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RESEARCH ARTICLE

Short title: *Developmental response to FPS down-regulation*

Suppressing Farnesyl diphosphate synthase alters chloroplast development and triggers a sterol-dependent induction Fe deficiency responses

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One sentence summary

Suppressing FPS expression affects chloroplast development, reducing cytosolic and plastidial isoprenoid levels including sterols

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Author contributions

DM and AF conceived and designed the research; DM, PA, DC, TA and MA, performed the research; DM and AF performed data analysis, collection and interpretation, and wrote the manuscript.

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Abstract

Farnesyl diphosphate synthase (FPS) catalyzes the synthesis of farnesyl diphosphate (FPP) from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). *Arabidopsis thaliana* contains two genes (*FPS1* and *FPS2*) encoding FPS. Single *fps1* and *fps2* knockout mutants are phenotypically indistinguishable from wild type plants, while *fps1/fps2* double mutants are embryo lethal. To assess the effect of FPS down-regulation at post-embryonic developmental stages, we generated Arabidopsis conditional knockdown mutants expressing amiRNAs devised to simultaneously silence both *FPS* genes. Induction of silencing from germination rapidly caused chlorosis and a strong developmental phenotype that led to seedling lethality. However, silencing of FPS after seed germination resulted in a slight developmental delay only, though leaves and cotyledons continued to show chlorosis and altered chloroplasts. Metabolomic analyses revealed also drastic changes in the profile of sterols, ubiquinones and plastidial isoprenoids. RNA-seq and RT-qPCR transcriptomic analysis showed that a reduction in FPS activity levels triggers misregulation of genes involved in biotic and abiotic stress responses, the most prominent one being the rapid induction of a set of genes related to the jasmonic acid (JA) pathway. Down-regulation of FPS also triggered a Fe-deficiency transcriptional response that is consistent with the Fe-deficient phenotype observed in FPS silenced plants. The specific inhibition of the sterol biosynthesis pathway by chemical and genetic blockage mimicked these transcriptional responses, indicating that sterol depletion is the primary cause of the observed alterations. Our results highlight the importance of sterol homeostasis for normal chloroplast development and function, and reveal important clues about how isoprenoid and sterol metabolism is integrated within plant physiology and development.

Introduction

Isoprenoids are the largest class of all known natural products in living organisms with tens of thousands of different compounds. In plants, isoprenoids perform essential biological functions, including maintenance of proper membrane structure and function (sterols), electron transport (ubiquinones and plastoquinones), posttranslational protein modification (dolichols serving as glycosylation cofactors and prenyl groups), photosynthesis (chlorophylls and carotenoids), and regulation of growth and development (abscisic acid, brassinosteroids, cytokinins, gibberellins (Croteau et al., 2000) and strigolactones (Al-Babili and Bouwmeester, 2015)). A large number of isoprenoids play also prominent roles as mediators of interactions between plants and their environment, including a variety of defense responses against biotic and abiotic stresses (Tholl and Lee, 2011). In fact, there is hardly any aspect of plant growth, development and reproduction not relying on isoprenoids or isoprenoid-derived compounds. In addition, many plant isoprenoids are of great economic importance because of their wide range of industrial and agricultural applications (Bohlman and Keeling, 2008; George et al., 2015).

All isoprenoids derive from the 5-carbon (C_5) building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). These precursors can be synthesized through two distinct pathways: the mevalonic acid (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (for a recent review see Hemmerlin et al., 2012). In contrast to most organisms, plants use both pathways to form IPP and DMAPP, which operate independently and are located in different cell compartments (Fig. 1A). The MVA pathway operates in the cytosol-endoplasmic reticulum (ER) while the MEP pathway localizes into plastids (Vranová et al., 2013). Both pathways are essential for plant viability (Rodríguez-Concepción et al., 2004). Then, different prenyltransferases catalyze a series of sequential head-to-tail condensations of IPP with DMAPP and the resulting allylic diphosphates to produce linear prenyl diphosphates of increasing chain length. Among these are the short chain *trans*- or (*E*)-prenyl diphosphates, geranyl diphosphate (GPP; C_{10}), farnesyl diphosphate (FPP; C_{15}) geranylgeranyl diphosphate (GGPP; C_{20}) and geranylfarnesyl diphosphate (GFPP; C_{25}) (Fig. 1A), so called because of the stereochemistry of double-bond established between IPP and the allylic substrates (Wang and Ohnuma, 2000; Vandermoten et al., 2009; Nagel et al., 2015). These linear prenyl diphosphates are further metabolized by terpene synthases leading to the large variety of cyclic and acyclic terpenoid carbon skeletons found in plants, which are subsequently decorated by oxidation, reduction, isomerization, conjugation or other secondary transformations

to produce the myriad different isoprenoids found in plants (Chen et al., 2011; Kumari et al., 2013).

FPP synthase (FPS; EC 2.5.1.10) catalyzes the condensation of MVA-derived IPP and DMAPP to form GPP, the allylic intermediate that undergoes a second condensation reaction with IPP to produce FPP (Poulter, 2006). Cytosolic FPP serves as common precursor of sterols, triterpenes and brassinosteroids, dolichols and polyprenols, sesquiterpenes, and the farnesyl moiety of heme groups and prenylated proteins (Fig. 1A) (Vranová et al., 2013). FPP formed in the mitochondria from MVA-derived IPP and DMAPP serves as a precursor of ubiquinones (Disch et al., 1998). On the contrary, GPP, GGPP and GFPP are mostly produced in plastids from MEP-derived IPP and DMAPP by GPP synthase (GPS), GGPP synthase (GGPS) and GFPP synthase (GFPS), respectively. GPP is the precursor of monoterpenes whereas GGPP is utilized for the biosynthesis of photosynthetic pigments such as chlorophylls and carotenoids, plastoquinones and other plastidial prenyl quinones, diterpenes, gibberellins, via oxidative carotenoid cleavage strigolactones and abscisic acid, and finally for protein geranylgeranylation (Vranová et al., 2013; Al-Babili and Bouwmeester, 2015; Huchelmann et al., 2016). GFPP is the suggested precursor of sesterterpenes (Fig. 1A) (Nagel et al., 2015). Although it is widely accepted that isoprenoids of cytosolic and plastidial origin are mostly formed from physically segregated pools of linear prenyl diphosphates, it is also well documented that under certain growth conditions and/or in specific plant tissues and species there is a limited exchange of precursors between the cytosol and plastidial isoprenoid pathways (Vranová et al., 2012; Opitz et al., 2014). IPP, DMAPP, GPP and FPP are considered as the most plausible intermediates to be transported across the plastidial membranes (Soler et al., 1993; Bick and Lange, 2003; Flügge and Gao, 2005), though the precise mechanisms by which this exchange occurs and is regulated, the identity of the putative prenyl phosphate transporters and the real capacity of plants to exchange intermediates between these compartments remains to be established (Opitz et al., 2014).

Arabidopsis thaliana contains two genes encoding FPS, namely *FPS1* (At5g47770) and *FPS2* (At4g17190) (Cunillera et al., 1996). *FPS1* encodes isoforms FPS1L and FPS1S, which differ by an N-terminal extension of 41 amino acids that targets FPS1L into mitochondria (Cunillera et al., 1997; Manzano et al., 2006) whereas FPS1S, like FPS2, localize to the cytosol (Fig. 1A) (Keim et al., 2012). The characterization of Arabidopsis mutants with either gain or loss of function of specific *FPS* genes revealed that *FPS1* and *FPS2* have highly overlapping but not completely redundant functions in isoprenoid biosynthesis. Overexpression of FPS1L or FPS1S cause a cell

death/senescence-like phenotype due to a metabolic imbalance that impairs cytokinin biosynthesis (Masferrer et al., 2002; Manzano et al., 2006) whereas over-expression of FPS2 has no apparent detrimental effect on plant growth and development (Bathia et al., 2015). On the other hand, *fps1* and *fps2* single knock-out mutants are almost indistinguishable from wild type plants, which is in sharp contrast with the embryo lethality of the *fps1*/*fps2* double mutant (Closa et al., 2010). This work and several other studies have demonstrated that normal functioning of the isoprenoid pathway in the cytosol is indispensable for plant viability. Genetic lesions affecting different enzymatic steps of this biosynthetic pathway result in male gamete impaired genetic transmission or early embryonic or post-embryonic developmental arrest (Schrick et al., 2000; Schrick et al., 2002; Kim et al., 2005; Babiychuk et al., 2008; Suzuki et al., 2009; Carland et al., 2010; Closa et al., 2010; Ishiguro et al., 2010; Jin et al., 2012). These lethal phenotypes have been primarily attributed to depletion of sterol levels, the major MVA-derived end products.

Plant sterols consist of a mixture of three major species, namely β-sitosterol (the most abundant one), stigmasterol and campesterol, and a variety of minor sterols that are biosynthetic precursors of main sterols (Schaller 2003; Benveniste 2004). β–Sitosterol, stigmasterol and campesterol are the bulk membrane sterols (Hartmann-Bouillon and Benveniste, 1987), and campesterol is also a precursor of the brassinosteroids (Fujioka and Yokota, 2003). Sterols are integral components of plant cell membranes that are found predominantly in the plasma membrane and in a much lower amount in the tonoplast, ER, mitochondria (Hartmann, 1998; Horvath and Daum, 2013) and the outer membrane of chloroplasts (Moeller and Mudd, 1982; Hartmann-Bouillon and Benveniste 1987; Lenucci et al., 2012). In addition to this key structural role, sterols also play pivotal roles in embryonic, vascular and stomatal patterning (Jang et al., 2000; Carland et al., 2002; Quian et al., 2013), cell division, expansion and polarity (He et al., 2003; Men et al., 2008), hormonal regulation (Souter et al., 2002; Kim et al., 2010), vacuole trafficking (Li et al., 2015) and cell wall formation (Schrick et al., 2012). Some recent reports also point towards a role for sterols in proper plastid development (Babiychuk et al., 2008; Kim et al., 2010; Gas-Pascual et al., 2015). As key components of cell membranes, sterols are dynamic modulators of their biophysical properties so that changes in the composition of sterols affect membrane fluidity and permeability (Roche et al., 2008; Grosjean et al., 2015) and therefore modulate the activity of membrane-bound proteins (Carruthers and Melchoir 1986; Cooke and Burden, 1990; Grandmougin-Ferjani et al., 1997) and the plant adaptive responses to different types of abiotic and biotic stress, including tolerance to thermal stress (Hugly

et al., 1990; Beck et al., 2007; Senthil-Kumar et al., 2013), drought (Posé et al., 2009; Kumar et al., 2015), metal ions (Urbany et al., 2013; Wagatsuma et al., 2015) and H_2O_2 (Wang et al., 2012a), and to bacterial and fungal pathogens (Griebel and Zeier, 2010; Wang et al., 2012b; Kopischke et al., 2013).

The embryo lethal phenotype of the Arabidopsis *fps1*/*fps2* double knock-out mutants makes it very difficult to assess the biological role of FPP biosynthesis in post-embryonic plant development. To overcome this drawback, we generated conditional knock-down Arabidopsis mutants using a chemically inducible amiRNA-based gene silencing approach to down-regulate *FPS* gene expression. We report here the results of the phenotypical, metabolomic and transcriptomic analysis of these mutants. Upon FPS silencing plants develop a chlorotic phenotype associated with important alterations in chloroplasts development and a marked alteration in the profile of the major cytosolic, mitochondrial and plastidial isoprenoids. In addition, we demonstrate that FPS down-regulation and the concomitant depletion of bulk membrane sterols trigger an early misregulation of genes involved in stress responses, being particularly remarkable the misregulation of genes related to the JA pathway and the maintenance of cellular Fe homeostasis. This transcriptional response is mimicked by the specific inhibition of the sterol biosynthesis pathway, suggesting that even though FPP serves as a precursor of a number of essential isoprenoid end products, sterol depletion is the primary cause of the observed alterations.

