Enzyme Inhibitor Studies Reveal Complex Control of Methyl-D-Erythritol 4-Phosphate (MEP) Pathway Enzyme Expression in *Catharanthus roseus*

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Abstract

In *Catharanthus roseus*, the monoterpene moiety exerts a strong flux control for monoterpene indole alkaloid (MIA) formation. Monoterpene synthesis depends on the methyl-D-erythritol 4-phosphate (MEP) pathway. Here, we have explored the regulation of this pathway in response to developmental and environmental cues and in response to specific enzyme inhibitors. For the MEP pathway entry enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS), a new (type I) DXS isoform, CrDXS1, has been cloned, which, in contrast to previous reports on type II CrDXS, was not transcriptionally activated by the transcription factor ORCA3. Regulation of the MEP pathway in response to metabolic perturbations has been explored using the enzyme inhibitors clomazone (precursor of 5-ketochlomazone, inhibitor of DXS) and fosmidomycin (inhibitor of deoxyxylulose 5-phosphate reductoisomerase (DXR)), respectively. Young leaves of non-flowering plants were exposed to both inhibitors, adopting a non-invasive *in vivo* technique. Transcripts and proteins of DXS (3 isoforms), DXR, and hydroxymethylbutenyl diphosphate synthase (HDS) were monitored, and protein stability was followed in isolated chloroplasts. Transcripts for *DXS1* were repressed by both inhibitors, whereas transcripts for *DXS2A* and *B*, *DXR* and *HDS* increased after clomazone treatment but were barely affected by fosmidomycin treatment. DXS protein accumulated in response to both inhibitors, whereas DXR and HDS proteins were less affected. Fosmidomycin-induced accumulation of DXS protein indicated substantial posttranscriptional regulation. Furthermore, fosmidomycin effectively protected DXR against degradation *in planta* and in isolated chloroplasts. Thus our results suggest that DXR protein stability may be affected by substrate binding. In summary, the present results provide novel insight into the regulation of DXS expression in *C. roseus* in response to MEP-pathway perturbation.

Introduction

*Catharanthus roseus* (Madagascar periwinkle) produces a variety of pharmacologically active monoterpene indole alkaloids (MIAs), e.g. ajmalicine and serpentine effective as antihypertensive agents, and the dimeric MIA derivatives vinblastine and vincristine used as anti-cancer drugs. Due to its pharmaceutical potential, *C. roseus* has become one of the best-studied medicinal plants with respect to secondary metabolism [1]. However, attempts towards upgrading MIA accumulation have as yet met with moderate success, and continuous efforts are directed to further elucidate the regulation of MIA biosynthesis [2,3]. MIA biosynthesis involves the condensation of tryptamine (indole moiety) with secologanin (monoterpene-secoiridoid moiety). Secologanin is derived from the basic isoprenoid units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), and supply of secologanin is considered to be rate-limiting for MIA biosynthesis [4–6]. Thus, isoprenoid precursor flux may impact on secologanin availability for MIA biosynthesis.

In higher plants, two pathways are used for the synthesis of the basic isoprenoid units, i.e. the cytosolic mevalonate (MVA) pathway generating precursors for sesqui- (C15) and triterpenes (C30), such as phytosteres, dolichols, and farnesyl residues for protein prenylation, and the plastidic methyl-D-erythritol 4-phosphate (MEP) pathway (Fig. 1) for the synthesis of carotenoids, plastoquinones, phytol conjugates (such as chlorophylls and tocopherols) and hormones (gibberellins and abscisic acid) [7,8]. Previous work has confirmed that secondary metabolites, such as MIAs derive their monoterpene moiety from the MEP pathway [9]. The MEP pathway operates in a broad range of organisms, including bacteria, certain protozoa, green algae, and higher plants. Extensive research has elucidated its biosynthetic steps, structure-function relationships of individual enzymes, and its role for terpenoid biosynthesis [7,10,11].

The first step of the MEP pathway is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS), converting pyruvate and glyceraldehyde-3-phosphate to 1-deoxy-D-xylulose 5-phosphate (DXP, Fig. 1). Previous studies have shown that DXS is highly regulated during plant development and in response to abiotic and biotic stress [12–15]. As the expression of DXS is closely correlated with accumulation and decrease of plastid isoprenoids, the DXS enzyme has been considered as a rate-limiting enzyme.
Recently, studies on MEP pathway enzyme expression in Arabidopsis thaliana have revealed the existence of posttranscriptional control(s) [23,25,26]. Thus, application of fosmidomycin led to an accumulation of DXS protein, apparently without increase in DXS transcript amount [23]. Furthermore, proteomic analysis of an Arabidopsis Cip protocast mutant revealed increased levels of MEP pathway enzyme proteins [26,32], suggesting that proteolytic turnover may be involved in fine tuning of MEP pathway enzyme levels. As yet, little is known about the multiple levels of MEP pathway regulation in C. roseus. Interestingly, overexpression of DXS in C. roseus hairy roots stimulated the accumulation of several MIAs [33], and DXS expression was induced in ORCA3 overexpression C. roseus cell lines (ORCA3: a jasmonate-responsive APETALA2 (AP2)-domain transcription factor activating MIA biosynthesis [34,35]). Besides, various analogues of the DXR inhibitor fosmidomycin inhibited MIA synthesis in C. roseus cells [36,37], indicating that MEP pathway flux may impact on MIA biosynthesis. In planta, expression of MEP pathway genes appears to be concentrated in internal phloem associated parenchyma (IPAP) cells [2,30]. Furthermore, several MEP pathway genes are induced upon stimulation of MIA biosynthesis [39,40–42]. However, the regulation of corresponding protein levels remains largely unknown.

Here, the expression of three MEP pathway enzymes in C. roseus, namely DXS (three isoforms), DXR, and HDS has been explored by using clomazone and fosmidomycin as tools to perturb MEP pathway flux. In particular, the following questions have been addressed: i) Do DXS isoforms respond differentially to depletion of MEP pathway intermediates (and/or downstream isoprenoid-derived products)? ii) Does interruption of MEP pathway flux either prior to or after 1-deoxy-D-xylulose 5-phosphate (DXP) result in differential responses for MEP pathway enzyme expression? iii) Do posttranscriptional control and/or protein turnover contribute to DXS enzyme protein accumulation? and iv) Does exposure of MEP pathway target enzymes to specific inhibitors impact on their proteolytic turnover in the chloroplast?

