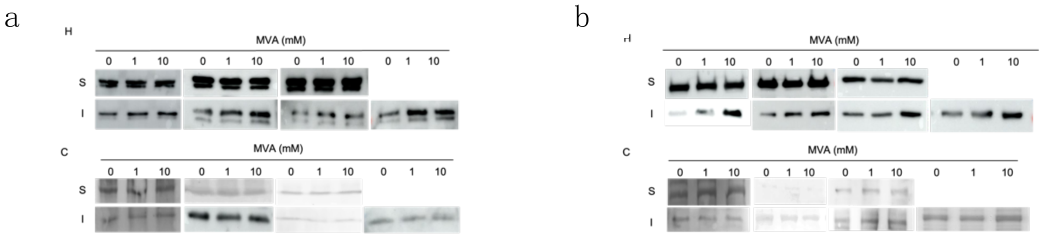
a

Rep.	MVA Concentration (mM)	Dimer	Monomer	Dimer/ Monomer	Relative ratio	Relative dimer	Relative monomer
1	0	71.834	94.453	0.761	1.000	1.000	1.000
	1	65.191	119.885	0.544	0.715	0.908	1.269
	10	12.568	132.114	0.095	0.125	0.175	1.399
2	0	50.559	67.908	0.745	1.000	1.000	1.000
	1	39.010	82.719	0.472	0.633	0.772	1.218
	10	10.118	122.750	0.082	0.111	0.200	1.808
3	0	37.788	86.555	0.437	1.000	1.000	1.000
	1	36.265	124.935	0.290	0.665	0.960	1.443
	10	27.177	157.356	0.173	0.396	0.719	1.818

b

Rep.	MVA Concentration (mM)	Dimer	Monomer	Dimer : Monomer	Relative ratio	Relative dimer	Relative monomer
1	0	101.943	69.870	1.459	1.000	1.000	1.000
	1	99.986	124.594	0.802	0.550	0.981	1.783
	10	105.299	177.574	0.593	0.406	1.033	2.541
2	0	104.820	103.749	1.010	1.000	1.000	1.000
	1	58.769	108.577	0.541	0.536	0.561	1.047
	10	45.399	98.142	0.463	0.458	0.433	0.946
3	0	77.000	59.247	1.300	1.000	1.000	1.000
	1	77.189	109.667	0.704	0.542	1.002	1.851
	10	40.131	121.959	0.329	0.253	0.521	2.058

Supplemental Figure 5. Immunoblot analysis of DXS monomers and dimers in bacteria. EcAM5-1 cells were transformed with constructs to express 6xHis-tagged versions of EcDXS (a) and SlDXS (b) enzymes and positive transformants were grown in media supplemented with the indicated amounts of MVA. Upper panels show representative images of immunoblot analyses with anti-6xHis antibodies. The position of dimers (D) and monomers (M) is indicated in the upper panels, which shows the result of independent representative experiments. The Table indicates the intensity of the bands in the images quantified with ImageJ, as well as the ratio of dimer to monomer and relative values compared to controls without MVA supplementation.



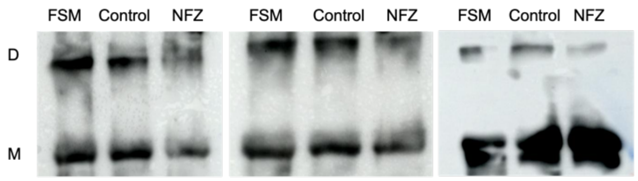
a

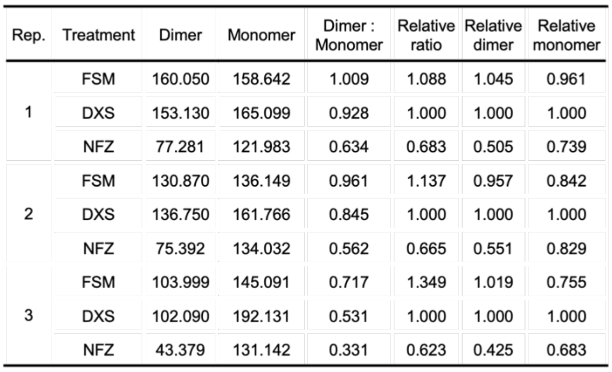
Rep.	MVA Concentration (mM)	Soluble	Control	Soluble : Control	Relative soluble	Insoluble	Control	Insoluble : Control	Relative insoluble	Insoluble : Soluble
1	0	176.953	60.128	2.943	1.000	117.252	56.761	2.066	1.000	1.000
	1	168.673	56.609	2.980	1.012	163.221	43.872	3.720	1.801	1.779
	10	175.182	58.257	3.007	1.022	176.730	52.305	3.379	1.636	1.601
2	0	183.829	56.349	3.262	1.000	95.094	49.936	1.904	1.000	1.000
	1	181.168	55.026	3.292	1.009	164.346	42.646	3.854	2.024	2.005
	10	185.287	60.108	3.083	0.945	158.181	38.427	4.116	2.162	2.288
3	0	219.326	103.764	2.114	1.000	112.081	162.403	0.690	1.000	1.000
	1	201.915	108.030	1.869	0.884	165.828	127.535	1.300	1.884	2.131
	10	209.963	103.292	2.033	0.962	196.708	145.660	1.350	1.957	2.035
4	0					132.990	62.441	2.130	1.000	
	1					197.811	59.536	3.323	1.560	
	10					195.211	55.714	3.504	1.645	