Results

Characterization of conditional Arabidopsis FPS knock-down mutants

Arabidopsis mutants harboring a single functional *FPS* gene show only slight phenotypic alterations that appear during the early stages of development, whereas simultaneous knockout of both *FPS* genes is embryo lethal (Closa et al., 2010). This makes it extremely difficult to assess the biological function of FPS beyond this stage of development. To overcome this limitation, we generated conditional Arabidopsis *FPS* knockdown mutant lines by combining an ecdysone-inducible promoter (Padidam et al., 2003) with the artificial microRNA (amiRNA) technology (Schwab et al., 2006). The Web MicroRNA Designer 3 tool (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) was used to generate a list of candidate amiRNAs specifically devised to simultaneously silence *FPS* genes. Two amiRNAs, referred to as amiFPSa and amiFPSb, were chosen from the top rank list. The sequence of these amiRNAs, their position on the FPS1 and FPS2 mRNA sequences, the hybridization energy, and the number and the position of the mismatches are shown in Figure 1B. The sequences

containing the amiFPSa and amiFPSb precursors were cloned into pB110-Red-284 binary vector harboring the ecdysone receptor-based inducible gene expression system (Padidam et al., 2003; Dietrich et al., 2008) and a DsRed constitutive expression cassette allowing for the identification of transgenic red fluorescent seeds. Arabidopsis Col-0 plants were then transformed using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Based on the segregation analysis of the fluorescent seed trait, several independent T3 homozygous lines harboring the amiFPS precursor constructs were generated, and one line for each amiRNA was selected for further characterization. Plants of the amiFPSa and amiFPSb selected lines grown on MS medium were indistinguishable from Arabidopsis Col-0 plants transformed with the pB110-Red-284 vector lacking the pre-amiRNA construct, further referred to as empty vector (EV) plants. However, plants germinated in the presence of 30 μM methoxyfenozide (MFZ), the ecdysone receptor agonist (Padidam et al., 2003; Koo et al., 2004), displayed a strong detrimental phenotype. Plants showed a severe size reduction of roots and the aerial part, chlorosis in the cotyledons, and failed to develop true leaves (Fig. 1C and D), which ultimately caused them to die. None of these effects was observed in EV plants treated with MFZ (Fig. 1C and D) or amiFPS and EV plants grown on MS (Fig. 1C).

The severe developmental phenotype observed when amiFPS plants were germinated in the presence of MFZ made impossible to distinguish between the effects specifically attributable to the silencing of FPS and those due to the developmental delay of FPS-silenced plants compared to control plants. To overcome this constraint, amiFPSa and amiFPSb plants were first grown for 3 days on MS and then transferred to MS supplemented with MFZ. Under these conditions, degreening symptoms started to appear in cotyledons between 2 and 3 days after induction of silencing, and became evenly spread over the whole cotyledons and leaves at 5 days after induction (Fig. 2A). Interestingly, the first pair of true leaves showed only a slight reduction in size. Thus, the developmental stage of amiFPS plants was considered comparable to that of the control plants (Fig. 2A), and these experimental conditions were selected to further characterize the response of plants to FPS silencing.

FPS gene expression analysis in silenced amiFPSa and amiFPSb plants revealed that FPS1 mRNA levels were reduced to 21% and 16%, respectively, of those in the EV plants treated with MFZ, while FPS2 mRNA levels were reduced to 26% and 35%, respectively (Fig. 2B). Such a drastic reduction of FPS transcript levels confirmed that both amiRNAs were highly effective in silencing *FPS* gene expression, although with a slightly different specificity towards their mRNA targets. Protein blot analysis using anti-

FPS antibodies (Manzano et al., 2006) and FPS activity measurements demonstrated a concomitant reduction of total FPS protein (Fig. 2C) and enzyme activity, which in amiFPSa and amiFPSb plants treated with MFZ dropped to below 40% and 50% the activity in the control plants, respectively (Fig. 2D). On the contrary, no significant differences in FPS mRNA, protein and enzyme activity levels were detected in amiFPS plants grown on MS compared to EV plants grown on MS or MS supplemented with MFZ (Fig. 2B, C and D). Constitutive overexpression of isoform FPS1S in amiFPSa plants harboring a *35S::FPS1S* transgene (Masferrer et al., 2002) (Figure S1A) fully complemented the phenotype of MFZ-treated amiFPSa plants (Figure S1B), demonstrating that the phenotype displayed by amiFPS plants upon treatment with MFZ was specifically due to down-regulation of FPS activity and not to undesired off-target gene silencing effects or to the inducible expression system used (compare EV results in Fig. 2).

Down-regulation of FPS leads to altered levels of major MVA-derived isoprenoids and triggers post-transcriptional up-regulation of HMGR activity

Quantitative analysis of sterols by GC-MS in amiFPSa, amiFPSb and EV plants grown with and without MFZ revealed a significant reduction in the levels of bulk membrane sterols when amiFPS plants were grown with MFZ. The levels of β-sitosterol in silenced amiFPSa and amiFPSb plants were, respectively, 36 and 25% lower than in MFZ-treated EV plants, whereas those of campesterol decreased by 42 and 30%, respectively (Fig. 3A). A much more prominent reduction was observed in the case of stigmasterol, with a decrease of 70 and 66%, respectively (Fig. 3A). Interestingly, these changes also led to a marked reduction of the stigmasterol to β-sitosterol ratio, which has a critical role in modulating cell membrane integrity and properties (Schuler et al., 1991; Grosjean et al., 2015) and the normal function of membrane-located proteins (Hartmann, 1998), and is known to influence plant disease resistance and affect the outcome of particular plant–pathogen interactions (Griebel and Zeier, 2010; Wang et al., 2012b). This ratio decreased by 53 and 54.5% in amiFPSa and amiFPSb plants treated with MFZ, respectively, compared to MFZ-treated EV plants (Fig. 3B), while the campesterol to β-sitosterol ratio remained unchanged. Quantitative analysis of ubiquinones using UPLC-MS showed that the levels of the major ubiquinone species (UQ-9) were decreased by 30% in silenced amiFPSa and amiFPSb plants when compared to EV plants grown on medium supplemented with MFZ, whereas the levels of UQ-10, a minor ubiquinone species in Arabidopsis, were drastically increased by 3 and 4 times, respectively (Fig. 3C).

Recent reports have shown that a selective blockage of the sterol pathway triggers a positive feedback regulatory response of HMGR, the main regulatory enzyme of the MVA pathway (Wentzinger et al., 2002; Babiychuk et al., 2008; Nieto et al., 2009; Posé et al., 2009). Such a regulatory response has also been reported in Arabidopsis seeds with reduced levels of FPS activity (Closa et al., 2010; Keim et al., 2012). HMGR activity measurements in amiFPSa, amiFPSb and EV plants grown with and without MFZ revealed that down-regulation of FPS activity at the early stages of plant development trigger a similar response. Treatment of amiFPSa and amiFPSb plants with MFZ resulted in a compensatory up-regulation of HMGR activity of 3.4-fold and 1.7-fold, respectively, compared to that in MFZ-treated EV plants, which in turn was similar to that in non-induced amiFPS and EV plants (Fig. 4A). Comparison of HMGR activity values (Fig. 4A) with results of protein blot analysis (Fig. 4B) using polyclonal antibodies raised against the catalytic domain of Arabidopsis HMGR1 (Masferrer et al., 2002) strongly suggested that up-regulation of HMGR activity in FPS silenced plants occurs at the post-translational level. This is supported by the observation that HMGR activity in extracts from amiFPSa and amiFPSb plants treated with MFZ was clearly higher than in control plants (Fig. 4A) even though HMGR protein levels in the same extracts were slightly or clearly lower than in the corresponding controls (Fig. 4B). Indeed, expression levels of the two Arabidopsis genes (*HMG1* and *HMG2*) encoding HMGR (Enjuto et al., 1994) were very similar in all the tested lines grown on MS supplemented or not with MFZ (Fig. 4C). Altogether, these data indicated that the activation of HMGR in response to silencing of FPS occurs through a post-translational regulatory mechanism.

The combined effect of FPS down-regulation and the activation of HMGR might lead to the accumulation of cytotoxic levels of pathway intermediaries upstream of FPP (Fig. 1A) that could be responsible for the phenotype of FPS silenced plants. However, the addition of 5 mM MVA to the growth medium did not enhance the phenotype of these plants (Fig. 4D), thus indicating that it is caused by the reduced accumulation of an FPP-derived compound. As mentioned above, the down-regulation of FPS leads to an important reduction of campesterol levels (Fig. 3A). This sterol is the biosynthetic precursor of brassinosteroids (Fujioka and Yokota, 2003), which raised the question of whether a deficiency of these hormones could trigger the phenotype of FPS silenced plants. However, our finding that the addition of 0.4 nM *epi*-brassinolide was unable to rescue the phenotype linked to down-regulation of FPS (Figure S2) ruled out this possibility.

Down-regulation of FPS alters chloroplast development and plastidial isoprenoid levels

The chlorotic phenotype displayed by FPS silenced plants strongly suggested that chloroplast structure and function might be affected in these plants. Confocal laser microscopy analysis of leaves revealed a strong reduction in the chloroplast area of amiFPSa and amiFPSb silenced plants compared to EV plants grown under the same conditions (i.e., about 55% and 38% of control chloroplast area, respectively) (Fig. 5A and C). A close-up inspection of the confocal microscope images also showed that chloroplasts of FPS silenced plants contained darker regions likely devoid of chlorophyll (Fig. 5A), suggesting that chloroplast ultrastructure was also affected. Indeed, chloroplasts of amiFPSa and amiFPSb plants treated with MFZ showed severe morphological alterations, including an irregular outer membrane envelope, disorganized and less abundant thylakoid membranes, massive accumulations of starch granules and a large number of electron-dense particles that are likely to correspond to plastoglobuli. On the contrary, chloroplasts of EV plants treated with MFZ showed normal structure, with well-organized thylakoid membranes and a regular shape (Fig. 5B), and looked indistinguishable from chloroplasts of amiFPSa, amiFPSb and EV plants grown on MS. Altogether, these data indicated that normal levels of FPS activity are essential for proper chloroplast development.

When the photosynthesis-related isoprenoid metabolites were quantified in all these plants, a drastic reduction of chlorophyll (a and b) and carotenoid levels (50 to 60%) was observed in MFZ-treated amiFPS plants compared to EV plants grown under the same conditions (Fig. 5D). A more detailed analysis of the carotenoid fraction confirmed a sharp decline in β-carotene, lutein, and violaxanthin-neoxanthin contents in MFZ-treated amiFPS plants compared to EV plants grown on medium with MFZ. The levels of these metabolites decreased, respectively, by 57, 48 and 53% in amiFPSa plants, and 45, 36 and 35% in amiFPSb plants (Figure S3A). Levels of other important plastidial isoprenoid compounds, such as prenylquinones, were also strongly altered. The levels of phylloquinone and plastochromanol-8 were markedly reduced in silenced amiFPS plants (42% in amiFPSa plants and 23 and 29%, respectively, in amiFPSb plants), while those of plastoquinone-9 were only slightly reduced (16% only in amiFPSa plants) (Figure S3B). On the contrary, the levels of tocopherols (phytyl-derived side chain compounds) were increased, as is the case of γ-tocopherol (3.2- and 2,4-fold in amiFPSa and amiFPSb plants, respectively), or remained unchanged, as observed in α- and δ-tocopherol levels (Figure S3C). Altogether these results

demonstrated that down-regulation of FPS activity has a strong negative impact on plastidial isoprenoid metabolism in addition to the detrimental effect on cytosol/ER isoprenoid biosynthesis.

Transcriptional profiling of plants silenced for FPS reveals misregulation of genes involved in JA pathway, abiotic stress response, Fe and redox homeostasis, and carbohydrate metabolism.