Results

cDNA cloning of a novel type I DXS isoform of C. roseus

Previous work had indicated the existence of two DXS genes in C. roseus (CrDXS, AJ011840; CrDXS2, DQ484672). However, phylogenetic analysis revealed that both genes group with the type II clade (Fig. S1). To explore the differential roles of DXS-encoding genes in C. roseus, we have cloned by RACE approach an additional DXS isoform which belongs to the type I clade (Fig. S1). This novel CrDXS cDNA, named CrDXS1 (KC625536), encompasses an ORF of 719 amino acids with a calculated Mr of 77.5 kDa, and includes 258 bp of 5'-UTR and 248 bp of 3'UTR, respectively. For consisency, we have renamed the previously cloned isoforms as CrDXS2A (CrDXS, AJ011840) and CrDXS2B (CrDXS2, DQ484672), respectively. When subjecting CrDXS1 to PSI-BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi), its protein sequence displayed high similarity (i.e. sequence identity of 80–87%) with type I DXS sequences from other plants, while comparison with CrDXS2A and CrDXS2B revealed only 73% identity. Moreover, like all previously cloned plant DXS proteins, CrDXS1 includes an N-terminal transit peptide of 57 amino acids (predicted by the ChloroP program, http://www.cbs.dtu.dk/services/ChloroP/), and the estimated Mr for mature CrDXS1 is 71.2 kDa.

Inhibitors of DXS and DXR have been used as an additional tool to study the regulation of isoprenoid production in plants [15,20,23–27]. Clomazone (2-[2-chlorophenyl] methyl]-4,4-di-methyl-3-isoxazolidinone), a soil-applied herbicide [28], is the precursor of 5-ketoclomazone, an inhibitor of DXS [29,30], and sensitivity of recombinant C. roseus DXS to 5-ketoclomazone has been demonstrated [29]. Fosmidomycin (3-N-formyl-N-hydroxyamino) propylphosphonic acid), also known as FR-31564, is a specific inhibitor of DXR [31].
Expression of DXS isofrom mRNAs and DXS protein respond to developmental and stress-related cues

The expression of DXS, DXR and HDS proteins in young leaves, mature (i.e. fully expanded) leaves and roots of 6-week-old non-flowering C. roseus plants was determined by immunoblotting (Fig. 2A), using polyclonal antisera against recombinant CrDXS2A, CrDXR and CrHDS proteins, respectively. While the latter two antisera are specific for their single gene target enzymes, the antiserum raised against CrDXS2A also detects CrDXS2B and CrDXS1 proteins, respectively, albeit with different intensity (2A/2B: 74%, 2A/1: 74%, 2B/1: 73%). The corresponding transcripts were quantified by qPCR (Fig. 2B).

The differential expression of CrDXS isoforms in young leaves, mature leaves and roots of 6-week-old non-flowering C. roseus plants was determined by immunoblotting (Fig. 2A), using polyclonal antisera against recombinant CrDXS2A, CrDXR and CrHDS proteins, respectively. While the latter two antisera are specific for their single gene target enzymes, the antiserum raised against CrDXS2A also detects CrDXS2B and CrDXS1 proteins, respectively, albeit with different intensity (2A/2B: 74%, 2A/1: 74%, 2B/1: 73%). The corresponding transcripts were quantified by qPCR (Fig. 2B).

The two single gene-encoded MEP pathway enzymes DXR and HDS were expressed to similar degrees in all three organs at protein and transcript level, respectively (Fig. 2). Conversely, DXS protein showed similar abundance in young leaves and roots, but only very weak expression in mature leaves, this being consistent with the corresponding transcript levels of CrDXS2A&2B (Fig. 2A,B). Note that the CrDXS2A antiserum detects, in addition to the main band at the predicted size of mature DXS2A proteins, a band of higher mobility (apparent Mr 60 kDa); this immune signal is suppressed by preincubation of the antiserum with recombinant CrDXS2A protein, indicative of a CrDXS2A degradation_processing product.

To explore the impact of oxidative stress on the expression of MEP-pathway enzymes, leaf discs (from mature leaves) were exposed to a 0.5 μM paraquat (methyl viologen) solution (Fig. 3). Leaf discs of control treatment did not show any bleaching over a period of 30 hrs, whereas paraquat-exposed leaf discs started to bleach after 10 hrs (Fig. S4). In the control treatment, CrDXS1 transcripts slightly increased during the first 24 hrs, followed by a decline to initial level, whereas in paraquat-treated leaf discs transcripts were expressed at very low level (Fig. 3B). Conversely, CrDXS2A&2B transcripts remained low in control discs but were strongly induced by paraquat treatment (Fig. 3B). While in control samples, DXS protein amount remained low and fairly unchanged, the paraquat-mediated induction of CrDXS2A&2B transcripts correlated with a strong increase in DXS protein (Fig. 3A). These results reveal that in response to paraquat treatment, the expression of DXS protein was largely controlled at the transcriptional level, i.e. strong induction of CrDXS2A&2B mRNAs, accompanied by a loss of CrDXS1 mRNA. Paraquat-induced oxidative stress also affected protein and transcript levels for DXR and HDS, respectively (Fig. 3A,B). While their transcripts appeared to be co-regulated with CrDXS2A&2B transcripts, DXR and HDS protein levels strongly declined, the protein loss being most pronounced for HDS. In summary, the data support the notion that in mature leaves, CrDXS1 performs a housekeeping function, whereas CrDXS2A&2B substitute under conditions of paraquat exposure.

CrDXS isoforms are differentially regulated by the transcription factor ORCA3

The differential expression of CrDXS1 versus CrDXS2A&B in different tissues and in response to paraquat exposure indicated distinct mechanisms for their transcriptional regulation. As previous work had shown that in cell culture, CrDXS2A is responsive to ORCA3 activation [34], we explored whether in planta the DXS isoforms respond differentially to this transcription factor. The promoters of all three CrDXS isoforms (including about 2 kb sequences upstream of translation start), were isolated and inserted into the multiple cloning site of pGreenII 0800-LUC vector [43] to drive the expression of firefly luciferase (LUC). After mobilization into Agrobacterium, the resulting constructs were co-infiltrated with Agrobacterium cells carrying a CaMV33S-ORCA3 construct into the first pair of fully expanded mature leaves of 6-week-old soil-grown C. roseus plants. Subsequently, promoter activities of DXS isoforms were analyzed by the dual luciferase assay [43]. In the presence of ORCA3, the DXS2A1 promoter activity was strongly (~3 fold) induced (Fig. 4), and the same was true for DXS2B, albeit to a low extent. Conversely, the promoter of CrDXS1 did not respond to ORCA3. As ORCA3-regulated genes are tightly linked to MIAs accumulation [34], the results suggest that CrDXS2A&B, but not CrDXS1, are involved in MIA biosynthesis. The differential affinity of DXS promoters to ORCA3 is consistent with the notion that only type II DXS