b

Rep.	MVA Concentration (mM)	Soluble	Control	Soluble : Control	Relative soluble	Insoluble	Control	Insoluble : Control	Relative insoluble	Insoluble : Soluble
1	0	183.648	60.716	3.025	1.000	52.149	124.537	0.419	1.000	1.000
	1	175.833	53.351	3.296	1.090	94.097	115.776	0.813	1.941	1.781
	10	154.724	54.084	2.861	0.946	170.305	110.507	1.541	3.680	3.891
2	0	198.631	74.541	2.665	1.000	121.690	62.663	1.942	1.000	1.000
	1	186.719	66.860	2.793	1.048	191.651	62.711	3.056	1.574	1.502
	10	193.074	68.900	2.802	1.052	206.426	65.500	3.152	1.623	1.543
3	0	165.390	145.044	1.140	1.000	157.290	120.841	1.302	1.000	1.000
	1	162.473	157.676	1.030	0.904	198.671	124.722	1.593	1.224	1.354
	10	173.540	156.927	1.106	0.970	201.579	124.866	1.614	1.240	1.279
4	0					103.203	207.849	0.497	1.000	
	1					136.963	208.470	0.657	1.323	
	10					206.680	209.953	0.984	1.983	

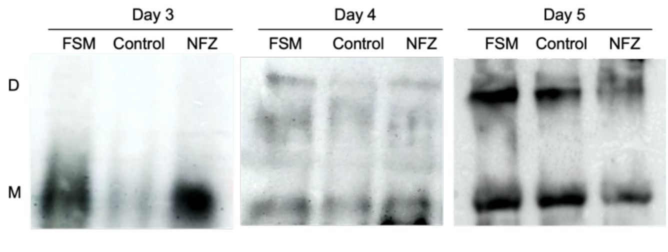
Supplemental Figure 6. Immunoblot analysis of DXS aggregates in bacteria. EcAM5 cells were transformed with constructs to express 6xHis-tagged versions of EcDXS (a) and SlDXS1 (b) enzymes and positive transformants were grown in media supplemented with the indicated amounts of MVA. Extracts corresponding to soluble (S) and insoluble/aggregated (I) proteins are indicated. (H) Immunoblot analyses with anti-6xHis antibodies. (C) Loading control by ponceau stain. The Table indicates the intensity of the bands in the images quantified with ImageJ, as well as ratios and relative values.



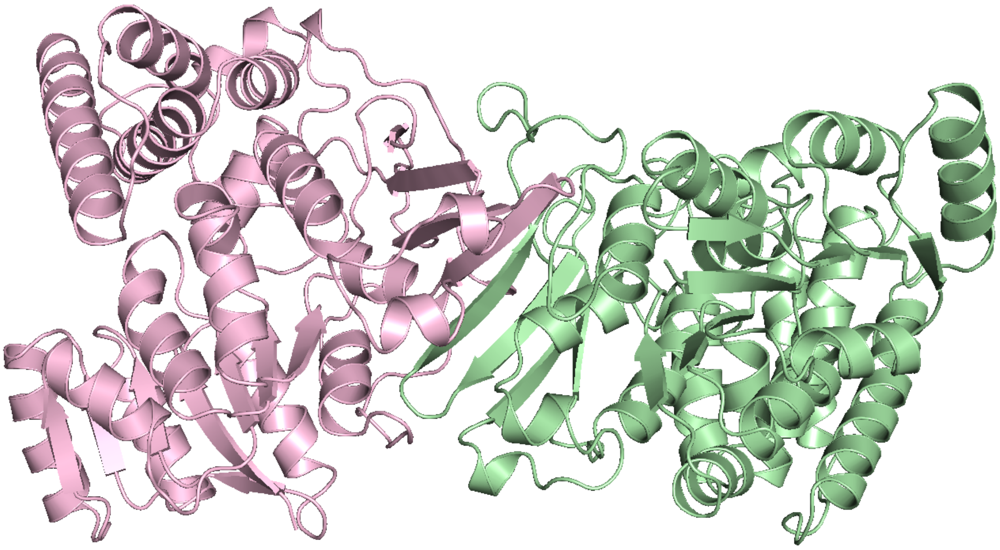


Rep.	Treatment	Dimer	Monomer	Dimer : Monomer	Relative ratio	Relative dimer	Relative monomer
1	FSM	160.050	158.642	1.009	1.088	1.045	0.961
	DXS	153.130	165.099	0.928	1.000	1.000	1.000
	NFZ	77.281	121.983	0.634	0.683	0.505	0.739
2	FSM	130.870	136.149	0.961	1.137	0.957	0.842
	DXS	136.750	161.766	0.845	1.000	1.000	1.000
	NFZ	75.392	134.032	0.562	0.665	0.551	0.829
3	FSM	103.999	145.091	0.717	1.349	1.019	0.755
	DXS	102.090	192.131	0.531	1.000	1.000	1.000
	NFZ	43.379	131.142	0.331	0.623	0.425	0.683