To get insight into the molecular phenotype of Arabidopsis plants with reduced levels of FPS activity, global changes of gene expression in FPS silenced plants relative to non-silenced plants were investigated using an RNA-Seq-based approach. Using RNA samples from three independent biological replicates of amiFPSa plants grown on MS supplemented or not with MFZ, two cDNA libraries were constructed and sequenced with an Illumina Hi-Seq 2000 platform. After trimming the obtained raw reads to remove adaptor sequences, empty reads and low-quality sequences, a total of 168,617,824 and 144,342,444 high-quality reads, designated as clean reads, were generated for amiFPSa plants grown on MS supplemented or not with MFZ, respectively, of which 99,29 and 98.18% were paired-end reads and 0,71% and 1,82% were single-end reads. Almost all clean reads (97% and 98%, respectively) were successfully mapped to the TAIR 10 version of *A. thaliana* reference genome. FPKM (fragments per kilobase of exon per million fragments mapped) normalized read counts were obtained from each of the samples and employed for differential gene expression analysis (DGEA). The resulting list of differentially expressed genes was filtered by $\log_2$ (fold_change) of ≥ 2 or ≤ -2 and a statistical value of $q = 0.05$. On this basis, a total of 168 differentially expressed genes were identified in FPS-silenced plants compared to non-silenced plants, including 116 up-regulated genes and 16 down-regulated genes. Additionally, we found 35 genes that were expressed only in FPS silenced plants (*switch on* genes) and one single gene that was expressed only in non-silenced plants (*switch off* gene) (Table S1). Classification of differentially expressed genes using Gene Ontology (GO) Biological Process (BP) functional domain revealed an overrepresentation of genes involved in stress responses. The biotic stress response, including the response to bacterial and fungal infections, signal transduction, regulation of systemic acquired resistance and response to salicylic acid (SA) stimulus (Fig. 6), was the most represented one. Remarkably, 15 genes were assigned to the category of jasmonic acid (JA) response, including genes involved in JA biosynthesis, signaling and homeostasis. Adding to this category six other genes reported to be specific targets of the JA response brought the total number of up-regulated JA-related genes to 19 (Table 1), i.e., 11.5% of the total of differentially expressed genes (Table S1). In the

abiotic stress category the main responses were related to wounding, salt, cold and water deprivation (Fig. 6), and genes included in this category were both up- and down-regulated (Table 1). Genes encoding proteins related to Fe homeostasis and redox functions were also prominent among the misregulated genes (Table 1). Interestingly, those associated to Fe storage, metabolism and transport were down-regulated whereas those coding for proteins involved in sensing and signaling of Fe deficiency were up-regulated. In the group of misregulated redox genes, all but one were up-regulated. Finally, we also identified a group of misregulated genes coding for proteins related to carbohydrate metabolism (Table 1), a transcriptional response consistent with the altered chloroplast development observed in the FPS silenced plants.

Inhibition of sterol biosynthesis mimics the transcriptional response to down-regulation of FPS

As mentioned previously, the phenotypic alterations displayed by FPS-silenced plants are not due to the accumulation of MVA or a derived compound upstream of FPP (Fig. 4D) but, rather, to the reduced levels of an FPP-derived compound other than brassinosteroids (Figure S2). This, together with the marked reduction of bulk-membrane sterols detected in these plants (Fig. 3A and B) and the suggested possible connection between impaired sterol biosynthesis and altered chloroplast development (Babiychuk et al., 2008; Kim et al., 2010), prompted us to investigate whether sterol depletion is responsible for the transcriptional response to FPS silencing. To this end, we compared the mRNA levels of 39 genes representative of the main physiological responses observed in FPS silenced plants (Table 1) with those in wild type plants treated with terbinafine (Tb), a specific inhibitor of squalene epoxidase (SQE) (Ryder, 1992), and *cvp1*/*smt3* mutant plants (Carland et al., 2010). SQE catalyzes the synthesis of 2,3-oxidosqualene from squalene, the first committed precursor of sterols. SMT2 and SMT3 are both sterol-C24-methyltransferases responsible for the methylation of 24-methylene lophenol to produce 24-ethylidene lophenol, the reaction that distinguishes the synthesis of structural sterols β-sitosterol and stigmasterol from campesterol and the signaling brassinosteroid derivatives (Schaller 2003; Carland et al., 2010) (Fig. 7A). Thus, we performed a real time RT-qPCR expression analysis for genes included in the categories of JA pathway (*LOX4, AOC1, AOC3, CLO-3, CYP94B3, ST2A, JAZ1, JAZ5, WRKY33, JAL23, ABCG40, VSP1, JR2* and *ATCLH1*), abiotic stress (*AKR4C8, AKR4C9, COR78, COR414, COR15B* and *RAP2.6L*), Fe homeostasis (*NEET, FER1, FER4*, *MLP329*, *BHLH038*, *BHLH039* and *BHLH100*), redox homeostasis (*GST22, GSTF6, GSTF12, Prx37,* and *WCRKC1*), carbohydrate metabolism (*DIN11, BMY1, SCORP, GPT2* and *FBA5*), and two genes not included in

the above categories (*ILL6 and MLP328*), using RNA from amiFPSa and EV plants treated with MFZ, wild type plants treated or not with 150 μM Tb, and *cvp1*/*smt3* mutant plants grown on MS. The fold-change in the expression levels of the selected genes for each treatment were calculated relative to their corresponding controls and the results represented as a heatmap (Fig. 7B). Overall, the comparison of the RT-qPCR and RNA-seq expression results in FPS silenced plants showed that all tested genes were misregulated in the same way (10 genes induced and 29 genes repressed), thus confirming the DGEA results obtained in the RNA-seq analysis (Table 1; Table S1). Additionally, there was a very high degree of qualitative correlation between gene expression changes in plants silenced for FPS and plants where the sterol pathway was inhibited chemically (terbinafine) or genetically (*cvp1/smt3* mutant). In both cases, 35 genes (i.e., 90% of the analyzed genes) were misregulated in the same way when compared to FPS silenced plants, while only 4 genes were misregulated in the opposite direction. Three of the latter (*COR78*, *COR414* and *COR15B*), unlike what happens in FPS silenced plants, were induced upon specific inhibition of the sterol pathway and belong to the same group of cold responsive genes (Table 1). The fourth differentially expressed gene was *WRCKC1* in plants treated with Tb and *AOC3* in the *cvp1/smt3* plants. In fact, these are the only 2 differentially regulated genes between plants treated with Tb and the *cvp1/smt3* plants, which means that 95% of the genes tested in these plants were misregulated in the same way. Altogether, these data indicated that inhibition of sterol biosynthesis triggers a transcriptional response highly similar to that observed in plants with a compromised synthesis of FPP, suggesting that depletion of sterols is the primary cause of the molecular and physiological phenotypes observed in FPS silenced plants.

Time course expression analysis reveals an early response of misregulated genes after FPS silencing.

To determine if the transcriptional responses observed in FPS silenced plants were a primary effect or a secondary consequence of FPS down-regulation, we conducted a time course expression analysis of a subset of genes representative of the different functional categories shown in Table 1. To this end, amiFPSa and EV plants were grown on MS for three days, transferred to MS supplemented with MFZ, and sampled at different time points (0, 4, 8, 12, and 24 hours) for RNA extraction. The JA-related transcriptional response was analyzed by quantifying the transcript levels of representative genes involved in JA biosynthesis (*LOX4*), signaling (*JAZ1* and *JAZ5*) and homeostasis (*ST2A*), as well as JA target genes (*JR2, VSP1* and *ABCG40/PDR12*) (Campbell et al., 2003; Wasternack and Hause, 2013). As shown in

Figure 8A, the mRNA levels of *LOX4*, *JAZ1* and *JAZ5* started to increase almost immediately after induction of silencing, suggesting that down-regulation of FPS triggers an early activation of JA biosynthesis and signaling pathways. This was further supported by the progressive increase of mRNA levels observed for the JA-responsive defense genes *JR2, ABCG40/PDR12* and *VSP1* after induction of FPS silencing, which reached a maximum at the end of the time course. Interestingly, mRNA levels of *ST2A*, a gene involved in JA homeostasis, were early down-regulated, suggesting a concomitant reduction of the JA catabolic turnover, which in the long term appears to be activated because 5 days after induction of silencing *ST2A* mRNA levels were clearly higher in the FPS silenced plants than in the non-silenced ones (Table 1 and Fig. 7B). The expression of three representative genes (*AKR4C8*, *AKR4C9* and *COR78*) known to be misregulated in response to different abiotic stresses including drought, heat, cold, salt and osmotic stress was also analyzed. *AKR4C8* and *AKR4C9* encode two members of the aldo-reductase family involved in detoxification of stress-induced reactive carbonyls (Sengupta et al., 2015) and *COR78* is a gene reported as responsive to cold (Nordin et al., 1991; Horvath et al., 1993). As shown in Figure 8B, in FPS silenced plants *AKR4C8* and *AKR4C9* mRNA levels were strongly increased from the very beginning and throughout the entire time course analysis compared to non-silenced plants. On the contrary, *COR78* mRNA levels were consistently lower throughout the entire time course, with the only exception of time point 12h. Overall, these results confirmed the close relationship between defective sterol biosynthesis and the induction of the JA pathway, and demonstrated that plants quickly perceive a disruption of sterol homeostasis as a stress signal that triggers an early misregulation of stress-related genes.

The Fe-deficiency transcriptional response to FPS silencing was investigated by measuring the mRNA levels of genes coding for proteins involved in Fe plastid storage (*FER1* and *FER4*) (Briat et al., 2010) and metabolism (*NEET*) (Nechushtai et al., 2012), as well as proteins involved in sensing and signaling of Fe deficiency (*bHLH038*, *bHLH039* and *bHLH100*) (Rodríguez-Celma et al., 2013) (Fig. 9A). *FER4* transcripts were markedly down-regulated throughout the time course analysis, whereas *FER1* mRNA levels showed a similar but milder initial depletion followed by a significant increase above the levels detected in control plants after 24h of FPS silencing. However, in the long term the *FER1* transcript levels were again lower than in control plants (Table 1 and Fig. 7B). The *NEET* transcript levels also decreased over the entire time course albeit to a different extent depending on the time point. The impact of FPS silencing on Fe homeostasis was further confirmed by quantifying the mRNA levels of

three members of the Ib subgroup of bHLH transcription factors that are induced by Fe deficiency. *bHLH038, bHLH39* and *bHLH100* mRNA levels changed in a very similar way, with an increase over the first 8h after FPS silencing followed by a reduction at time points 12 and 24h (Fig. 9A). After 5 days of silencing the mRNA levels of these genes in FPS-silenced plants were again higher than in control plants (Table 1 and Fig. 7B). Overall, the long term Fe-related gene expression changes pointed to a Fe deficiency, which was confirmed by the finding that Fe levels in MFZ-treated amiFPS plants were 30% lower than in EV plants grown on MFZ (Fig. 9B). Moreover, the rapid and highly coordinated response of all these Fe-related genes strongly suggests that the perturbation of Fe cellular homeostasis (Fig. 9B) is an early consequence of FPS silencing and sterol depletion.

To analyze the time course of the oxidative stress-related transcriptional response, we investigated the expression of genes coding for the glutathione S-transferases GST6, GST12 and GST22 (Dixon et al., 2010; Foyer and Noctor, 2011), and the peroxidase Prx37 (Shin et al., 2005). Interestingly, the mRNA levels of these genes showed the same qualitative pattern of changes throughout the time course analysis (Figure S4A). Silencing of FPS led to decreased mRNA levels of all four genes from the very beginning throughout the entire time course, being *GST6* and *GST12* the most deeply repressed, with the only exception of time point 12h. The early down-regulation of these antioxidant genes is in sharp contrast to their long-term response, since 5 days after induction of silencing the mRNA levels of all four genes were much higher than in non-silenced plants (Table 1 and Fig. 7B). This antioxidant response might account for the absence of oxidative stress symptoms in the leaves of FPS silenced plants compared to control plants (Figure S4B). Either way, the results are indicative of an early disturbance of the cellular redox homeostasis in response to FPS silencing.