Figure 2. Tissue-specific expression of MEP pathway genes in young leaves, mature leaves and roots of 6-week-old non-flowering C. roseus plants, at protein and transcript level, respectively. Soil-grown periwinkle (Catharanthus roseus) plants were cultivated in a growth chamber at 25°C with 14 hrs light period (170 μmol m−2 s−1) and 22°C during dark period. (A) DXS, DXR, and HDS proteins were detected by immunoblot with polyclonal antisera raised against recombinant CrDXS2A, DXR and HDS proteins. Equal sample loading was confirmed by Coomassie staining. The arrow marks the position of mature CrDXS2A protein. (B) transcript amounts for DXS isoforms (1, 2A & 2B), DXR, and HDS were determined by qPCR (the experiment was performed 3 times, values from a representative experiment are presented ± SD), relative to the geometric mean of multiple reference genes according to Vandesompele et al. [60]. doi:10.1371/journal.pone.0062467.g002
During the chosen time interval. As even the injected (fully expanded) leaves did not show any bleaching (Fig. S5), indicating rapid systemic spreading of the compound. Extended areas of the expanding leaves were strongly bleached 30 hrs after clomazone injection (not shown), and after 78 hrs emerging young leaves, bleaching was observed as early as at transcript and protein level, respectively (Fig. 5A,B). In young leaves (Fig. 5A) revealed a dramatic increase in DXS protein accumulation (Fig. 5A), starting as early as 10 hours after clomazone application. At transcript level, a block of the entire pathway, we have monitored the effects of an enzyme inhibitor for DXS. Clomazone (50 μM, aqueous solution) was applied to the first two pairs of fully expanded mature leaves of 6-week-old C. roseus plants. Leaf discs (5 mm diameter) were collected from fully expanded leaves and floated on distilled water in the presence or absence of 0.5 μM paraquat for the indicated time intervals at 25°C under continuous light (270 μmol m⁻² s⁻¹). (A) DXS, DXR and HDS proteins were detected by immunoblot. Equal sample loading was confirmed by Coomassie staining. The arrow marks the position of mature CrDXS2A protein. (B) transcript amounts for DXS (isoforms 1, 2A & 2B), DXR and HDS were determined by qPCR relative to the geometric mean of multiple reference genes according to Vandesompele et al. [60]. The experiment was performed 3 times; values from a representative experiment are presented ± SD. doi:10.1371/journal.pone.0062467.g003

isoforms contribute to biosynthesis of secondary metabolites [14,44,45].

Clomazone strongly induces DXS protein and a coordinate up-regulation of transcripts for DXS2A/B, DXR and HDS

To explore the response of MEP pathway enzyme expression to a block of the entire pathway, we have monitored the effects of an enzyme inhibitor for DXS. Clomazone (50 μM, aqueous solution) was applied to the first two pairs of fully expanded mature leaves of 6-week-old non-flowering C. roseus plants (Fig. S5) via injection with a needleless plastic syringe, thereby flooding the entire intracellular space with clomazone solution. Tissue samples from young leaves were taken at 0 to 102 hrs after clomazone application and analyzed for MEP pathway enzyme expression at transcript and protein level, respectively (Fig. 5A,B). In emerging young leaves, bleaching was observed as early as 30 hrs after clomazone injection (not shown), and after 78 hrs extended areas of the expanding leaves were strongly bleached (Fig. S5), indicating rapid systemic spreading of the compound. Conversely, fully expanded leaves did not show any bleaching during the chosen time interval. As even the injected (fully expanded) leaves showed little bleaching over time, it is concluded that in mature leaves pigment turnover is too slow to reveal inhibition of MEP pathway flux, in marked contrast to young growing leaves, where de novo synthesis of leaf pigments is blocked. However, it cannot be excluded that transport efficiencies for clomazone may differ according to the developmental stage of the leaf.

The effect of clomazone on MEP pathway enzyme expression in young leaves (Fig. 5A) revealed a dramatic increase in DXS protein accumulation (Fig. 5A), starting as early as 10 hours after clomazone application. At transcript level, CrDXS1 expression slightly declined, whereas CrDXS2A&B showed a moderate increase, paralleled by comparable up-regulation of DXR and HDS transcripts (Fig. 5B); however, DXR and HDS protein levels showed only a moderate increase. To confirm that clomazone treatment induces DXS protein to a similar degree as the active derivative 5-ketoclomazone, a similar experiment was performed with 5-ketoclomazone (Fig. S7). The effect of a treatment with 50 μM 5-ketoclomazone on DXS protein accumulation was slightly less pronounced than observed for clomazone, although 5-ketoclomazone caused more bleaching.

To address possible effects of clomazone treatment on DXS protein turnover, the time course of DXS protein decline in isolated chloroplasts from control and clomazone-treated plants was monitored. Three days after clomazone treatment, chloroplasts were isolated and incubated at 25°C for up to 60 min and

Figure 3. Effect of paraquat treatment on the expression of MEP pathway genes at protein and transcript level, respectively, in leaf discs obtained from mature leaves of 6-week-old C. roseus plants. Leaf discs (5 mm diameter) were collected from fully expanded leaves and floated on distilled water in the presence or absence of 0.5 μM paraquat for the indicated time intervals at 25°C under continuous light (270 μmol m⁻² s⁻¹). (A) DXS, DXR and HDS proteins were detected by immunoblot. Equal sample loading was confirmed by Coomassie staining. The arrow marks the position of mature CrDXS2A protein. (B) transcript amounts for DXS (isoforms 1, 2A & 2B), DXR and HDS were determined by qPCR relative to the geometric mean of multiple reference genes according to Vandesompele et al. [60]. The experiment was performed 3 times; values from a representative experiment are presented ± SD. doi:10.1371/journal.pone.0062467.g003

Figure 4. Activation of DXS isoforms’ promoters (CrDXS1p, CrDXS2Ap and CrDXS2Bp) by the transcription factor ORCA3, analyzed via transient expression of promoter-luciferase fusions in leaves of 6-week old C. roseus plants. Results are expressed as fold change of promoter activation, based on four independent assays. The promoter activity was determined by the ratio of luciferase (LUC) activity to renilla expression. Dual CaMV 35S promoter was employed as a positive control showing high LUC activity, while promoterless LUC control (pGreenII 0800-LUC vector) reflected the LUC background value. DXS promoter sequences were fused to the firefly luciferase and transiently co-transformed with ORCA3 or without (mock control). Agrobacteria-mediated transient transformation was carried via leaf infiltration of 6-week-old soil-grown C. roseus plants. Two days after transformation, infiltrated leaves were harvested and subjected to dual-luciferase reporter assay. Error bars represent the standard deviation of four independent experiments for promoter activity analysis. The asterisks represent significant difference (** p<0.001, * p<0.1 by student’s t-test). doi:10.1371/journal.pone.0062467.g004
presented was performed 3 times, values from a representative experiment are reference genes according to Vandesompele et al.\[60\]. The experiment determined by qPCR relative to the geometric mean of multiple transcript amounts for DXS isoforms (1, 2A & 2B), DXR and HDS were respectively.