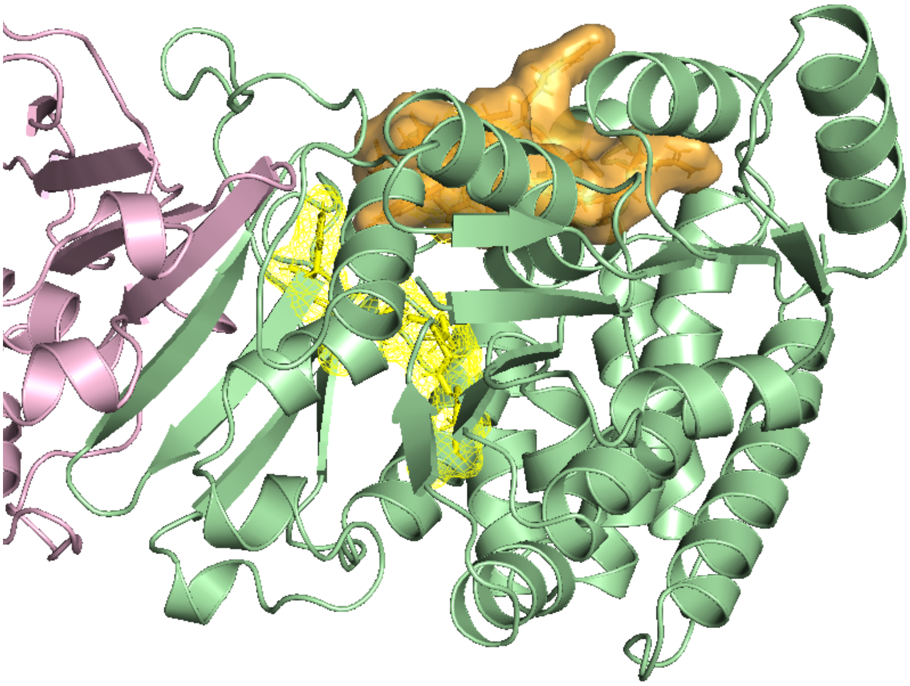
Supplemental Figure 7. Immunoblot analysis of DXS monomers and dimers in plant cells.
Leaves from *N. benthamiana* plants were infiltrated with constructs to produce SlDXS1 together with either water (control) or the indicated inhibitors, and samples were collected 5 days later. Immunoblot analyses of protein extracts were carried out with anti-DXS antibodies. The position of dimers (D) and monomers (M) is indicated in the upper panels, which shows the result of three independent representative experiments. The Table indicates the intensity of the bands in the images quantified with ImageJ, as well as the ratio of dimer to monomer and relative values compared to water controls.



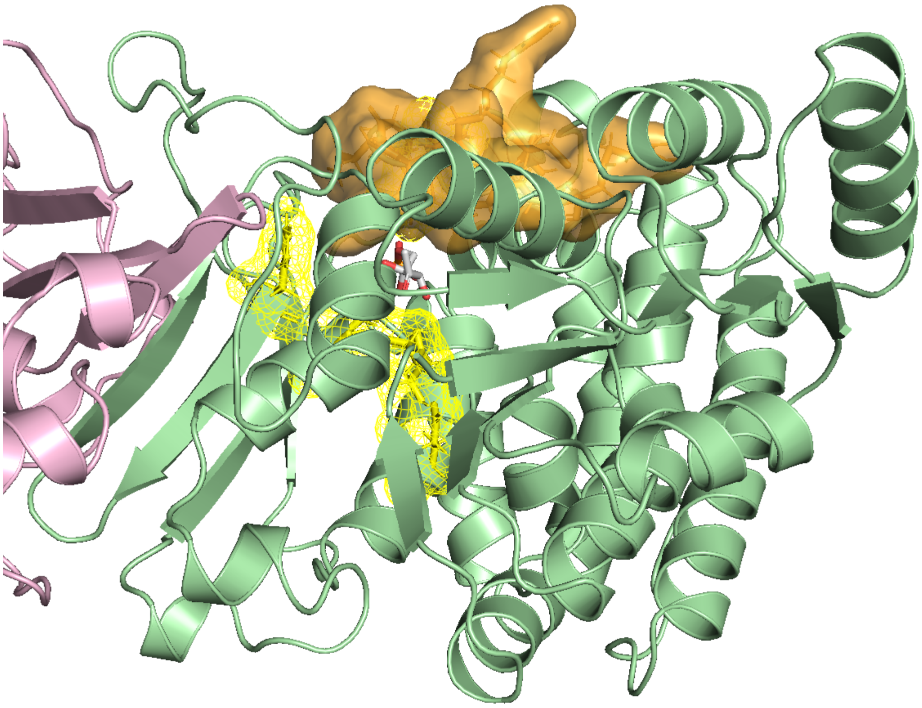
Supplemental Figure 8. Immunoblot analysis of SlDXS1 levels in N. benthamiana leaves at different days post-infiltration. Leaves from *N. benthamiana* plants were infiltrated with constructs to produce SlDXS1 together with either water (control) or the indicated inhibitors, and samples were collected after 3, 4 and 5 days. Immunoblot analyses with anti-DXS antibodies of protein extracts from representative experiment are shown. The position of dimers (D) and monomers (M) is indicated.



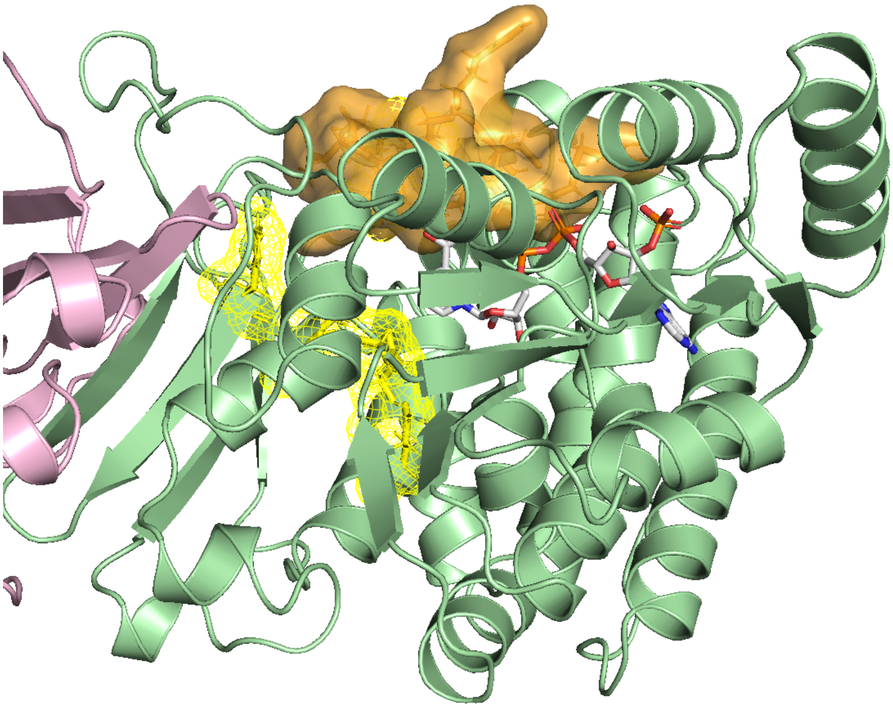
Supplemental Figure 9. The crystal structure of EcDXR (PDB: 1jvs)