Lastly, we investigated the expression of selected genes coding for proteins related to carbohydrate metabolism, including a defensin-like protein predicted to be involved in maltose and starch metabolism (*SCORP*), an aldolase superfamily protein involved in the sucrose signaling pathway (*FBA5*) (Lu et al., 2012), a glucose 6-phosphate/phosphate translocator that imports glucose 6-phosphate from cytosol to chloroplast and is induced in response to impaired carbon metabolism or its regulation (*GPT2*) (Dyson et al., 2015), a 2-oxoacid-dependent dioxygenase repressed by sugar (*DIN 11*) (Fujiki et al., 2000; Fujiki et al., 2001), and β-amylase 5 (*BMY1*), the major form of β-amylase in Arabidopsis (Laby et al., 2001). Results showed that FPS silencing triggered an early down-regulation of *SCORP*, *FBA5* and *GPT2* mRNA levels and a mild but steady up-regulation of *DIN11* mRNA content, but had no effect on

BMY1 mRNA content over the first 24h (Figure S5). Interestingly, changes in the mRNA levels of all five genes were much more pronounced at the end of the time course, with a strong up-regulation of *BMY1*, *DIN11*, *GPT2* and *SCORP* mRNA levels, and a marked depletion of *FBA5* mRNA (Table 1 and Fig. 7B). Altogether these results suggest that carbohydrate metabolism is also altered by the down-regulation of FPS.

Discussion

Down-regulation of FPS activity in Arabidopsis amiRNA-based conditional knockdown mutants

Arabidopsis *fps1* and *fps2* single knockout mutants are viable and almost indistinguishable from wild type plants while *fps1*/*fps2* double knockout mutants are embryo-lethal (Closa et al., 2010). This makes it impossible to investigate the biological function of FPS at postembryonic stages using *fps* knockout mutants. To overcome this limitation, we obtained and characterized Arabidopsis mutant lines expressing amiRNAs targeting simultaneously both Arabidopsis *FPS* genes (Fig. 1B) under the control of an ecdysone-inducible expression promoter system (Padidam et al., 2003; Dietrich et al., 2008). When the expression of the amiFPS-coding transgenes was induced after seed germination, amiFPS plants were able to develop the first pair of true leaves, which showed only a slight reduction in size compared to control plants, and displayed a chlorotic phenotype (Fig. 2A). Molecular and biochemical analyses in amiFPS seedlings grown under these conditions showed a strong down-regulation of FPS1 and FPS2 transcripts that resulted in a pronounced reduction of total FPS protein and enzyme activity levels (Fig. 2). The finding that expression of both amiRNAs caused the same phenotype, together with the fact that it was fully complemented by constitutive expression of isoform FPS1S (Masferrer et al., 2002) (Figure S1), confirmed that the phenotype was specifically due to down-regulation of FPS and not to undesired side effects caused by the misregulation of unpredicted off-targets and/or artifacts derived from the inducible silencing system used. It is worth noting that full reversion of the phenotype was achieved even though FPS mRNA levels in the double transgenic plants amiFPSa/35S::FPS1 were not fully restored to wild type levels (Figure S1C), which is in agreement with the notion that wild type levels of FPS activity are not limiting in the isoprenoid pathway (Manzano et al., 2004). Interestingly, FPS2 mRNA levels in the double transgenic plants were also higher than in amiFPSa plants (Figure S1C), most likely because large amounts of amiFPSa (Figure S1D) are diverted to silence both endogenous and ectopic *FPS1* gene expression at the expense of the amount of amiFPSa available for silencing of *FPS2* expression.

Previous studies have shown that both the genetic and pharmacological block of the sterol biosynthesis pathway leads to a compensatory up-regulation of HMGR activity (Wentzinger et al., 2002; Babiychuk et al., 2008; Nieto et al., 2009; Posé et al., 2009; Closa et al., 2010). In agreement with these observations, HMGR activity was also enhanced in FPS silenced plants (Fig. 4A), though neither the amount of HMGR protein nor the levels of *HMG1* and *HMG2* transcripts (Fig. 4B and C) were simultaneously increased, indicating that the mechanism behind HMGR up-regulation is post-translational. This is fully consistent with the hypothesis that variations of HMGR activity in response to changes in the flux of the MVA-derived isoprenoid pathway occur mainly via post-translational control (Nieto et al., 2009), although the precise nature of this regulatory mechanism remains to be established. HMGR activity levels in FPS silenced plants may be up-regulated through changes in the phosphorylation status of the enzyme (Dale et al., 1995; Douglas et al., 1997; Leivar et al., 2011) and/or protein degradation mechanisms involving the ERAD protein quality control system (Doblas et al., 2013; Pollier et al., 2013). In any case, an enhancement of HMGR activity concomitant to a reduction of FPS activity could lead to the accumulation of MVA pathway intermediates upstream of FPP that might cause the observed phenotype. For example, MVA at high concentrations has mild inhibitory effect on growth of cultured tobacco BY-2 cells (Crowell and Salaz 1992) and the accumulation of high levels of IPP and DMAPP appear to have a cytotoxic effect (Martin et al. 2003; Sivy et al., 2011). Alternatively, these available prenyl diphosphates could be metabolized by a prenyltransferase other than FPS leading to the accumulation of toxic levels of pathway derivatives. In both metabolic scenarios, an external supply of MVA would be expected to exacerbate the phenotype associated to down-regulation of FPS. However, no changes in the intensity of the phenotype were observed (Fig. 4D), which clearly ruled out the above possibilities, thus demonstrating that the phenotype of FPS silenced plants is due to the reduced accumulation of an FPP-derived product.

Down-regulation of FPS activity disturbs both cytosolic and plastidial isoprenoid metabolism

Analysis of the major FPP-derived end products in FPS silenced plants revealed a similar quantitative decrease of the total amount of bulk sterols (i.e., campesterol, stigmasterol and β-sitosterol) and UQ-9 (i.e., 30-38% and 30% of control sterol and UQ-9 levels, respectively) (Fig. 3A and C). This is consistent with previous results showing that *fps1-1* and *fps2-1* single knockout mutants display a similar, although overall less pronounced, decrease of these isoprenoids (i.e., 10-17% and 20% of wild type sterol and UQ-9 levels, respectively) (Closa et al., 2010). Therefore, our results

599 confirm that a limited supply of FPP has a fairly similar quantitative impact on the two
600 major branches of the MVA-derived isoprenoid pathway, which suggests that precise
601 regulatory mechanisms are operating to ensure a balanced distribution of FPP between
602 these two competing pathways, particularly considering that Arabidopsis contains, by
603 mass, about one order of magnitude more sterols than ubiquinones (Closa et al., 2010)
604 and hence a much higher amount of FPP needs be allocated to sterol than to
605 ubiquinone formation. The limited availability of FPP also led to qualitative changes in
606 the profile of bulk sterols and ubiquinones. In particular, the reduction of stigmasterol
607 was much more pronounced than that of β-sitosterol and campesterol (Fig. 3B). Such
608 changes resulted in a drastic reduction of the stigmasterol to β-sitosterol ratio (i.e. more
609 than 50% lower than in control plants) whereas the campesterol to β-sitosterol ratio
610 remained unchanged. Similar qualitative changes in the profile of bulk sterols have
611 been described in roots of Arabidopsis seedlings silenced for acetoacetyl CoA thiolase
612 2 (Jin et al., 2012), which catalyzes the initial reaction of the MVA pathway, and leaves
613 of *W. somnifera* plants silenced for squalene synthase (SQS) (Singh et al., 2015),
614 which converts FPP into squalene, the first committed precursor of sterols and
615 brassinosteroids (Fig. 1A). Collectively, these observations suggest that when sterol
616 precursors become limiting, plants keep the relative levels of campesterol and β-
617 sitosterol properly balanced at the expense of stigmasterol to minimize the negative
618 impact that major changes in the campesterol to β-sitosterol ratio might have on growth
619 and development (Schaeffer et al., 2001). Such metabolic adaptation may involve the
620 concerted action of highly regulated enzymes of the post-squalene segment of the
621 sterol pathway, such as SMT2 and SMT3, the branch point isozymes starting the
622 synthesis of structural sterols (Schaeffer et al., 2001; Carland et al., 2010), and the
623 sterol C22 desaturase (CYP710A1) that converts β-sitosterol into stigmasterol
624 (Morikawa et al., 2006; Arnqvist et al., 2008).

625 Silencing of FPS also provoked a significant change in the relative abundance of UQ-9
626 and UQ-10. These UQ species differ only in the length of the polyprenyl side chain,
627 which consists of nine (C_{45}; solanesyl) and ten (C_{50}; decaprenyl) isoprenyl units in UQ-9
628 and UB-10, respectively. Upon silencing of FPS the level of UQ-10 rose up to 15% of
629 the total UQ content while in control plants it accounts for only 3%. Importantly, this
630 change is due to a decrease of UQ-9 levels concomitant to a sharp increase in the
631 content of UQ-10 (Fig. 3C). The side chain of UQs is synthesized by *trans*-long-chain
632 prenyl diphosphate synthases that elongate the allylic diphosphates FPP or GGPP by
633 successive condensation of IPP units (Hirooka et al., 2003). The *trans*-long-chain
634 prenyl diphosphate synthase that synthesizes the solanesyl side chain of UQ-9 in

Arabidopsis mitochondria has recently been identified and characterized (Ducluzeau et al., 2012). The enzyme shows broad product specificity with regard to chain length depending on the allylic diphosphate used as a substrate (Hsieh et al., 2011). Thus, a certain shift of the enzyme's product specificity towards longer polyprenyl diphosphates might explain the relative changes of UQ-9 and UQ-10 levels associated to FPS silencing. The limited availability of its preferred allylic substrate FPP could increase the use of GGPP as allylic substrate for successive condensation of IPP units, thus favoring the synthesis of polyprenyl side chains of ten units. A very recent study has demonstrated the mitochondrial localization of a GFPP-forming isoprenyl diphosphate synthase (AtIDS1) that appears also to be able to catalyze the synthesis of significant amounts of GGDP in addition to its major reaction product GFPP (Nagel et al., 2015). A similar effect on the solanesyl diphosphate synthase product chain length could be induced by a change in the ratio of the substrates FPP and IPP (Ohnuma et al., 1992; Pan et al., 2002), which is a likely consequence of down-regulation of FPS activity. Finally, we cannot exclude the possibility that an as yet uncharacterized *trans*-long-chain prenyl diphosphate synthase specialized in the synthesis of the UQ-10 prenyl side chain could be up-regulated in response to depletion of UQ-9 levels. This could be the case in tomato plants showing a 3-fold increase of UQ-10 levels in response to solanesyl diphosphate synthase silencing (Jones et al., 2013). Whatever the biochemical reason behind the change in the relative abundance of Arabidopsis UQ species, our results demonstrate that depletion of UQ-9 content due to a limited supply of FPP triggers a compensatory antioxidant response involving enhanced UQ-10 synthesis.

The chlorotic phenotype displayed by Arabidopsis plants silenced for FPS is most likely the result of the severe morphological and ultrastructural defects observed in their chloroplasts (Fig. 5A and B), whose appearance resembled that of chloroplasts subjected to photooxidative damage. This is consistent with the strong depletion of chlorophylls, carotenoids, phylloquinone, plastochromanol-8 and, to a lesser extent, plastoquinone-9 observed in these plants (Fig. 5C and Figure S3). On the contrary, the levels of tocopherol species were unaffected or even increased, e.g., the γ-tocopherols (Figure S3), although this does not argue against the above since it is becoming increasingly evident that tocopherols play a number of important biological functions beyond their role in protecting plants from photooxidative stress, which appears to be more limited than originally assumed (Falk and Munné-Bosch, 2010). The observed increase of tocopherol content is consistent with the ultrastructural changes of chloroplasts and the concomitant chlorotic phenotype displayed by FPS silenced

671 plants. In fact, previous studies have indicated that the phytyl side chain of the
672 tocopherols accumulated during chlorotic stress and leaf senescence is not
673 synthesized “de novo” but formed through a salvage pathway that recycles phytol
674 released from chlorophyll breakdown (Ischebeck et al., 2006; vom Dorp et al., 2015).
675 The tocopherols accumulated in plastoglobuli of chloroplasts of plants under stress
676 have been suggested to serve as a transient sink for the deposition of phytol, which in
677 their free form might destabilize the bilayer membrane of thylakoids due to their
678 detergent-like characteristics (Gaude et al., 2007). The strong impact of a limited
679 supply of cytosolic FPP on chloroplast structure and isoprenoid metabolism further
680 reinforces the connection between cytosolic isoprenoid biosynthesis and proper plastid
681 functioning. Therefore, plants silenced for FPS should be added to the growing list of
682 MVA-derived isoprenoid/sterol pathway mutants displaying altered plastid-related
683 phenotypes (Nagata et al., 2002; Suzuki et al., 2004; Posé et al., 2009; Babiychuk et
684 al., 2008; Bouvier-Navé et al., 2010; Ishiguro et al., 2010; Kim et al., 2010).