Coomassie staining

Figure 5. Time course of in vivo clomazone treatment on the expression of MEP pathway genes, and subsequent degradation of DXS and DXR proteins in isolated chloroplasts. For each plant, two pairs of mature leaves were injected with a 50 μM clomazone solution (or water for control) until the entire leaf blades were fully soaked, using a 1 ml needleless syringe applied to the lower epidermis. For each time point, young leaves were pooled from three independent plants and processed for MEP pathway protein and transcript analysis, respectively. (A) DXS, DXR and HDS proteins were detected by immunoblot. Equal sample loading was confirmed by Coomassie staining. The arrow marks the position of mature CrDXS2A protein. (B) transcript amounts for DXS isoforms (1, 2A & 2B), DXR and HDS were determined by qPCR relative to the geometric mean of multiple reference genes according to Vandesompele et al[60]. The experiment was performed 3 times, values from a representative experiment are presented ± SD. (C) chloroplasts were isolated from young leaves of 6-week-old soil-grown C. rosues plants as described above. Again, tissue samples from young leaves were collected at 0 to 102 hrs after inhibitor application and analyzed for MEP pathway enzyme expression at protein and transcript level, respectively (Fig. 6A,B). Similar to the results obtained with clomazone application, fosmidomycin caused a dramatic accumulation of DXS protein (Fig. 6A), while expression of GDXSI mRNA declined (Fig. 6B). However, in contrast to clomazone treatment, inhibition of DXR had only a modest effect on the expression of GDXS2A&B mRNAs. Likewise, transcript amounts for MEP pathway enzymes DXR and HDS were only weakly and transiently affected, showing an increase at 54 hrs after fosmidomycin application and declining thereafter (Fig. 6B).

Cycloheximide strongly inhibits fosmidomycin-induced accumulation of DXS protein

To block MEP pathway flux at the level of DXR enzyme activity, i.e. allowing the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) while blocking the remaining MEP pathway, a 50 μM aqueous solution of fosmidomycin was applied to the first two pairs of fully expanded mature leaves of 6-week-old C. rosues plants as described above. Again, tissue samples from young leaves were collected at 0 to 102 hrs after inhibitor application and analyzed for MEP pathway enzyme expression at protein and transcript level, respectively (Fig. 6A,B). Similar to the results obtained with clomazone application, fosmidomycin caused a dramatic accumulation of DXS protein (Fig. 6A), while expression of GDXSI mRNA declined (Fig. 6B). However, in contrast to clomazone treatment, inhibition of DXR had only a modest effect on the expression of GDXS2A&B mRNAs. Likewise, transcript amounts for MEP pathway enzymes DXR and HDS were only weakly and transiently affected, showing an increase at 54 hrs after fosmidomycin application and declining thereafter (Fig. 6B).

In a previous study with Arabidopsis seedlings, an induction of DXS protein after fosmidomycin treatment was observed without any increase of DXS transcript [23]. As this study did not monitor possible differences between DXS isoforms, we have repeated this experiment by individually monitoring the transcription levels of the two expressed DXS isoforms AtDXS1 and AtDXS2 in 8-day-old Arabidopsis seedlings (for AtDXS2, no transcripts were detected; see Fig. S6). We could confirm that DXS protein was significantly increased 6 hrs after fosmidomycin treatment. For both isoforms, transcripts transiently increased only after 24 hrs, confirming the notion that the early increase of DXS protein was not due to transcriptional up-regulation (Fig. 7A,B). Thus, comparing the response of C. rosues young leaves and Arabidopsis seedlings (cotyledon stage) to fosmidomycin treatment, the results indicate that in both species the observed up-regulation of DXS protein is uncoupled from the level of DXS transcripts.
blocked cytosolic translation with cycloheximide. A 100 μM cycloheximide solution was applied either alone or in combination with 50 μM fosmidomycin or clomazone as described above. Tissue samples from young leaves were taken at 0 to 78 hrs after inhibitor application and analyzed for MEP pathway enzyme expression at protein level (Fig. 8A). Note that application of cycloheximide resulted in slight wilting and anthocyanin accumulation (‘‘violet phenotype’’, not shown), and when applied together with fosmidomycin or clomazone, cycloheximide retarded leaf development and prevented the fosmidomycin or clomazone-associated bleaching phenotype.

Treatment with cycloheximide alone caused a rapid decline of DXS, DXR and HDS proteins, revealing not only an efficient block of translation but also indicating high protein turnover. When applying cycloheximide together with fosmidomycin, the decline of DXS and HDS proteins followed a similar time course, indicating that the strong accumulation of DXS protein after fosmidomycin treatment results from increased de novo protein synthesis, presumably by increased polysome-recruitment of CrDXS2A&B transcripts (see above).

Unsurprisingly, DXR protein level remained relatively stable when fosmidomycin and cycloheximide were applied simultaneously (Fig. 8A). Thus, fosmidomycin apparently prevented the degradation of DXR protein observed upon cycloheximide treatment. Considering the mode of action of fosmidomycin [47], it may be speculated that its binding to DXR might change enzyme conformation, rendering it resistant to protein turnover via chloroplast-localized proteases (e.g. Clp protease). It is noteworthy that the presence of clomazone (which is converted in planta to the specific inhibitor 5-ketoclomazone, see above) did not protect DXS protein from turnover in the presence of cycloheximide (Fig. 8A).
in the presence of fosmidomycin, supporting the hypothesis that specific binding of fosmidomycin to DXR protein protects the latter against degradation via plastidic protease(s).

**Discussion**

The molecular basis for higher plant MEP pathway regulation at different levels has been the focus of several recent studies [17,20,23,25,26]. As this chloroplast-localized but nuclear-encoded pathway delivers not only precursors for photosynthetic components (chlorophylls, carotenoids), plant hormones (abscisic acid, gibberellic acid) and antioxidants (tocopherols), but also for monoterpenoids and derived secondary metabolites of high pharmacological value [8,48], understanding the different modes of its regulation is of fundamental importance for basic and applied research.

Hitherto, molecular studies have largely focused on the model plant *Arabidopsis*, unraveling several aspects of posttranscriptional regulation of MEP pathway enzyme expression [23,25,26], and on the differential regulation of DXS isoforms and their specific roles in housekeeping functions for photosynthesis (i.e. type I) and stress-related functions (i.e. type II), respectively, with a major focus on transcriptional control [15,45,49,50]. With the present study, we provide evidence for both transcriptional and posttranscriptional controls affecting the expression of DXS and DXR, respectively, in the pharmacologically important plant species *C. roseus*.

As an important initial step of the present study, a type I DXS isoform was cloned from *C. roseus* (Fig. S1). Since comprehensive analysis of all primary RT-PCR products did not deliver any related type I DXS sequence, the cloned *CdDXS1* cDNA apparently represented the only housekeeping leaf-expressed DXS isoform. For further confirmation of its housekeeping function, we compared the expression of all three *CdDXS* isoforms in different plant tissues (Fig. 2) and in leaf discs exposed to oxidative stress via paraquat treatment (Fig. 3). The results reveal that *CdDXS1* transcripts are prevalent in young and mature leaves, however with lower *CdDXS2A&B* mRNA levels also being detected in young leaves, whereas roots exclusively express *CdDXS2A&B* transcripts (Fig. 2), in agreement with expression patterns observed in other dicot and monocot species [15,45]. Furthermore, in leaf tissue oxidative stress induces only *CdDXS2A&B* transcript accumulation, whereas *CdDXS1* mRNA declines (Fig. 3); similar observations were reported for plants under biotic and abiotic stress conditions [12,15,44,50]. Note that in most plant species the consensus thiamine pyrophosphate (TPP)-binding domain, a highly conserved asparagine (N236 in CrDXS2A) residue in type II DXS isoforms is replaced by aspartic acid in type I DXS isoforms (Fig. S2). Whether this substitution is of functional significance remains to be shown.