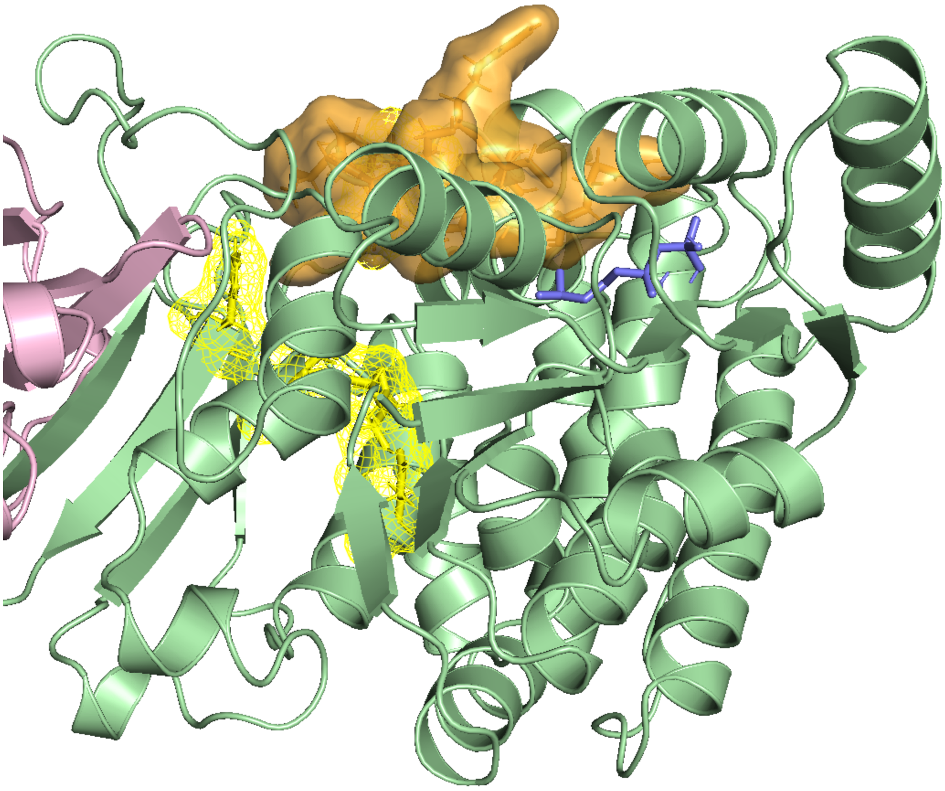
Supplemental Figure 10. The active site and lid of EcDXR. The flexible 'lid' (residues 206-216) is shown in orange, and the active site (conserved residues Asp150, Glu152, His 209, Glu231, Glu234 and His257) is in yellow.



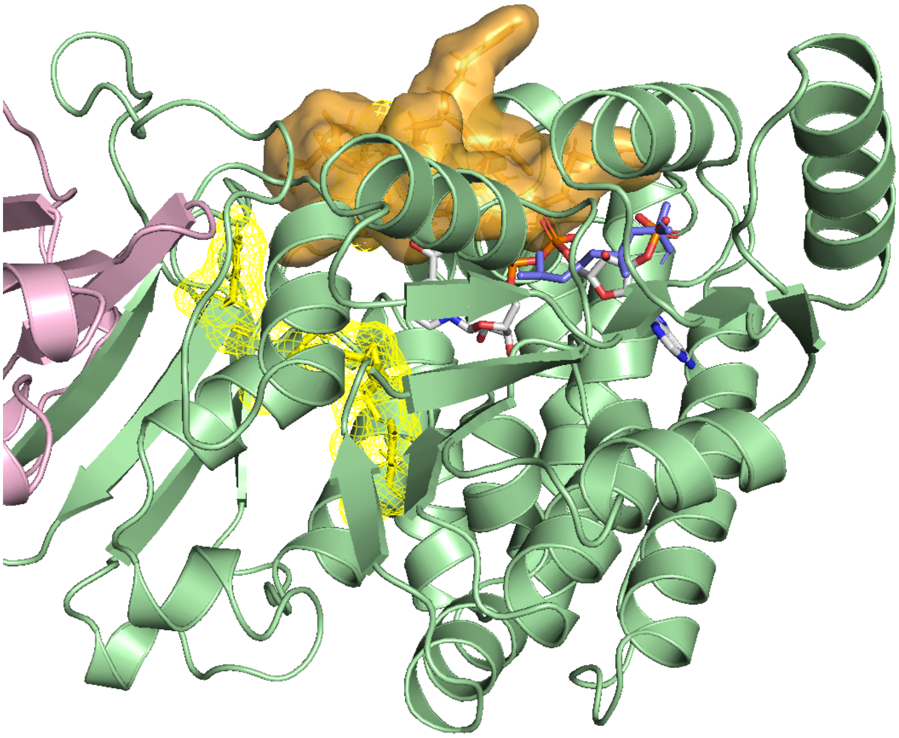
Supplemental Figure 11. DXP binding site of EcDXR