685 Under the premise that the phenotype of chlorosis is due to the reduced levels of an
686 FPP-derived isoprenoid and not to the accumulation of a toxic compound upstream of
687 FPP, the possibility that the limited availability of an isoprenoid other than sterols may
688 be the causative agent of such phenotype seems unlikely. As indicated above, plant
689 sterols are by far the most abundant FPP-derived isoprenoid products. Moreover, the
690 external supply of BL does not restore the wild type phenotype to FPS silenced plants
691 (Figure S2), indicating that campesterol levels (Fig. 3A) are sufficient to sustain normal
692 brassinosteroid biosynthesis, which is in keeping with the fact that mutants defective in
693 brassinosteroid biosynthesis and signaling are not chlorotic but darker green than wild
694 type plants (Fujioka et al., 1997; Li and Chory, 1997). Finally, the phenotypes of
695 Arabidopsis mutants affected in the synthesis of other essential isoprenoids are clearly
696 different to those of FPS silenced plants. Plants with impaired biosynthesis of the
697 benzenoid moiety of UQ displaying a much higher reduction in UQ content (62%) than
698 FPS silenced plants (Fig. 3C) are indistinguishable from wild type plants (Block et al.,
699 2014). Similarly, depletion of dolichol content up to 85% the wild type levels triggers a
700 leaf wilting phenotype in the margin of old leaves, but the plant growth and size are not
701 significantly altered (Zhang et al., 2008), and Arabidopsis *era1-2* mutant plants lacking
702 farnesyl transferase activity display enlarged organs instead of smaller ones (Yalovsky
703 et al., 2000). These observations indicate that neither a depletion of dolichols nor
704 defective protein farnesylation are involved in the phenotype of plants silenced for FPS.
705 Thus, even though it is certainly possible that the flux through the branches of the
706 isoprenoid pathway leading to FPP-derived isoprenoids other than sterols and

ubiquinones may also be affected by a limited supply of FPP, the levels of the corresponding end products appear to be sufficient to sustain normal plant growth and development. The above considerations raise the question as to why changes in the sterol profile may have such an important impact on chloroplast structure and function. The still controversial possibility that sterols might be required for proper biogenesis of the chloroplast membrane network should not be overlooked, as several reports claim the existence of sterols in the plastidial outer membrane of different plant species (Brandt and Benveniste, 1972; Moeller and Mudd, 1982; Hartmann-Bouillon and Benveniste, 1987; Lenucci et al., 2012), and very recently compelling evidence supporting a direct role of sterols in chloroplast membranes has been presented in the microalga *Nannochloropsis oceanica* where, despite sterols are exclusively derived from the plastidial MEP pathway, the specific inhibition of the post-squalene sterol pathway leads to severely altered chloroplasts and depressed photosynthetic efficiency (Lu et al., 2014). Assuming that a plastid-specific sterol biosynthesis pathway in plants is highly unlikely, chloroplasts should acquire sterols from another subcellular compartment (Corbin et al., 2001) such as the ER. In fact, an extensive exchange of chemically diverse metabolites, including lipids, between ER and plastids has been reported in many plant species, involving in some cases the establishment of physical contact sites between the membranes of these organelles (Wang and Benning, 2012; Mehrshahi et al., 2014). Thus, depletion of sterols in plant cell membranes, including the ER network, might compromise chloroplast development, either directly because of a limited availability of sterols for deposition into plastid membranes or indirectly by altering the structure of the plastid-ER connection domains and negatively affecting the exchange of metabolites required for correct plastid metabolism and development.

Plants perceive a reduction of major sterols as a stress signal

The quantitative transcriptomic analyses undertaken to characterize the molecular response to down-regulation of FPS revealed that plants perceive a reduction in the flux through the MVA-derived isoprenoid pathway and the resulting decline of sterol levels as a stress signal that causes a rapid misregulation of genes involved in both biotic and abiotic stress responses (Table 1; Fig. 6, 7B and 8). This notion is supported by the high overall degree of consistency observed when the expression changes of a targeted set of stress-related genes in response to down-regulation of FPS were compared to those observed when the whole sterol branch of the isoprenoid pathway was inhibited with terbinafine or the synthesis of structural sterols was genetically blocked (*cvp1/smt3* mutant) (Fig. 7A). Thus, our RNA-seq and RT-qPCR transcriptomic analyses further strengthen the connection between distinct biotic and abiotic stresses

and changes in sterol composition, in particular those affecting the stigmasterol to β-sitosterol ratio (Grunwald 1978; Whitaker, 1994; Griebel and Zeier, 2010; Wang et al., 2012; Senthil-Kumar et al., 2013; Sewelam et al., 2014), which declines sharply upon silencing of FPS (Fig. 3B). Amongst the stress-related transcriptional responses, the most prominent was the induction of a set of genes related to the JA-pathway, which accounted for 11.5% of the total of misregulated genes and included JA biosynthesis, homeostasis, signaling and target genes (Table 1; Fig. 7B and 8A). This particular stress response could also be involved in the development of chlorosis associated to chloroplast disorganization, either by playing a primary role as causal agent or acting synergistically with the decreased provision of sterols. JA is considered to play an important role in the initiation and progression of natural senescence, a process characterized by a gradual de-greening due to loss of chlorophyll and thylakoid membranes, and an increase in plastoglobuli (Kim et al. 2015). However, there is still unclear whether JA is a signal that triggers senescence or a byproduct of senescence. It has been suggested that JA production during senescence is a consequence of increased thylakoid membrane turnover rather than the causal agent (Seltmann et al., 2010a, 2010b), and more recently compelling evidence for recruitment of JA biosynthetic enzymes to plastoglobuli in structurally disorganized chloroplasts has been reported in Arabidopsis mutants lacking both plastoglobule-localized kinases ABC1K1 and ABC1K3 under light stress (Lundquist et al., 2013). Thus, further work is needed in order to elucidate the actual contribution of the JA pathway to the development of the phenotypic alterations associated to down regulation of FPS.

In addition to the JA-related stress response, silencing of FPS also triggered changes in the expression of genes involved in abiotic stress responses (Table 1; Fig. 7B and 8B). Interestingly, comparison of our results with those of transcriptomic and proteomic analyses carried out in the Arabidopsis *cyp51A2* mutant defective in the obtusifoliol-14α-demethylation step of the sterol pathway (Kim et al., 2005; Kim et al., 2010) revealed important differences. The JA-related stress response was not induced in this sterol-deficient mutant, which on the other hand exhibited an enhancement of ethylene biosynthesis and signaling, and ROS accumulation. These responses, which have been suggested to be partly involved in the postembryonic lethality of the *cyp51A2* seedlings (Kim et al., 2005; Kim et al., 2010), were not activated in plants silenced for FPS (Table 1; Figure S4B). On the contrary, genes involved in ROS detoxification were misregulated in both mutants. Thus, it can be speculated that changes in the expression of antioxidant genes in the FPS silenced plants are able to keep ROS production and scavenging properly balanced. In any case, the observation that

779 perturbation of sterol homeostasis may result in substantially different molecular
780 responses is not unprecedented. Silencing of SQS in *W. somnifera* results in reduced
781 sterol levels and leads to decreased expression of both JA-dependent PR3 and SA-
782 dependent PR1 and PR5 transcripts (Singh et al., 2015) while expression of *Brassica*
783 *juncea* HMG-CoA synthase (HMGS) in Arabidopsis increases the sterol content and
784 activates the expression of the SA-dependent PR1, PR2 and PR5 transcripts with no
785 involvement of the JA pathway in the defense response (Wang et al., 2012). However,
786 neither the SA nor the JA defense pathways are activated in *Pseudomonas syringae*
787 resistant Arabidopsis mutants lacking sterol C22 desaturase (CYP710A1) activity
788 (Griebel and Zeier, 2010). So far the reasons behind these differential stress responses
789 to changes in the profile of sterols are unknown, but it may reflect specific differences
790 in the relative abundance of individual sterols in each of these mutants. The precise
791 mechanism by which the relative amounts of plant sterols, in combination with other
792 membrane lipids, finely control the physicochemical properties of membranes and their
793 biological function is still unclear, but it is known that the contribution of individual
794 sterols to the biophysical properties and the functionality of cell membranes and the
795 associated proteins differs greatly (Schuler et al., 1991; Grandmougin-Ferjani et al.,
796 1997; Hartmann, 1998; Hodzic et al., 2008; Grosjean et al., 2015). The fact that
797 particular sterol imbalances may have specific consequences could also explain the
798 minor differences observed in the gene expression patterns depending on whether
799 sterol biosynthesis was inhibited at the level of FPS or downstream in the pathway, and
800 even the intriguing observation that some sterol biosynthetic mutants do not display
801 plastid-related phenotypes (Carland et al., 2010; Jin et al., 2012).

802 **Reduced levels of major sterols alters Fe homeostasis**

803 In plants silenced for FPS, transcripts coding for proteins involved in Fe storage,
804 metabolism and/or intracellular trafficking were down-regulated whereas those coding
805 for group Ib bHLH transcription factors known to play essential roles in activating iron-
806 deficiency responses and uptake were up-regulated (Table 1 and Fig. 7B). This Fe
807 deficiency transcriptional response is consistent with the reduced levels of Fe
808 measured in FPS silenced plants (Fig. 9B) and was also observed when the sterol
809 branch of the isoprenoid pathway was inhibited (Fig. 7B). These results confirmed the
810 existence of a close relationship between plant sterol metabolism and Fe homeostasis,
811 a connection that is well established in other organisms like fungi (Craven et al., 2007;
812 Blatzer et al., 2011; Hosogaya et al., 2013; Thomas et al., 2013; Chung et al., 2014;
813 Vasicek et al., 2014) but is much less known in plants (Urbany et al., 2013). In general,
814 depletion of sterol biosynthesis has been correlated with both higher membrane

permeability and increased uptake of metal ions in roots, though the underlying molecular mechanism remains unknown (Diener et al., 2000; Khan et al., 2009; Urbany et al., 2013; Wagatsuma et al., 2015). These observations argue against the possibility that the Fe deficiency-related gene expression response may be a direct consequence of the impact of reduced sterol levels on membrane permeability. The suggested role of JA as a negative regulator of Fe deficiency gene expression (Hindt and Guerinot 2012) further reinforces this hypothesis, as silencing of FPS triggers a JA-related transcriptional response. Thus, considering that the vast majority of leaf cellular Fe is stored in the chloroplasts associated with metalloproteins of the thylakoid electron transfer chain (Briat et al., 2007), a most plausible hypothesis could be that the altered Fe homeostasis in response to down-regulation of FPS results from the strong impact of this metabolic perturbation on chloroplast size and structural integrity (Fig. 5A and B). The impact of FPS down-regulation on chloroplasts might also be the underlying cause of changes in the expression of genes coding for proteins involved in carbohydrate metabolism (Table 1; Fig. 7B).

Conclusions

The presented results indicate that Arabidopsis plants silenced for FPS develop a chlorotic phenotype associated to important morphological and structural alterations of chloroplasts, and a marked change in the profile of both cytosolic and plastidial isoprenoids, including a depletion of the bulk membrane sterols, which is perceived by plants as a stress signal that induces early transcriptional stress responses including JA signaling and Fe homeostasis. This supports the view that plant sterol levels must be stringently controlled, and indicates that changes in the composition of sterols are rapidly sensed by plants cells, which in turn activate a series of adaptive responses aimed at coping with the new metabolic scenario. Further studies are required to determine the role and mode of action of individual sterol species, paying special attention to the emerging connection between sterol metabolism and chloroplasts, which appear to be a key player of this complex regulatory response.

Methods

Plant growth conditions and treatments.