Interpretation of the oxidative stress experiment (Fig. 3) has to acknowledge that leaf disc preparation causes a wound response also in the “control” treatment, thus the comparison is between wounding and wounding with simultaneous chloroplast-derived oxidative stress. Interestingly, wounding as such did not cause significant changes of MEP pathway gene transcripts, and, likewise, MEP pathway protein levels remained rather stable, in marked contrast to additional oxidative stress, which strongly but differentially affected protein levels of CrDXS, DXR and HDS (Fig. 3). Rivasseau et al. [22] demonstrated that in spinach leaves oxidative stress damaged HDS, an α2-hypersensitive [Fe2-S4]-protein, providing a possible explanation for the massive loss in HDS protein (Fig. 3). In line with an important role of MEP pathway imbalance for stress signaling, Xiao et al. [51] have

**Figure 8. Time course of in vivo cycloheximide treatment as compared to simultaneous treatment with fosmidomycin or clomazone, respectively, and subsequent turnover of DXS and DXR proteins in isolated chloroplasts.** (A) For each plant, two pairs of mature leaves were injected with 100 μM cycloheximide solution alone, or in combination with 50 μM fosmidomycin or 50 μM clomazone, respectively. For each time point, young leaves were collected from three independent plants and processed for immunoblot analysis of DXS, DXR and HDS, respectively. (B) Chloroplasts from young leaves of 6-week-old soil-grown *C. rosues* plants, treated with 50 μM fosmidomycin, 50 μM clomazone, or water (control) were collected 78 hours after leaf injection (see Fig. 3). Chloroplasts were incubated for 1 h in the light (100 μmol m⁻² s⁻¹) at 25°C in the presence of 5 mM ATP and fosmidomycin or clomazone (50 μM). Aliquots were taken at 0, 15, 30 and 60 minutes and used for protein extraction. DXS and DXR proteins were detected by immunoblot. To obtain similar signal intensity at time point 0, the loading amount of protein from control samples (chloroplast isolated from water infiltrated plants) was twice that of fosmidomycin or clomazone samples (see also Fig. 5C). doi:10.1371/journal.pone.0062467.g008

**Fosmidomycin protects DXR enzyme from proteolytic turnover in the chloroplast**

To confirm the hypothesis that fosmidomycin protects DXR protein from degradation, chloroplasts were isolated 78 hrs after treatment from control plants, fosmidomycin-treated plants and clomazone-treated plants, respectively. For the *in vitro* protein degradation experiment, chloroplasts were incubated for 1 h in the light at 25°C in the presence of 5 mM ATP (as described above). To compensate for fosmidomycin/clomazone loss/dilution during the chloroplast isolation procedure, the final incubation solution also contained 50 μM fosmidomycin/clomazone. Samples were taken at 0, 15, 30, 60 min and analyzed by immunoblotting for DXS and DXR protein, respectively (Fig. 8B). While turnover of DXS was not affected by fosmidomycin or clomazone (for comparison see also Fig. 5C), DXR protein was clearly stabilized...
demonstrated that abiotic stress causes an increase in the HDS substrate methylethylketone cyclophosphate (MeEPP), which in turn acts as retrograde signal inducing the expression of nuclear-encoded stress genes.

Previously, it has been shown that CrDXS expression was stimulated in ORCA3 overexpression C. roseus cell lines [34] and plants [35], however, those reports addressed only the CrDXS2A isoform. Here, we have compared the response of all three DXS promoters (CrDXS1p, CrDXS2Ap&Bp) to the transcript factor ORCA3, using the dual luciferase assay for reporting in vivo promoter activities. The results indicate that only type II DXS isoforms are regulated by ORCA3. The only moderate induction of DXS by ORCA3 in leaf tissue, which is known to synthesize MIAs [35], suggests that other transcription factors may be involved in the regulation of DXS.

Having demonstrated the differential regulation of DXS isoforms in C. roseus in response to ORCA3 and chloroplast-derived oxidative stress, we have used this information to study the expression of MEP pathway enzymes in response to selective inhibition of MEP pathway flux at DXS and DXR level, respectively (Fig 5s. -Fig 6, Fig 7, Fig 8). Using MEP pathway efficient inhibitors 5-ketoclomazone (or its immediate precursor clomazone) and fosmidomycin, an efficient in vivo application technique (which is non-invasive for the targeted tissue, i.e. young leaves) has been established. The method for in vivo delivery of the DXS and DXR inhibitors (and cycloheximide, see below) via remote injection of inhibitor to fully expanded leaves relies on the fact that the injected compounds are phloem-mobile [52,53] and are therefore rapidly transported with the assimilation stream to the shoot apex and young ‘sink’ leaves. Mock leaf infiltration with deionized water did not cause any visible phenotype in the young leaves, nor did it induce changes in gene expression. Thus, the chosen protocol appears to be a highly efficient method for non-invasive (in planta) inhibitor delivery. While clomazone blocks the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) and thus also prevents precursor flux towards thiamine biosynthesis [54], fosmidomycin allows the accumulation of DXP but interrupts the downstream MEP pathway. Note that for DXR of Plasmodium falciparum, the structure of a fosmidomycin-DXR complex has already been solved, indicating tight binding of fosmidomycin to the active site of DXR enzyme [47]. While the exact binding mechanism of 5-ketoclomazone to DXS remains to be elucidated, a recent study on DXS from Haemoplasma influenzae has demonstrated that the inhibitor’s binding site differed from the binding sites for DXS enzyme substrates pyruvate and D-glyceraldehyde 3-phosphate, respectively [55].

The first important conclusion from in vivo treatments with clomazone/5-ketoclomazone (Figs. 5A, S6A) and fosmidomycin (Fig. 6A) is that, like in Arabidopsis and maize seedlings [15,23,46], the amount of DXS protein is increased, C. roseus showing a particularly strong response. However, while in Arabidopsis and maize, the increase of DXS protein in response to both inhibitors occurred in the absence of any change in DXS transcript amount, accumulation of DXS protein in C. roseus in response to clomazone correlated with a moderate increase of CrDXS2A&CrDXS2B transcripts (Fig. 5); in contrast, fosmidomycin treatment caused only a slight and transient increase of CrDXS2A&CrDXS2B mRNAs (Fig. 6B). Interestingly, in Arabidopsis the fosmidomycin-induced DXS protein increase is due to accumulation of ArDXS1 protein (=AtCLCA1), which belongs to clade I [see Fig. S1], consistent with the induction of AtCLCA1 protein in response to clomazone [Kobayashi et al., [46]]. It is noteworthy that, although containing 3 DXS genes in its genome, Arabidopsis appears to be exceptional with respect to the absence of a type II DXS [49].