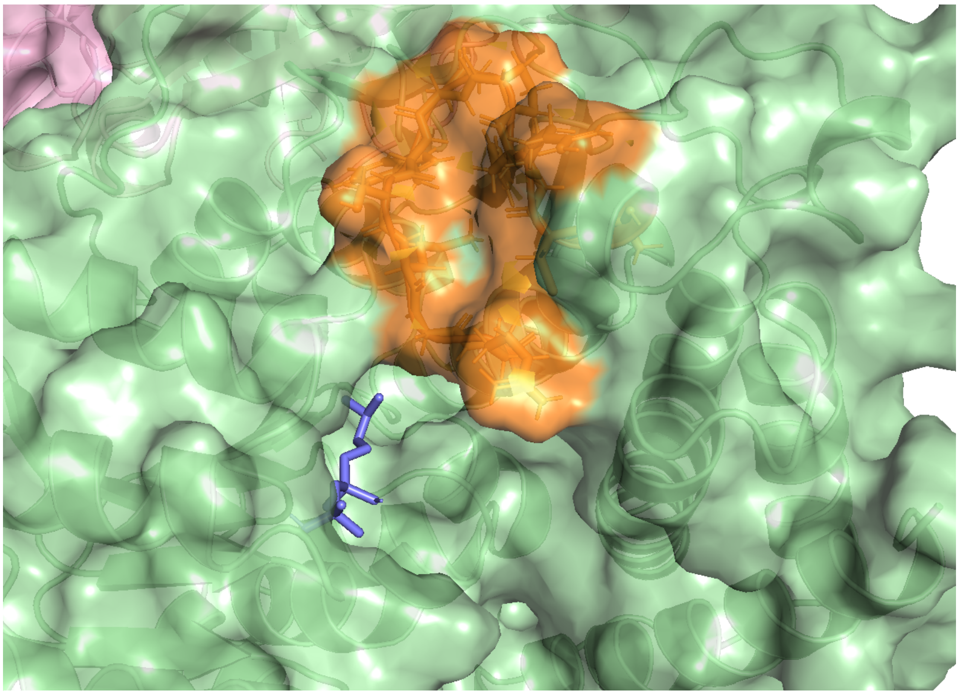
Supplemental Figure 12. NADPH binding site of EcDXR



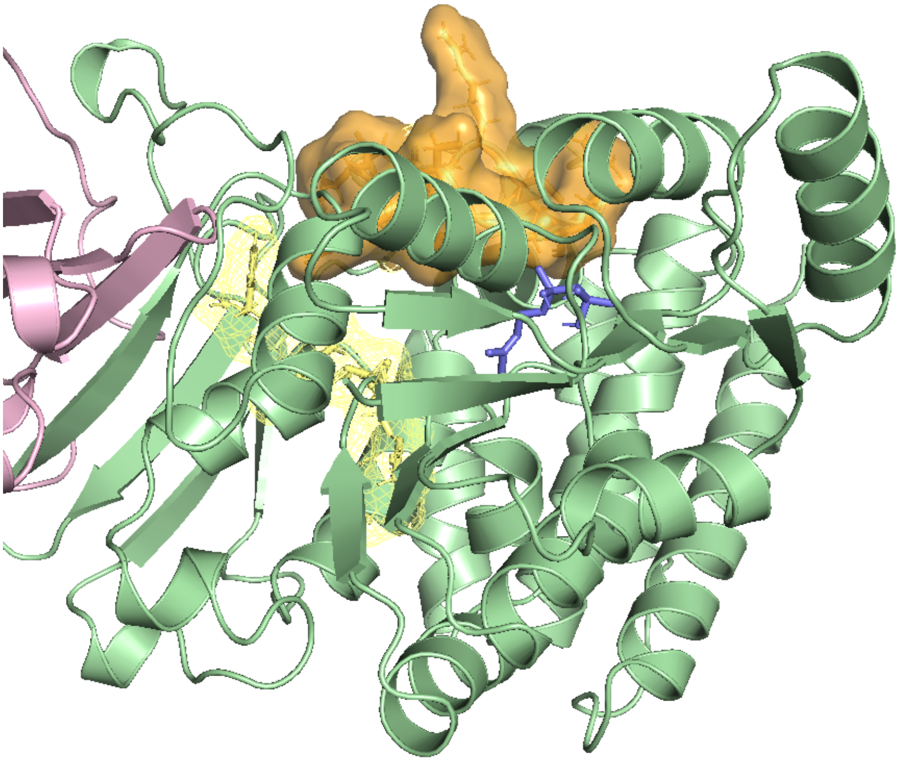
Supplemental Figure 13. IPP binding site of EcDXR prediction