Arabidopsis thaliana plants ecotype Columbia-0 (Col-0) were used throughout this study. For *in vitro* culture, seeds were surface-sterilized with ethanol and sown on Petri dishes containing Murashige and Skoog (MS) medium (MS salts, 1% sucrose (w/v) and 0,8% agar (w/v), pH 5.7). After stratification at 4ºC for 2 days, plates were transferred

into a growth chamber set for long day conditions (16h light/8h darkness) at 150 µmol^{-2}s^{-1} and 22ºC. To induce *FPS* gene silencing, seedlings were germinated and grown on MS medium supplemented with 30µM methoxyfenozide (MFZ) (Runner®, Bayer CropScience) or germinated and grown for three days on sterile filter papers that were first placed on MS plates and subsequently transferred onto new MS plates supplemented or not with 30 µM MFZ. For phenotype reversion experiments, seedlings were germinated and grown as indicated above on growth media supplemented with 5 mM mevalonate (MVA; Sigma M4667) or 0.4 nM *epi*-brassinolide (BL; Wako®, Japan). For chemical inhibition of the sterol pathway, three-day-old seedlings grown on filter papers on MS were transferred to new MS plates supplemented or not with 150 µM terbinafine (Tb; Sigma, T8826). The *cvp1*/*smt3* double mutant plants (Carland et al., 2010) kindly provided by Dr. Francine Carland, were grown for 8 days in the same conditions. In all cases, seedlings were grown for the desired time intervals under the light and temperature conditions indicated above. Seedling samples were collected in three biological replicates, quickly frozen in liquid nitrogen and stored at -80ºC until further processing.

Generation of pre-amiRNA DNA vectors and plant transformation

Two amiRNA sequences (amiFPSa and b) were designed to target simultaneously *FPS1* and *FPS2* genes (Fig. 1B) using the Web MicroRNA Designer (WMD3; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) (Schwab et al., 2006; Ossowski et al., 2008). The amiRNA foldback fragments were generated by overlap PCR using pRS300 plasmid as a template and the following oligonucleotides:
FPSaI: 5'-gaTATTGCGAAGTAGAATCGCGTtctctcttttgtattcc-3', FPSaII:
5'-gaACGCGATTCTACTTCGCAATAtcaaagagaatcaatga-3', FPSaIII:
5'-gaACACGATTCTACTACGCAATTtcacaggtcgtgatatg-3' and FPSaIV:
5'-gaAATTGCGTAGTAGAATCGTGTtctacatatatattcct-3' for amiFPSa, and FPSbI:
5'-gaTAGGTCAACATAGTAAGCCTTtctctcttttgtattcc-3', FPSbII:
5'-gaAAGGCTTACTATGTTGACCTAtcaaagagaatcaatga-3', FPSbIII:
5'-gaAAAGCTTACTATGATGACCTTtcacaggtcgtgatatg-3', and FPSbIV:
5'-gaAAGGTCATCATAGTAAGCTTTtctacatatatattcct-3', for amiFPSb.
The amplified fragments were gel-purified and cloned into pGEM-T easy vector (Promega). After sequencing to exclude amplification artifacts, the pre-amiFPSa and pre-amiFPSb constructs were digested with *Sal*I and *Not*I, and subcloned into the pENTR3C gateway entry vector (Life technologies). The resulting pre-amiFPS constructs were then transferred into pB110-Red-2844 binary vector harboring the ecdysone-inducible receptor based-system (Padidam et al., 2003), using Gateway LR

Clonase II Enzyme mix (Invitrogen). An empty version of pB100-Red-2844 (EV) to generate EV plants that were used as a control in FPS silencing experiments was generated as follows. Plasmid pENTR3C was digested with *Eco*RI, religated to eliminate the *ccdB* gene and subsequently recombined into pB100-Red-2844 using LR clonase reaction to yield pB100-Red-EV. The recombinant binary plasmids were transferred to *Agrobacterium tumefaciens* strain GV3101 and the resulting strains were used to transform Arabidopsis Col-0 plants by the floral dip method (Clough and Bent, 1998). Transformed plant lines homozygous for the corresponding transgenes were selected on the basis of the segregation of the red fluorescence trait in transgenic seeds. Transgenic Arabidopsis homozygous for the pre-amiFPSa MFZ-inducible transgene were retransformed with a 35S::FPS1S gene construct devised for constitutive overexpression of Arabidopsis FPS1S isoform (Masferrer et al., 2002) as indicated above. Double homozygous transgenic lines were selected using the kanamycin resistance trait. T3 plants were genotyped by PCR for the presence of 35S::FPS1S transgene using primer pair 35S-3'F: 5'-CACTGACGTAAGGGATGACG-3' and FPS1Srv: 5'-CTGTGGATGTGATTGCGAAGTAG-3', and for the presence of the amiFPSa construct using primer pair FPSaII and FPSaIII. PCR was performed using genomic DNA with a Tm for the annealing step of 55ºC and 35 cycles. The amiFPSa RNA levels were quantified in double and single transgenic plants by RT-PCR as described elsewhere (Varkonyi-Gasic et al., 2007) using the following oligonucleotides: RT-amiRFPSa: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGCGA-3 (for amiRNA specific RT) and microUNI-rv: 5′-CCAGTGCAGGGTCCGAGGTA-3′ and amiRFPSa-fw: 5'-CAGGCATATTGCGAAGTAGAATC-3' (for PCR amplification).

Laser confocal microscopy

For chloroplast observation, whole seedlings were examined by confocal laser-scanning microscopy using a Leica SP5II microscope (Leica Microsystems) and a water-immersion objective (HCX PL Apo 63x/1.20 W). Chlorophyll fluorescence was excited with an argon laser at 488 nm and detected using a 640- to 680-nm band-pass filter. LAS-AF Lite 2.6.0 software was used for image capture. Chloroplast area was measured using confocal microscope images and Image J Software (Image Processing and Analysis in Java; http://imagej.nih.gov/ij/). Thirty-six independent chloroplast measurements were made for each plant line and treatment.

Transmission electron microscopy

For electron microscopy, the first pair of true leaves from seedlings were cut into small pieces and fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) overnight at 4ºC. Samples were then washed with PB, post-fixed with 2% OsO4 (w/v) for 2 h, and sequentially washed again with milliQ water and PB during 10 min each. Sections were dehydrated at 4ºC through a series of increasing acetone concentrations (50, 70, 90, 96, 100%), prior to being progressively (25%, 50%, 75%, 100%) embedded in Epon 812 epoxy resin. The resin was polymerized at 60 ºC during 48 h. Sections with a thickness of 50 nm were cut with an ultramicrotome UC6 (Leica Microsystems, Vienna) and placed on TEM grids (Formvar carbon-coated Cu grids). Finally, grids were further contrasted with uranyl acetate and lead citrate. All electron micrographs were obtained with a Jeol JEM 1010 MT electron microscope (Jeol, Japan) operating at 80 kV. Images were recorded with AnalySIS on a Megaview III CCD camera.

Western blot analysis and enzyme activity assays

Seedling extracts for HMGR and FPS activity assays were obtained as described in Campos et al. (2014) and Arró et al. (2014), respectively. HMGR activity was determined as described in Campos et al. (2014) and is reported as picomols of 3-hydroxy-3-methyglutaryl coenzyme A converted into MVA per min and mL of protein extract at 37ºC. FPS activity was measured as described in Arró et al. (2014) and is reported as picomols of IPP incorporated into acid-labile products per minute and mL of protein extract at 37ºC. Immunoblot analysis was performed as described in Keim et al. (2012) in the same protein extracts used for enzyme activity assays. Protein samples corresponding to 7µL and 2µL of each of the extracts analyzed for FPS and HMGR activity levels, respectively, were loaded into gel lanes. Rabbit polyclonal antibodies raised against FPS1 (Masferrer et al., 2002) and HMGR1 (Manzano et al., 2004) were used at 1/8000 and 1/20000 dilution, respectively. The secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) was used at a 1/50000 dilution. The FPS and HMGR antibody complexes were visualized using the ECL Advance Western blotting system (GE Healthcare) according to the manufacturer's instructions. Protein concentration was determined as described by Bradford (1976) and the amount of protein on the blotted membranes was assessed by Coomassie blue staining.

Metabolite analysis

A pool of 100 mg of fresh weight of seedlings per genotype and treatment was used per each measurement and all the measurements were carried out in three independent biological replicates. Quantification of sterols was performed by GC-MS

as previously described (Closa et al., 2010). For photosynthetic pigment analysis, seedling samples were immediately frozen in liquid nitrogen and ground to a fine powder using TissueLyser equipment. Photosynthetic pigments were extracted in 1.0 ml of 80% acetone in the dark at 4ºC for 1h. Plant extracts were centrifuged for 5 min at 13000 rpm at 4ºC and the supernatant was subjected to spectrophotometric analysis at 470, 646 and 663 nm. The chlorophyll a and b, total chlorophyll and carotenoid content was calculated as described by Lichtenthaler (1987). Quantitative analysis of prenylquinones and carotenoids was performed by UPLC-MS as described using 30-45 mg of seedlings (DW) (Martinis et al., 2011; Kessler and Glauser, 2014). Absolute quantification of phylloquinone, plastochromanol-8, plastoquinone-9 and tocopherol levels was made using the corresponding standards, and values are reported in micrograms per g dry weight. Ubiquinone-9, ubiquinone-10, β-carotene, lutein, and viola-neoxanthin levels are reported as relative amounts that were calculated using the value of the signal divided by the mass of samples.

RNA-seq and differential gene expression analysis

RNA preparation and Illumina Sequencing. For RNA-seq, amiFPSa plants were grown on MS, or MS medium supplemented with 30μM MFZ. Three independent pools of seedlings (100 mg of FW) were collected per each treatment. Seedlings were ground to a fine powder using TissueLyser equipment and used for extraction of RNA using NucleoSpin® RNA Plant (Macherey-Nagel) according to the manufacturer's instructions, including the DNase step. The quality and quantity of total RNA samples were assessed using a Bionalyzer Expert 2100 Instrument (Agilent Technologies) and an equimolar mixture of RNA samples from each treatment was prepared. The RNA samples (3 μg) were fragmented and ligated with adaptors prior to cDNA synthesis and PCR amplification. The cDNA libraries were prepared according to the Illumina protocols and sequenced using an Illumina HiSeq2000 machine to perform 2x100 paired-end sequencing.

Mapping and Differential Gene Expression (DGE) analyisis. Quality of the reads obtained by HiSeq2000 sequencing was checked with FastQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Preprocessing of the reads was performed with fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and aScidea specific perl scripts property of aScidea (http://www.ascidea.com) in order to filter regions of low quality. Adaptors and low quality bases at the ends of sequences and reads with undetermined bases or with 80% of their bases with less than 20% quality score were trimmed and raw reads that passed the quality filter threshold were

mapped using Tophat 2.0.7 (Trapnell et al., 2009) and Bowtie 2 2.0.6 (Langmead et al., 2009; Langmead and Salzberg, 2012) to generate read alignments for each sample. The reference genome used was the *Arabidopsis thaliana* genome TAIR10 and genomic annotations were obtained from TAIR database (http://www.arabidopsis.org) in general feature format 3 (GFF3). The inner distance between mate pairs used was 50 bp and the rest of the parameters were used with the default values. The transcript isoform level and gene level counts were calculated and FPKM normalized using Cufflinks 2.0.2 software (Trapnell et al., 2010). Differential transcript expression was then computed using Cuffdiff (Trapnell et al., 2013). The resulting lists of differentially expressed genes were filtered by $\log_2$(fold_change) >2 and < -2 and a q-value of 0.05. The analysis of Biological Significance was based on a Gene Ontology (Ashburner et al., 2000) using the hypergeometric statistical test and the Bonferroni multi-test adjustment method considering a significance level cutoff of 0.05. Gene Ontology was performed by using GeneCodis (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009) web services. Main statistical analyses were performed using the free statistical language R and the libraries developed for data analysis by the Bioconductor Project (www.bioconductor.org). Raw data of the experiment can be downloaded at GEO (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE79412.

Iron quantification

For quantification of Fe levels, seedlings were collected and freeze-dried overnight. About 30-45 mg of dry material was digested with 1 ml HNO_3 and 0,5 ml H_2O_2 overnight at 90ºC. After cooling down, 10ml of ultra-pure water was added. Iron content was measured using an Inductively Coupled Plasma Optical Emission Spectrometer (Optima 3200 RL, Perkin Elmer). Analyses of samples followed external calibration with diluted single Fe element. Total Fe concentration of seedlings of the different lines analyzed was determined in biological triplicates from independent experiments. One-way ANOVA test was performed using Graphpad Prism (v5) software.