The stability of CrDXS protein in isolated chloroplasts of clomazone-exposed leaves was not increased (but rather reduced (Fig. 5C)), supporting the notion that the protein accumulation observed in vivo is the result of de novo synthesis. Whether the increase in CrDXS2A&CrDXS2B mRNAs as such fully accounts for the massive CrDXS protein accumulation, or if increased translation efficiency is also involved remains to be shown. The observation that blocking MEP pathway flux at either DXS or DXR enzyme level caused a comparable accumulation of CrDXS protein, while only for clomazone treatment CrDXS2A&CrDXS2B transcripts showed a substantial increase (in contrast to fosmidomycin treatment) argues in favor of a common mechanism (at least partially) independent of transcript increase. For other stress-factors, e.g. anaerobiosis, substantial transcript uploading and de novo recruitment of stress-induced transcripts strongly contributes to reprogramming gene expression [56]. Thus, CrDXS2A&CrDXS2B transcripts may have been preferentially recruited to polysomes under stress conditions.

To address the in vivo turnover of MEP pathway enzymes, the same experimental setup used for DXS and DXR inhibitors (see above) was used to deliver cycloheximide to the young developing leaves (Fig. 8A). For an incubation of up to 70 hrs, plants showed only mild visible stress syndromes (slight wilting; visible anthocyanin accumulation), but further shoot growth was retarded. Unexpectedly, analysis of protein expression levels revealed that simultaneous application of fosmidomycin and cycloheximide protected DXR protein from turnover, this effect being specific for DXR when compared with turnover of DXS and HDS protein, respectively. As this apparent protection of DXR from degradation could be confirmed in isolated chloroplasts (Fig. 8B), we hypothesize that the substrate-like tight binding of fosmidomycin to the active site of DXR [47] either shields protease-sensitive sites or causes a conformational change of DXR rendering it resistant to chloroplast protease(s), e.g. Clp protease [26,32]. Thus, fosmidomycin inhibits but also stabilizes the DXR enzyme. The apparent absence of a fosmidomycin-mediated stabilization and subsequent accumulation of DXR protein in vivo (Fig. 6A) may be due to the fact that in intact cells (control treatment) it is the substrate DXP which stabilizes the DXR protein, whereas in isolated chloroplasts the supply of DXP is interrupted. Regarding the observed substantial turnover of MEP pathway enzymes in vivo (Fig. 8A) and in isolated chloroplasts (Figs. 5C&8B), it is tempting to speculate that in vivo the level of enzyme substrates may impact on the stability of MEP pathway enzymes. Recent proteomic studies have confirmed that impaired Clp-protease function causes accumulation of MEP pathway enzyme proteins, e.g. DXR and HDS [32].

In summary, the present study provides new insight into the regulation of MEP pathway gene expression, in particular into mechanisms affecting the steady state levels of the MEP pathway enzymes DXS and DXR in a plant species of high pharmaceutical importance. Clearly, posttranscriptional mechanisms are involved which are likely to include increased protein synthesis (e.g. polysome recruitment) and modulation of protein turnover by chloroplast proteases (e.g. Clp protease). As the biosynthesis of MIAs in C. roseus is highly regulated and involves several cell types [57,58,59], the novel non-invasive protocol for inhibitor delivery will allow to explore the in planta consequences of changed MEP pathway flux for overall MIA accumulation. In particular, the localization of CrDXS2A mRNA in internal phloem parenchyma cells [39] and the pronounced differential expression of CrDXS isoforms in response to MEP pathway perturbation (this study) may reflect the plant’s metabolic strategy to redirect MEP pathway flux under stress exposure and in response to developmental cues.
Materials and Methods

C. roseus plant cultivation and chemical treatment procedures

Madagascar periwinkle (Catharanthus roseus [L.] G. Don, Apocynaceae) plants were grown in a growth chamber at 25 °C with 14 hrs light period (fluorescent white light, 170 μmol m⁻² s⁻¹), and 22 °C in the dark. If not indicated otherwise, 6-week-old plants (i.e. before onset of flowering) were used for chemical treatments.

For oxidative stress experiments, leaf discs (diameter 5 mm) were collected from fully expanded leaves, pooled on de-ionized water, and subsequently distributed to petri dishes to float on de-ionized water (for control) or 0.5 μM paraquat solution. Leaf discs were allowed to equilibrate with this solution in the dark for 1 h, and were thereafter transferred to continuous light in a SANYO growth cabinet at 25 °C with continuous fluorescent white light (270 μmol m⁻² s⁻¹). Leaf discs were harvested at 0, 10, 16, 24, 30 hrs after treatment and further processed for protein and RNA isolation.

For inhibitor experiments (i.e. 50 μM clomazone, Sigma; 50 μM fosmidomycin, Invitrogen; 50 μM 5-ketoclozamone, a gift from K. Grossmann, BASF; 100 μM cycloheximide, Sigma; all in aqueous solution), inhibitor solution (approx. 1 ml total) was applied to the lower epidermis of each of the first two pairs of fully expanded leaves via a 1 ml needleless syringe; control plants were injected with de-ionized water. Note that based on initial screening experiments with different inhibitor dosage, inhibitor concentrations were chosen such as to cause a visible phenotype after 48 hrs. Injection of inhibitor solution was stopped when the solution had visibly penetrated the entire leaf. Per sampling point, young leaves of two to three independent plants were collected at 0, 4, 10, 16, 30, 54, 78 and 102 hrs after chemical treatment. The pooled plant material was homogenized with a Retsch MM301 mill under liquid nitrogen and stored at −80 °C until further use.

RNA isolation and cDNA synthesis

Total RNA was extracted from frozen and homogenized tissue with the GeneMATRIX Universal RNA purification Kit (Roboklon, Berlin, Germany), according to the manufacturer’s instructions. RNA quality was verified by agarose gel electrophoresis in addition to the absorbance ratios A₂₆₀/A₂₈₀. For qRT-PCR experiments, first strand cDNA synthesis was performed immediately after DNase (AppliChem, Germany) treatment by PCR experiments, first strand cDNA synthesis was performed using AMV-Reverse Transcriptase (Roboklon, Berlin, Germany), according to the manufacturer’s instructions. For RACE cDNA amplification, total RNA extracted from C. roseus leaves was used. The cDNA for 5’-RACE was synthesized using a modified lock-docking oligo (dT) primer and the SMART II A oligo, whereas the reverse primer and the reverse primer 5’-RACE cDNA was synthesized using a traditional reverse transcription procedure, but with a special oligo (dT) primer provided with the kit. For details see the user manual of Smart™ Race cDNA amplification kit (Clontech, Germany).