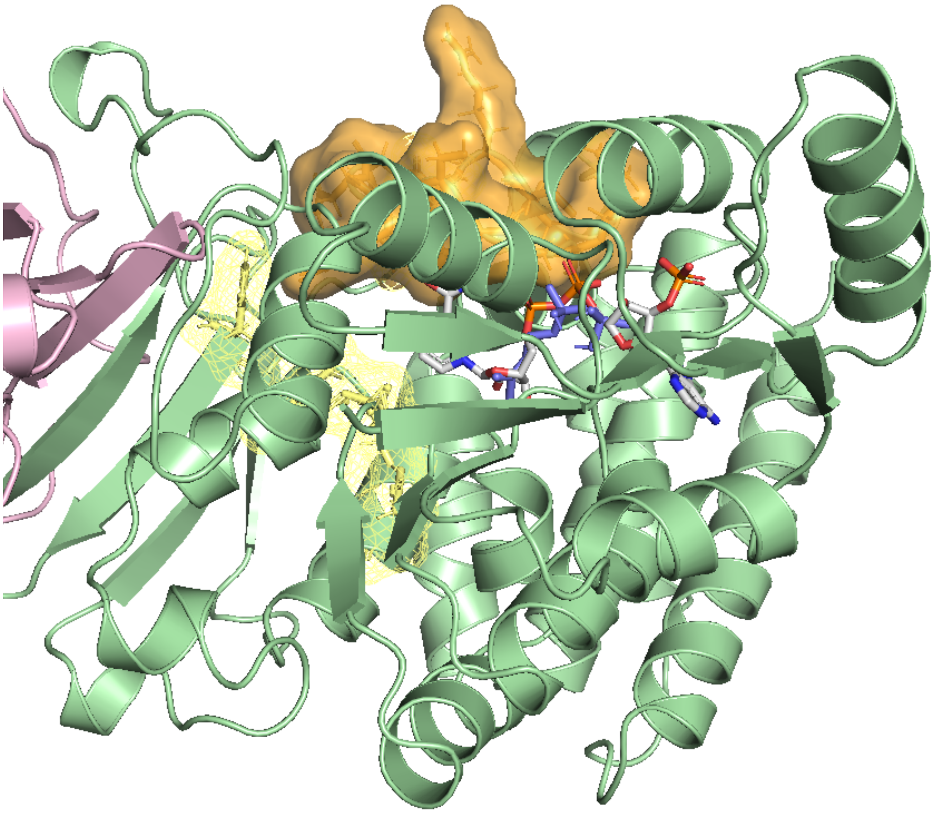
Supplemental Figure 14. IPP and NADPH bind to EcDXR at similar site



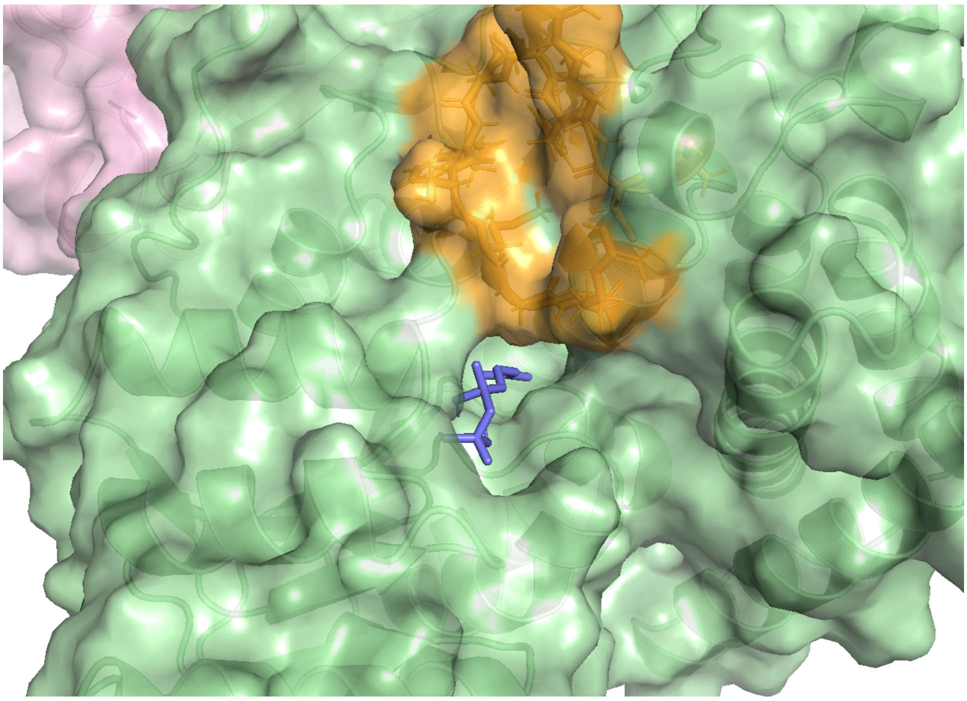
Supplemental Figure 15. IPP binding site of EcDXR (showed as surface)



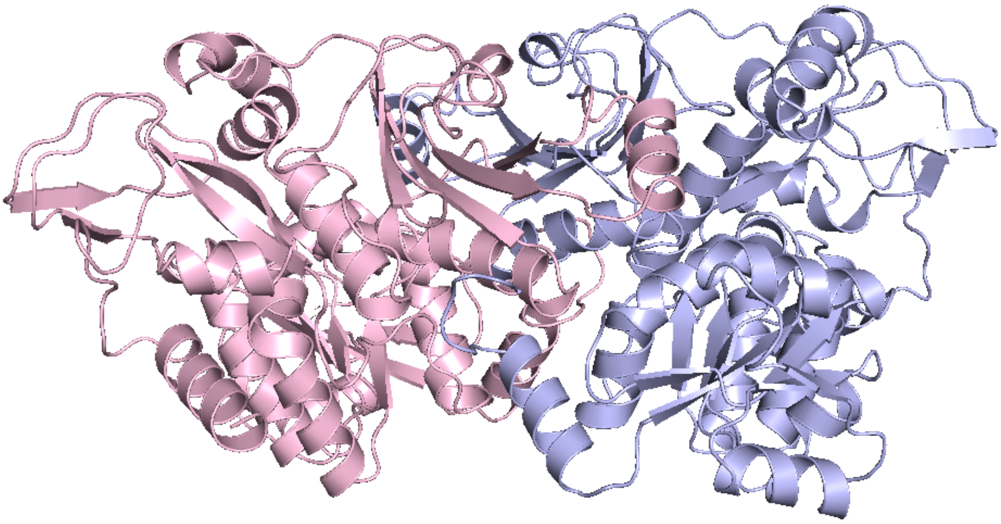
Supplemental Figure 16. DMAPP binding site of EcDXR prediction