RT-qPCR analysis of HMG and FPS gene expression

Total RNA was extracted from seedling samples (100 mg of FW) using the PureLink® RNA Mini Kit (Ambion, Life Technologies) following the manufacturer's instructions. The RNA samples were treated with DNase I (DNA-free Kit, Ambion) in a final reaction volume of 25 µl, and cDNA was synthesized from 1 µg of total RNA using Superscript™ III Reverse Transcriptase (Invitrogen) and oligo-(dT) primers. Real time PCR was performed using a LightCycler 480 equipment (Roche Diagnostics). The raw PCR data from LightCycler Software 1.5.0 were used in the analysis. Amplification

curves were analyzed using Second Derivative Maximum method and crossing points were determined for each curve. For efficiency determination, a standard curve of six serial dilution points (ranging from 200 to 6,25 ng) was made in triplicate. Specific primer pairs for HMG1 and HMG2 mRNAs were described previously (Nieto et al., 2009). The following specific primer pairs were used for FPS1 and FPS2 mRNAs: qFPS1fw (5'-AAA GTC TCA GCC CTC AAA AAT TTC-3'), qFPS1rv (5'- CAA GAA TAA AAG TGA GGC AGG TTT-3'), qFPS2fw (5'-CGT TTT ATT CTT CTG ACA TTT ATG TAT-3') and qFPS2rv (5'-AAT CTC AAA TTC TAT TTT CGG AAG G-3'). Quantification of transcript levels was done in three independent biological replicates and for each biological replicate three technical replicates were performed. *PP2AA3* (At1g13320) was used as a housekeeping gene with previously designed oligonucleotides (Hong et al., 2010). The ΔCT was calculated as follows: ΔCT= CT (Target)-CT (PP2A). The fold change value was calculated using the expression 2-ΔCT (Livak and Schmittgen, 2001).

High-throughput RT-qPCR gene expression analysis.

RNA extraction and cDNA synthesis was performed as described above for standard RT-qPCR analysis. Transgene expression was quantified by real-time PCR using the Biomark™ HD instrument (Fluidigm, www.fluidigm.com) and 2 x SsoFast EvaGreen Supermix with low Rox (Bio-Rad, www.bio-rad.com) dye. The cDNA samples were diluted from 0,15 to 6 ng/µl and pre-amplified using TaqMan PreAmp Master Mix (Applied Biosystems, Lifetechnologies). Primers were used at a final concentration of 500 nM. After pre-amplification, cDNAs were treated with Exonuclease I to remove leftover primers. Primer pairs for each gene candidate were designed using PrimerQuest (http://eu.idtdna.com/PrimerQuest/Home/Index) (see Table S2 for a complete list of oligonucleotides). The PCR efficiency for each primer pair used was calculated according to a dilution series from a pooled cDNA sample including all biological treatments. Expression fold was calculated using Data Analysis Gene (DAG) Expression software (http://www.dagexpression.com/dage.zip) (Ballester et al., 2013) through the construction of standard curves for relative quantification and multiple reference genes for sample normalization. Quantification of transcript levels was done in three independent biological replicates and for each biological replicate two technical replicates were performed. Three different reference genes previously reported as stably expressed during plant development and throughout time course experiments were selected: *PP2AA3* (At1g13320), *UBC9* (At4g27960) (Hong et al., 2010) and *UBC* (At5g25760) (Czechowski et al., 2005).

Hierarchichal clusterization

Hierarchical clusterization of the genes was performed by using the TM4 suite (http://www.tm4.org/mev.html). The clusterization was performed by using the Euclidean distance of the gene expression profiles across the different comparisons, the “average” clusterization method was used (Eisen et al., 1998).

Data analysis

Statistical significance of changes between amiFPS and EV plants grown on MS supplemented with MFZ was calculated by using paired student’s t-test.

List of supplemental materials

1) Supplemental material file including the following items:
   Figure S1. Overexpression of FPS1S restores the wild type phenotype to plants silenced.
   Figure S2: Brassinosteroids fail to complement the phenotypes caused by silencing of FPS.
   Figure S3. Effect of FPS silencing on carotenoid and prenylquinone levels.
   Figure S4. Silencing of FPS leads to misregulation of genes involved in maintaining redox homeostasis but does not trigger symptoms of oxidative stress.
   Figure S5. Time course expression analysis of genes coding for enzymes involved in carbohydrate metabolism.
   Table S2. List of oligonucleotides used in high-throughput qPCR gene expression analysis (BioMark™ System from Fluidigm).

2) Table S1. Transcriptomic analysis of FPS silenced plants using Illumina RNA-seq technology.

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support with the Differential Gene Expression analysis software. We also thank the greenhouse facilities and the Scientific and Technical Services at CRAG and the Centres Científics i Tecnològics (CCiTUB) of the University of Barcelona. This work was supported by grants from the Spanish Government (BIO2009-06984 and AGL2013-43522-R) to AF, and the Generalitat de Catalunya (2014SGR-1434). Finally, we thank Manuel Rodríguez-Concepción and Norma Fàbregas for critical reading of the manuscript and Berta Esteve and Antonella Pavoni for technical help.

Figure legends

Figure 1. Silencing of Arabidopsis *FPS* gene expression using amiRNA technology alters plant shoot and root phenotypes. (A) Simplified scheme of the isoprenoid biosynthetic pathways. The reactions catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and farnesyl diphosphate synthase (FPS) are indicated. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GFPP, geranylfarnesyl diphosphate; GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate. (B) Main features of the amiRNAs designed for *FPS1* and *FPS2* gene silencing. (C) Shoot phenotype of 8-day-old EV, amiFPSa and amiFPSb seedlings grown on MS medium (top images) and MS supplemented with 30 μM MFZ (bottom images). (D) Root phenotype of 10-day-old EV, amiFPSa and amiFPSb seedlings grown on MS medium supplemented with 30 μM MFZ.

Figure 2. Expression of amiFPSa and amiFPSb leads to reduced levels of FPS mRNA, protein and enzyme activity. (A) Phenotype of EV, amiFPSa and amiFPSb seedlings grown for 8 days on MS medium (top images) or 3 days on MS medium and 5 days on MS medium supplemented with 30 μM MFZ (bottom images). (B) RT-qPCR analysis of *FPS1* and *FPS2* transcripts using RNA from EV, amiFPSa and amiFPSb seedlings grown on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 μM MFZ (grey bars). Transcript levels were normalized relative to the mRNA levels of the *PP2AA3* gene. (C) Western blot analysis of FPS protein (top panel) and Coomassie brilliant blue stained large subunit of RuBisCO (bottom panel) in extracts of EV, amiFPSa and amiFPSb plants grown as indicated above. Image shows the result of a representative experiment. (D) FPS activity in the same extracts used for western blot analysis obtained from plants grown on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 μM MFZ (grey bars). Values in (B) and (D) are means ± SD (n=3). Asterisks show the values that are significantly different (**$p < 0.005$) compared to those in the EV control plants.

Figure 3. Down-regulation of FPS activity leads to altered profiles of sterols and ubiquinones. (A) Bulk membrane sterols, (B) stigmasterol to β-sitosterol ratio, and (C) ubiquinones UQ-9 and UQ-10 in EV, amiFPSa and amiFPSb plants grown for 8 days on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 μM MFZ (grey bars). Values are means ± SD (n=3). Asterisks show the values that are significantly different (**$p < 0.005$) compared to those in the EV control plants.

Figure 4. Down-regulation of FPS activity triggers post-translational up-regulation of HMGR activity. (A) HMGR activity measured in extracts from EV, amiFPSa and amiFPSb plants grown for 8 days on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (grey bars). Values are means ± SD (n=3). Asterisks show the values that are significantly different (**p < 0.005) compared to those in the EV control plants. (B) Western blot analysis of HMGR protein (top panel) and Coomassie brilliant blue stained large subunit of RuBisCO (bottom panel) in the same extracts used for HMGR activity determination. Image shows the result of a representative experiment. (C) Quantitative RT-PCR analysis of *HMG1* and *HMG2* mRNA levels using RNA from EV, amiFPSa and amiFPSb seedlings grown on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (grey bars). Transcript levels were normalized relative to the mRNA levels of the *PP2AA3* gene. Values are means ± SD (n=3). (D) Phenotype of 8-day-old EV, amiFPSa and amiFPSb seedlings grown with or without 5 mM mevalonate (MVA) on MS (left side images) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (right side images).

Figure 5. Down-regulation of FPS activity alters chloroplast development as well as chlorophylls and carotenoid levels. (a) Laser confocal microscopy, (b) transmission electron microscopy (bars indicate 10 µm and 1 µm, respectively), and (c) area (μm^2) of chloroplasts in leaves of plants grown for 3 days on MS and 5 days on MS supplemented with 30 µM MFZ. Chloroplast area values are expressed as means ± SD (n=36) (d) Chlorophylls (total, a and b) and carotenoid contents in EV, amiFPSa and amiFPSb plants grown for 8 days on MS or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ. Values are means ± SD (n=3). Asterisks show the values that are significantly different (**p < 0.005) compared to those in the EV control plants.

Figure 6. Gene ontology (GO) classification of the differentially expressed genes in FPS silenced plants. The 168 genes showing at least a $\log_2$ (fold_change) of ≥ 2 or ≤ −2 and q-value <0,05 (Supplemental Table 1) were classified by "Biological Process" category using GeneCodis tool (http://genecodis.cnb.csic.es/) (Carmona-Saez et al., 2007)**.**

Figure 7. Inhibition of sterol biosynthetic pathway mimics the transcriptional response of FPS silenced plants. (A) Simplified scheme of the post-MVA sterol biosynthesis pathway. The position of reactions catalyzed by farnesyl diphosphate synthase (FPS), squalene synthase (SQS), squalene epoxidase (SQE), cycloartenol synthase (CAS), sterol methyltransferases (SMT1, SMT2 and SMT3), and sterol C22-desaturase (CYP710A1) is shown. Dashed arrows represent multiple enzymatic steps. (B) Heatmap showing the expression changes of a selection of 39 representative genes (Table 1) in seedlings silenced for FPS (amiFPSa) and seedlings where the sterol pathway was inhibited chemically with 150 µM Tb (Col-0 Tb) or genetically (*cvp1*/*smt3*). Color scale indicates the level of gene expression change with values ranging from $\log_2$ fold change (FC) -2.0 (lower expression-blue color) to 4.0 (higher expression-yellow color). Hierarchical clustering was made using the Euclidean distance. Rows represent genes and columns the FC of mRNA levels for each gene under each experimental condition compared to its corresponding control: amiFPSa

RNAseq: amiFPSa seedlings grown for 3 days on MS and 5 days on MS supplemented with 30 µM MFZ *vs* amiFPSa seedlings grown for 8 days on MS; amiFPSa RT-qPCR: amiFPSa *vs* EV seedlings grown both for 3 days on MS and 5 days on MS supplemented with 30 µM MFZ; Terbinafine RT-qPCR; Col-0 seedlings grown for 8 days on MS supplemented with 150 µM Tb *vs* Col-0 seedlings grown on MS; *cvp1*/*smt3* RT-qPCR: *cvp1*/*smt3* seedlings *vs* Col-0 seedlings grown for 8 days on MS.

Figure 8. Plants perceive a reduction in bulk membrane sterols as a stress signal that triggers an early up-regulation of genes involved in both biotic and abiotic stress responses. Three-day-old amiFPSa and EV seedlings grown on MS were transferred to MS supplemented with 30 µM MFZ and tissue samples were collected at the indicated time points from the start of MFZ treatment. The mRNA levels of genes involved in (A) the JA pathway, including JA synthesis (*LOX4*), signaling (*JAZ1* and *JAZ5*), homeostasis (*ST2A*) and target genes (*JR2*, *VSP1* and *ABCG40*), and (B) different abiotic stress responses (*AKR4C8*, *AKR4C9* and *COR78*), were quantified by RT-qPCR using RNA samples from seedlings collected at the indicated time points. Data are expressed as the mean of normalized quantity values ± SD (n=3) calculated using three independent housekeeping genes (*UBC*, *UBC9* and *PP2A*) (Ballester et al., 2013). Asterisks show the values that are significantly different (*$p < 0.05$ and **$p < 0.005$) compared to those in the EV control plants.