Cloning of CrDXS1 full-length cDNA by RACE (Acc. No. KC625536)

Degenerate oligonucleotides were derived from conserved motives in type I DXS sequences from Capsicum annuum (Y15782), Lycopersicon esculentum (AADD38941), Nicotiana tabacum (CB120099) and Arachis hypogea (Y127099). The forward primer DXSI FW: 5’- CCHATGACATASRYTKCGCCDAAAGTDGATG - 3’ and the reverse primer DXSI Rev: 5’- GCCTTACAVG-CYARNCCCHGAC -3’ were derived from the amino acid sequences PMHELAAKVD and AAGLACEG, respectively. This primer pair was used to amplify a core fragment of 504 bp by standard gradient (from 40 °C to 80 °C) PCR amplification from C. roseus leaf cDNA. After gel purification, the PCR product was cloned into the pGEM-T plasmid (Promega) and sequenced. The sequence confirmed fragment was subsequently used to design gene specific primers for the cloning of the full-length cDNA of CrDXS1 by RACE according to Smart™ Race cDNA amplification kit (Clontech, Germany). The cDNA templates were synthesized as described above.

Primers used for each RACE PCR reactions were as follows: 5’RACE UPM (universal primer mix): 5’-CTATACGACTGCA-TATAGGGCAAGCAGTGTATCAACGCAAGAGT; 5’RACE DXS1 GSP (gene specific primer): GGCTTCACAC-CAGTCCTGCCCCTGTC; 5’RACE nested UPM: 5’-AAC- CAGTGGTATCAACCGCAGAGT; 5’RACE DXSI nested GSP: GCCAGTCCCGGCAAGAAGTTACAGGA; 3’RACE DXS1 GSP: ATATTTGTGCATTACTCGA; 3’-RACE Primer A: AAGCAGTGGTATCAACCGCAGAGTAC. Primers 5’- and 3’-RACE PCR reaction were performed using Jumpstart Taq DNA polymerase (Sigma). PCR conditions for 5’-RACE PCR were as follows: initial denaturation at 95 °C for 4 min followed by 35 cycles of 95 °C for 1 min, 68 °C (annealing) for 1 min, 72 °C (extension) for 2 min, and a single final incubation for 10 min at 72 °C. This primary PCR product was diluted (1:50) and used as template for the nested 5’-RACE PCR with 4 min initial denaturation at 95 °C, followed by 35 cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and a single final incubation for 10 min at 72 °C. PCR conditions for 3’-RACE were similar except that the annealing temperature was set at 50 °C. Because the bands of 3’-RACE PCR products in the agarose gel were very sharp, no nested 3’-RACE PCR was performed. PCR products were agarose gel-purified (NucleoTrap Gel Extraction Kit; Clontech, Germany), cloned into pGEM-T vector (Promega), and 6 independent clones were selected for sequencing. Contiguous sequences were checked for a continuous open reading frame (ORF) and homology with known plant type I DXS sequences. Sequences of the cloned 5’- and 3’-RACE products were assembled using the Vector NTI software (Invitrogen). Finally the sequence of the initially amplified DXSI fragment was joined to the 5’- and 3’-RACE sequences at the overlapping sites.

Isolation of DXS promoter sequences

Seven blunt-end cutting restriction enzymes (EcoRI, DruI, PvuII, SphI, SacI and SalI) were used individually to completely digest C. roseus genomic DNA. Each batch of digested genomic DNA was purified and ligated overnight at 16 °C to the adaptors provided by the Universal GenomeWalker kit (Clontech, USA). Amplification of PCR products from DXS promoters by primary and secondary walk with appropriate primers (listed in Table S1) was performed according to the manufacturer’s protocol.

Plasmid construction for promoter analysis

A 2146 bp region of the CrDXS1 promoter (Acc. No. KC625533; restricted with BamHI and NotI), a 2348 bp region of the CrDXSI promoter (Acc. No. KC625534; restricted with NotI and SacI), and a 2053 bp region of the CrDXSI2 promoter (Acc. No. KC625533; restricted with BamHI and NotI), were cloned into the multiple cloning site (MCS) of pGreen II 0800-LUC. The open reading frame of ORCA3 (EU072424) was cloned into pB7GW2 by the Gateway technology (Invitrogen).
Agrobacteria-mediated transformation of C. roseus leaves

Electrocompetent Agrobacterium C58C51 cells were transformed with empty pGreen II 0800-LUC vector, empty pB7GW2 vector, or pGreen II 0800-LUC, containing the respective DXS promoter sequence, or pB7WG2 vector containing ORCA3. Single colonies were inoculated into 5 ml of liquid YEB medium containing the appropriate selection antibiotics, and grown overnight at 28°C with vigorous shaking (180–200 rpm). 0.5 ml of the overnight culture were used to inoculate 50 ml of YEB medium including 5 μM acetylxylosyngone and appropriate antibiotics, incubated at 28°C under vigorous shaking until reaching the stationary phase. Bacteria were harvested by centrifugation at 3,000 g for 30 min at room temperature. Cells were resuspended in 10 ml of infiltration buffer (10 mM MgCl2, 10 mM MES (pH 5.8), 150 mM acetosyringone), supplemented with 10 μl L-1 Silver L-77. Concentration of bacterial suspension was measured by spectroscopy and adjusted to OD600 1.5 with infiltration buffer, incubated at room temperature for additional 2 hrs. Bacteria with DXS promoter constructs and ORCA3, respectively, were mixed at a 1:1 ratio, and the resulting bacterial suspension was infiltrated into leaves via the lower epidermis with a needleless syringe.

Dual-luciferase reporter assay

The dual-luciferase reporter assay was performed according to the procedure reported by Hellens and coauthors [43]. Approximately 10 mg of grinded leaf material was resuspended in 40 μl passive lysis buffer (PLB, Promega). Subsequently, Firefly and Renilla luciferase activities were determined using the Beetle and Renilla GlowJuice buffer, containing the corresponding substrates (PKJ, Kleinblittersdorf, Germany). Briefly, 20 μl of extract was mixed with 50 μl Beetle and Renilla Juice buffer in separate tubes and Firefly/Renilla activities were determined with the Lumat LB9507 Luminometer (Berthold Technologies). Relative luciferase activities were calculated as the ratio between the Firefly and the Renilla luciferase activities. All transfection experiments were performed four times.

Protein extraction and immunoblotting

Total protein was extracted from frozen and homogenized plant materials (same starting material as used for quantitative real-time PCR, see below) in freshly prepared extraction buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM MgCl2, 1 mM DTT, and 1 mM PMSF, followed by centrifugation for 30 min at 16,000 x g and 4°C. The protein concentration in the supernatant was determined with the Bradford reagent (Bio-Rad, USA) and centrifuged for 30 min at 16,000 x g and 4°C. The protein concentration in the supernatant was determined with the Bradford reagent (Bio-Rad, USA). Bacteria were harvested by centrifugation at 3,000 g and the resulting bacterial suspension was infiltrated into leaves via the lower epidermis with a needleless syringe.