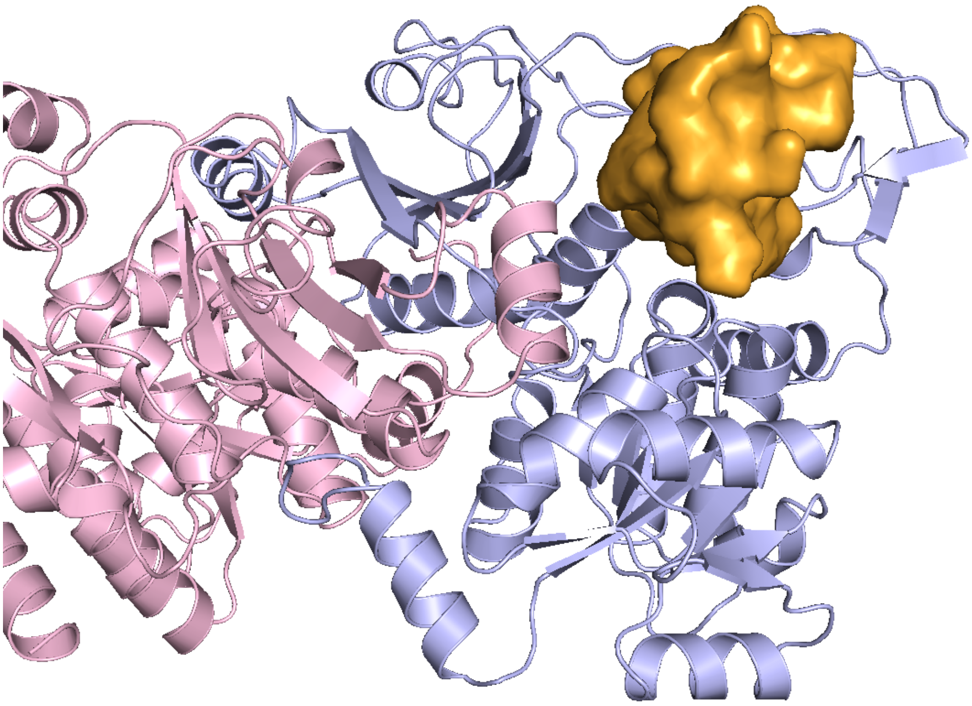
Supplemental Figure 17. DMAPP and NADPH bind to EcDXR at similar site



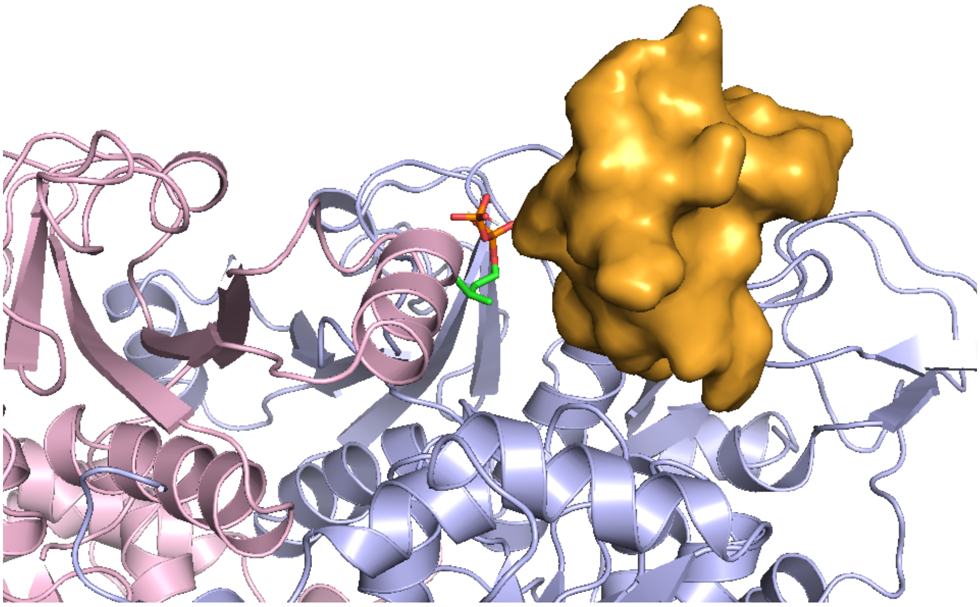
Supplemental Figure 18. DMAPP binding site of EcDXR (showed as surface)



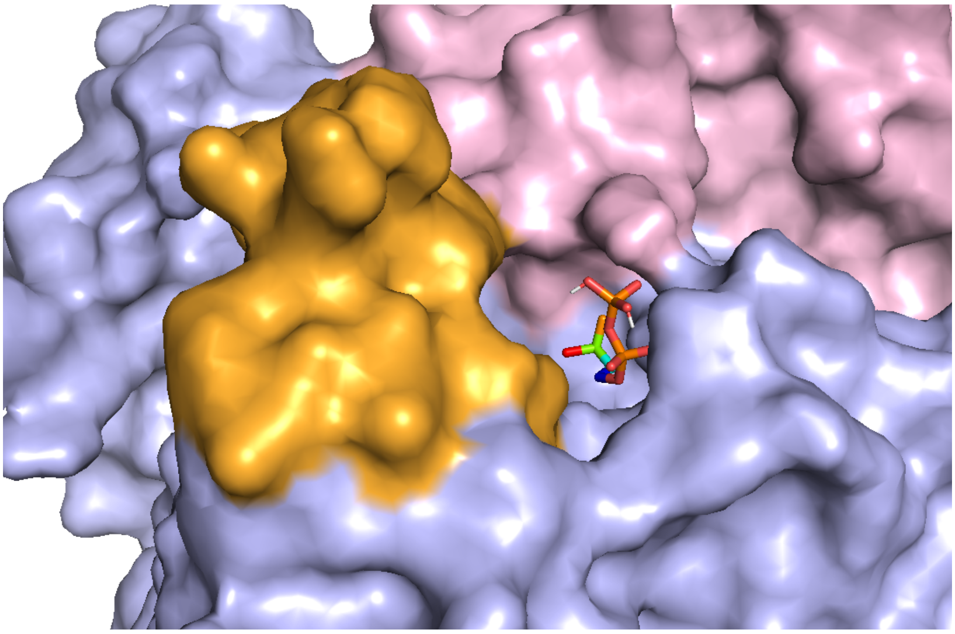
Supplemental Figure 19. The crystal structure of BaDRL (PDB: 3upy)



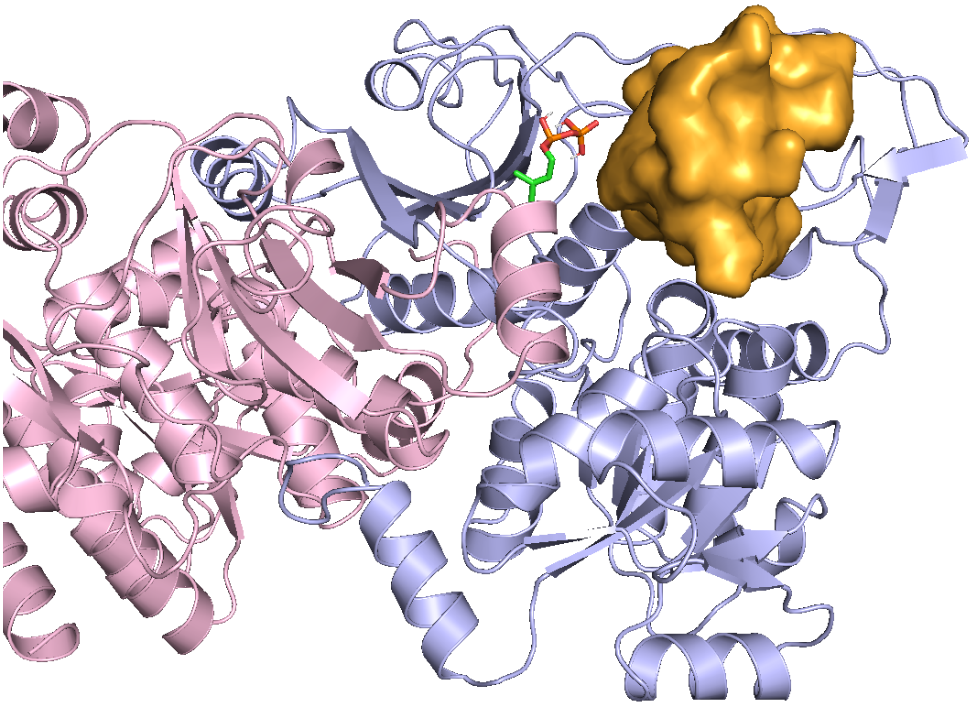
Supplemental Figure 20. The flexible 'lid' (orange) of BaDRL



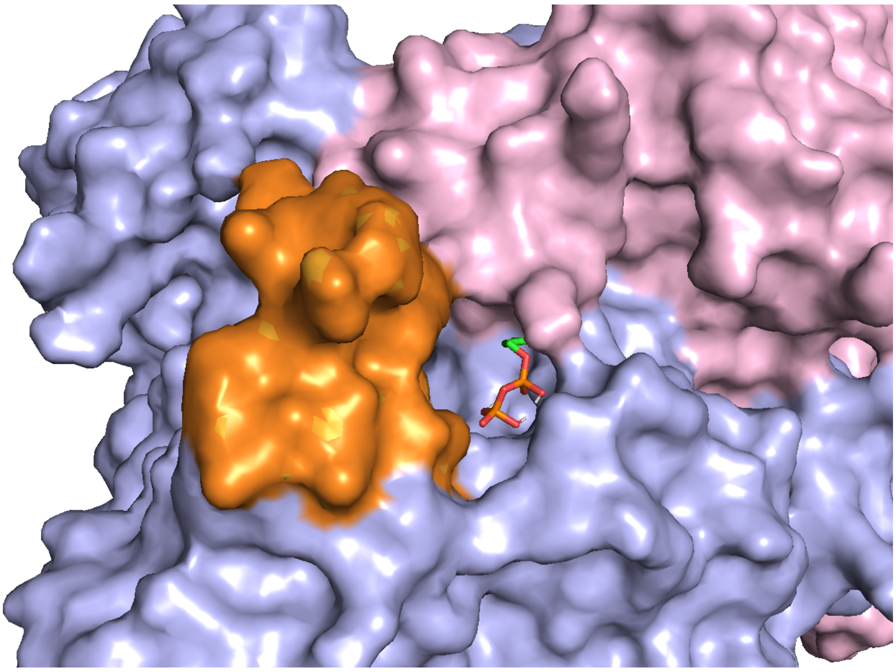
Supplemental Figure 21. IPP binding site of BaDRL prediction



Supplemental Figure 22. IPP binding site of BaDRL (showed as surface) prediction



Supplemental Figure 23. DMAPP binding site of BaDRL prediction



Supplemental Figure 24. DMAPP binding site of BaDRL (showed as surface) prediction