Transcript assessment by quantitative real time PCR (qPCR)

PCR reactions were performed in 96-well plates with the iCycler thermocycler (Biorad) using SYBR Green (S7563, Invitrogen) to monitor dsDNA synthesis. Each reaction contained a mixture of 5 μl diluted cDNA sample (equivalent to 15 ng RNA input), 0.5 μl of each primer (10 μM), 1.5 μl 10 × PCR buffer, 0.3 μl dNTPs (10 mM each), 0.15 μl Jumpstart Taq DNA polymerase (Sigma), 0.15 μl diluted (1:400) SYBR Green, 0.6 μl diluted FITC (1:4000, Bio-Rad) and water in a final volume of 15 μl. The gene-specific sets of primers are presented in Table S2 (C. roseus) and Table S3 (A. thaliana). The qRT-PCR reactions were carried out following the recommended thermal profile: 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 58°C for 20 s and 72°C for 20 s. After 40 cycles, melting curves were determined by heating from 55°C to 95°C with a ramp speed of 0.5°C min −1. To determine primer efficiency, serial dilutions of the templates were conducted for all primer combinations. Each reaction was performed in triplicate, and the amplification products were examined by agarose gel electrophoresis and melting curve analysis. The expression stability of reference genes (Actin, Ubiquitin, GAPDH and EF1x) was assessed using geNorm algorithms, and the relative gene expression level was calculated by normalizing to the geometric mean of the reference genes according to a previously described method [60].

Cultivation and fosmidomycin treatment of Arabidopsis thaliana seedlings

A. thaliana ecotype Ler seeds were germinated and grown under sterile conditions for 8 days (cotyledons fully expanded), and treated with 100 μM fosmidomycin solution as reported by [23].

Chloroplast isolation and in vitro protein turnover experiments

Intact chloroplasts were isolated from young leaves of 6-week-old C. roseus plants, treated with clomazone or fosmidomycin via leaf infiltration (as described above), according to a previously described procedure [61]. Chloroplast protein degradation experiments were carried out according to the method described by Flores-Perez et al. [26]. In time course experiments, turnover of DXS and DXR proteins was monitored by separating proteins via SDS-PAGE, followed by immunoblot analysis as described above.

Supporting Information

Figure S1 Phylogenetic tree of plant DXS enzymes based on protein sequences, showing the separation into three clades. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [62]. The following plant sequences were included: CaTKT2 (Capsicum annuum, accession number CAAT75778, LeDXS (Lycopersicon esculentum, AAD38941), NiDXS (Nicotiana tabacum, CBA12909), EgDXS (Elaeis guineensis, AAS99388), CrDXS1 (Catharanthus roseus, KC625536), MtDXS1 (Medicago truncatula, CAD22530), AaDXS (Artemisia annua, AAD56909), AtCL1 (Arabidopsis thaliana, AAC49368), AtDXS2 (A. thaliana, NP_350629), ApDXS1 (Andrographis paniculata, AAP14353), GbDXS1 (Ginkgo biloba, AAS89341), PaDXS1 (Picea abies, ABS0518), PfDXS1 (Pinus densiflora, ACC54557), OsDXS1 (Oryza sativa, NP_00155524), AdDXS3 (A. thaliana, NP_001078507), VnDXS1 (Vitis vinifera, XP_002277919), MpDXS (Mentha piperita, AAC35313), TeDXS (Tagetes erecta, AAG29432), CrDXS2B (C. roseus, AB155993), PaDXS2A (P. abies, ABS0519), PfDXS2 (P. densiflora, AAS89341) and VvDXS1 (V. vinifera, XP_001295870). The expression stability of reference genes (Actin, Ubiquitin, GAPDH and EF1x) was assessed using geNorm algorithms, and the relative gene expression level was calculated by normalizing to the geometric mean of the reference genes according to a previously described method [60].
ACC54554), GbDXS2 (G. biloba, AAR56969), PaDXS2B (P. abies, AB583518), NpDXS (Narcissus pseudonarcissus, CAC08450), MtDXS2 (M. truncatula, CAD22531), SrDXS2 (Sisera rhodaniana, CAD22153), CrDXS2A (C. roseus, CA009094) and LhDXS2 (Lycopersicon hirsutum, AAT97962), OsDXS2 (O. sativa, NP_001059086), VvDXS2A (V. vinifera, XP_002260925), VvDXS2B (V. vinifera, CBI17763), VvDXS2C (V. vinifera, XP_002270336), VvDXS2D (V. vinifera, XP_002271505), OsDXS3 (O. sativa, BAA83576), VvDXS3 (V. vinifera, XP_002282420). CrDXS isoforms are indicated with close circles. Bootstrap values are based on 1000 replications. (DOCX)

Figure S2 The transketolase consensus thiamine pyrophosphate (TPP) binding domain of DXS proteins. The arrow marks the presence of negative charged amino acid aspartic acid (D) in type I DXS except for the gymnosperms Picea abies, Pinus densiflora and Ginkgo biloba, replaced by asparagin (N) in type II and III DXS. (DOCX)

Figure S3 Determination of DXS antiserum affinity to different DXS isoforms by immunoblot. Purified His-tagged DXS1, 2A and 2B protein were subjected to SDS-PAGE, transferred to the PVDF membrane, and detected by immunoblot with a polyclonal antiserum raised against recombinant CrDXS2A protein. The upper panel shows DXS specific immune signals. The lower panel indicates the loading of DXS1, 2A and 2B protein, respectively, at 500, 250, 125, 62.5 and 25 ng by silver staining. (DOCX)

Figure S4 Phenotypic changes (bleaching) in 0.5 µM paraquat-treated leaf discs from mature leaves of C. roseus. Control discs did not show visible bleaching over 30 hrs, whereas bleaching started in paraquat-treated discs after 16 hrs. (DOCX)

Figure S5 Phenotypic changes of 6-week-old C. roseus plants at 78 hrs after treatment with 50 µM clomazone or 50 µM fosmidomycin. Clomazone or fosmidomycin solution was applied to the first two pairs of mature leaves with a 1 ml needleless syringe to the lower epidermis. Arrows mark the injected leaves. Note, that only developing leaves and still growing leaf tissues are bleached. (DOCX)

Figure S6 RT-PCR amplification products of Arabidopsis DXS isoforms separated on agarose. Semi-quantitative RT-PCR amplification with isoform-specific primer sets. Note that AtDXS2 transcripts level was extremely low in 8-day-old seedlings of Arabidopsis thaliana. (DOCX)

Figure S7 Effect of 5-ketocloazone on expression of MEP pathway proteins and phenotypic changes in young leaves of 6-week-old C. roseus plants. For each plant, the first two pairs of mature leaves were injected with a 50 µM 5-keto clomazone solution (or water for control) via a 1 ml needleless syringe to the lower epidermis. For each time point, young leaves from three independent plants were harvested and processed for MEP pathway protein analysis. (A) DXS, DXR and HDS proteins were detected by immunoblot with corresponding polyclonal antisera. (B) Phenotypic changes in 5-keto clomazone and clomazone treated plants at 30, 54 and 78 hrs. (DOCX)

Table S1 Primers used for isolation of DXS promoters. (DOCX)

Table S2 Primers for qPCR analysis in C. roseus. (DOCX)

Table S3 Primers for qPCR analysis in A. thaliana. (DOCX)

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Author Contributions

Conceived and designed the experiments: MH SCH JB YZ ZA TR. Performed the experiments: MH SCH TS. Analyzed the data: MH TR. Wrote the paper: MH SCH TR.

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