Figure 9. Down-regulation of FPS causes early misregulation of genes involved in maintaining Fe homeostasis. (A) The mRNA levels of genes encoding proteins involved in Fe storage (*FER1* and *FER4*), metabolism (*NEET*), and sensing and signaling (*BHLH038*, *BHLH039* and *BHLH100*) were quantified by RT-qPCR using RNA samples from seedlings collected at the indicated time points. Data are expressed as the mean of normalized quantity values ± SD (n=3) calculated using three independent housekeeping genes (*UBC*, *UBC9* and *PP2A*) (Ballester et al., 2013). Asterisks show the values that are significantly different (*$p < 0.05$ and **$p < 0.005$) compared to those in the EV control plants. (B) Fe levels in samples of EV, amiFPSa and amiFPSb seedlings grown for 8 days on MS or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ were determined using an Inductively Coupled Plasma Optical Emission Spectrometer. Values are means ± SD (n=3). Asterisks show the values that are significantly different (*$p < 0.05$) compared to the EV control plants.

Supplemental figure legends

Figure S1. Overexpression of FPS1S restores the wild type phenotype to plants silenced for FPS. (A) PCR-based genotype analysis of EV, amiFPSa and amiFPSa/35S::FPS1S seedlings using primer pairs targeting specific regions of the 35S::FPS1S and pre-amiFPSa transgenes. (B) Phenotype of EV, amiFPSa and amiFPSa/35S::FPS1S seedlings grown for 8 days on MS supplemented with 30 µM MFZ (top images) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (bottom images). (C) Quantitative RT-PCR analysis of FPS1 and FPS2 transcripts using RNA from EV, amiFPSa and amiFPSa/35S::FPS1S seedlings grown for 3 days on MS and 5 days on MS supplemented with 30 µM MFZ. Transcript levels were normalized relative to the mRNA levels of the *PP2AA3* gene. Values are means ± SD (n=3). (D) RT-PCR analysis of amiFPSa expression levels in EV, amiFPSa and

amiFPSa/35S::FPS1S seedlings grown for 3 days on MS and 5 days on MS supplemented with 30 μM MFZ.

Figure S2. Brassinosteroids fail to complement the phenotypes caused by silencing of FPS. Phenotype of 8-day-old EV, amiFPSa and amiFPSb seedlings grown with or without 0.4 nM brassinolide (BL) on MS medium (left side images) or 3 days on MS and 5 days on MS supplemented with 30 μM MFZ (right side images).

Figure S3. Effect of FPS silencing on carotenoid and prenylquinone levels. The contents of phytoene-derived compounds: β-carotene, lutein and viola-neoxanthin (orange bars), solanesyl diphosphate (C45)-derived compounds: plastoquinone-9 and plastochromanol-8 (blue bars), and phytyl-derived side chain compounds: phylloquinone (vitamin K), α-, δ- and γ-tocopherol (green bars), were measured in EV, amiFPSa and amiFPSb seedlings grown for 8 days on MS or 3 days on MS and 5 days on MS supplemented with 30 μM MFZ. Values are means ± SD (n=3). Asterisks show the values that are significantly different (**p < 0,005) compared to those in the EV control plants.

Figure S4. Silencing of FPS leads to misregulation of genes involved in maintaining redox homeostasis but does not trigger symptoms of oxidative stress. (A) Time course analysis of mRNA levels of genes coding for enzymes involved in maintaining redox homeostasis. Three-day-old amiFPSa and EV seedlings grown on MS were transferred to MS supplemented with 30 μM MFZ. The mRNA levels of genes encoding the glutathione S-transferases GST6, GST12, GST22 and the peroxidase Prx37 were quantified by real time RT-qPCR using RNA samples from seedlings collected at the indicated time points from the start of MFZ treatment. Data are expressed as the mean of normalized quantity values ± SD (n=3) calculated using three independent housekeeping genes (*UBC*, *UBC9* and *PP2A*) (Ballester et al., 2013). Asterisks show the values that are significantly different (*p < 0.05 and **p < 0.005) compared to those in the EV control plants. (b) Leaves of EV, amiFPSa and amiFPSb seedlings grown as indicated above were stained for reactive oxygen species (ROS) either with Nitroblue terazolium (NBT), which reacts with O_2^- developing a blue precipitate (top images), or Diaminobenzidine (DAB), which develops a brown precipitate after reacting with H_2O_2 (bottom images).

Figure S5. Time course expression analysis of genes coding for enzymes involved in carbohydrate metabolism. Three-day-old amiFPSa and EV seedlings grown on MS were transferred to MS supplemented with 30 μM MFZ. The mRNA levels of genes encoding SCORP (defensin-like protein involved in maltose and starch metabolism), FBA5 (aldolase superfamily protein involved in the sucrose signaling pathway), GPT2 (glucose 6-phosphate/phosphate translocator that imports glucose 6-phosphate from cytosol to chloroplast), DIN11 (2-oxoacid-dependent dioxygenase repressed by sugar), and BMY1 (the major form of β-amylase in Arabidopsis) were quantified by real time RT-qPCR using RNA samples from seedlings collected at the indicated time points from the start of MFZ treatment. Data are expressed as the mean of normalized quantity values ± SD (n=3) calculated using three independent housekeeping genes (*UBC*, *UBC9* and *PP2A*) (Ballester et al., 2013). Asterisks show the values that are significantly different (*p < 0.05 and **p < 0.005) compared to those in the EV control plants.

Tables

Table 1. Selection of genes that are differentially expressed between amiFPSa plants grown for 8 days on MS or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ.

Table S1. Transcriptomic analysis of FPS silenced plants using Illumina RNA-seq technology. List of differentially expressed genes between FPS silenced (AR) and non-silenced plants (AM). The transcript level and gene level counts were calculated and FPKM normalized using Cufflinks 2.0.2 software. Differential transcript expression was computed using Cuffdiff. The resulting list of differentially expressed transcripts were filtered by ln(fold_change) >2 and < -2 and a q-value of 0.05. A total of 168 genes were differentially expressed including 116 up-regulated genes and 16 down-regulated genes. In addition, the expression of some genes was detected only in one experimental condition, either in FPS silenced plants (35 *switch on genes*) or in FPS non-silenced plants (1 *switch off* gene).

Table S2. List of oligonucleotides used in high-throughput qPCR gene expression analysis (BioMark™ System from Fluidigm).

Gene Name	Gene Symbol	log2_fc	p_value	q_value	Gene Description
Jasmonate synthesis					
AT2G26560	PLA-IIA	3.9	5,00E-05	0.011	PHOSPHOLIPASE A 2A
AT1G72520	LOX4	3.4	0.00035	0.047	PLAT/LH2 domain-containing lipoxygenase family protein
AT3G25760	AOC1	2.9	5,00E-05	0.011	allene oxide cyclase 1
AT3G25780	AOC3	2.8	5,00E-05	0.011	allene oxide cyclase 3
Jasmonate homeostasis					
AT3G48520	CYP94B3	4.7	0.0003	0.042	cytochrome P450, family 94, subfamily B, polypeptide 3
AT5G07010	ST2A	2.9	0.0003	0.042	sulfotransferase 2A
Jasmonate signaling					
AT1G17380	JAZ5	4.5	0.0002	0.032	jasmonate-zim-domain protein 5
AT1G19180	JAZ1	3.6	5,00E-05	0.011	jasmonate-zim-domain protein 1
AT5G13220	TIFY9	3.6	5,00E-05	0.011	jasmonate-zim-domain protein 10
AT1G70700	JAZ9	2.6	5,00E-05	0.011	TIFY domain/Divergent CCT motif family protein
AT1G72450	JAZ6	2.3	0.0001	0.019	jasmonate-zim-domain protein 6
AT3G56400	WRKY70	4.1	5,00E-05	0.011	WRKY DNA-binding protein 70
AT2G38470	WRKY33	2.2	0.00015	0.025	WRKY DNA-binding protein 33
Jasmonate targets					
AT2G39330	JAL23	6.7	5,00E-05	0.011	jacalin-related lectin 23
AT1G15520	ABCG40	6.2	5,00E-05	0.011	pleiotropic drug resistance 12
AT5G24780	VSP1	5.4	5,00E-05	0.011	vegetative storage protein 1
AT2G34810	AT2G34810	3.8	5,00E-05	0.011	FAD-binding Berberine family protein
AT4G23600	JR2	3.7	5,00E-05	0.011	Tyrosine transaminase family protein
AT1G19670	ATCLH1	2.8	5,00E-05	0.011	chlorophyllase 1
Abiotic stress					
AT5G13330	Rap2.6L	3.8	0.0001	0.019	related to AP2 6l
AT2G37770	AKR4C9	3.7	5,00E-05	0.011	NAD(P)-linked oxidoreductase superfamily protein
AT2G33380	CLO-3	3	5,00E-05	0.011	Caleosin-related family protein
AT4G02330	ATPMEPCRB	2.5	5,00E-05	0.011	Plant invertase/pectin methylesterase inhibitor superfamily
AT2G37760	AKR4C8	2.2	0.00015	0.025	NAD(P)-linked oxidoreductase superfamily protein
AT5G52310	COR78	-2.4	0.00035	0.047	cold regulated 78
AT1G29395	COR414-TM1	-3.1	5,00E-05	0.011	cold regulated 314 thylakoid membrane 1
AT2G42530	COR15B	-3	5,00E-05	0.011	cold regulated 15b
Iron homeostasis					
AT3G56970	BHLH038	4	5,00E-05	0.011	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT2G41240	BHLH100	3.8	5,00E-05	0.011	basic helix-loop-helix protein 100
AT3G56980	BHLH039	3.5	0.0001	0.019	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT2G01530	MLP329	-2.4	5,00E-05	0.011	MLP-like protein 329
AT2G40300	FER4	-2.4	0.0001	0.019	ferritin 4
AT3G25190	VTL5	-2.4	0.0001	0.019	Vacuolar iron transporter (VIT) family protein
AT5G01600	FER1	-2.6	0.00025	0.037	ferretin 1
AT5G51720	NEET	-4	5,00E-05	0.011	2 iron, 2 sulfur cluster binding
Redox homeostasis					
AT1G69880	ATH8	4.5	0.0002	0.032	No Description Available
AT2G29460	GST22	4.2	0.00035	0.047	glutathione S-transferase tau 4
AT1G02930	ATGSTF6	3.7	5,00E-05	0.011	glutathione S-transferase 6
AT1G02920	GST11	3	5,00E-05	0.011	glutathione S-transferase 7
AT4G08770	Prx37	2.8	0.0001	0.019	Peroxidase superfamily protein
AT5G17220	ATGSTF12	2.7	0.0001	0.019	glutathione S-transferase phi 12
AT3G49110	ATPCA	2.2	0.00025	0.037	peroxidase CA
AT5G06690	WCRKC1	-2.3	0.0001	0.019	WCRKC thioredoxin 1
CHO metabolism					
AT3G49620	DIN11	5.6	5,00E-05	0.011	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT3G60140	DIN2	5.2	5,00E-05	0.011	Glycosyl hydrolase superfamily protein
AT4G15210	BMY1	4.2	0.00025	0.037	beta-amylase 5
AT2G43530	SCORP	3.2	5,00E-05	0.011	Scorpion toxin-like knottin superfamily protein
AT1G61800	GPT2	3.1	5,00E-05	0.011	glucose-6-phosphate/phosphate translocator 2
AT5G24420	PGL5	2.5	0.0001	0.019	6-phosphogluconolactonase 5 OPP shunt
AT4G26530	FBA5	-3.1	0.0001	0.019	Aldolase superfamily protein
Others					
AT1G44350	ILL6	2.8	0.0001	0.019	IAA-leucine resistant (ILR)-like gene 6
AT2G01520	MLP328	-2.4	0.00015	0.025	MLP-like protein 328

1289 **Table 1**

1290

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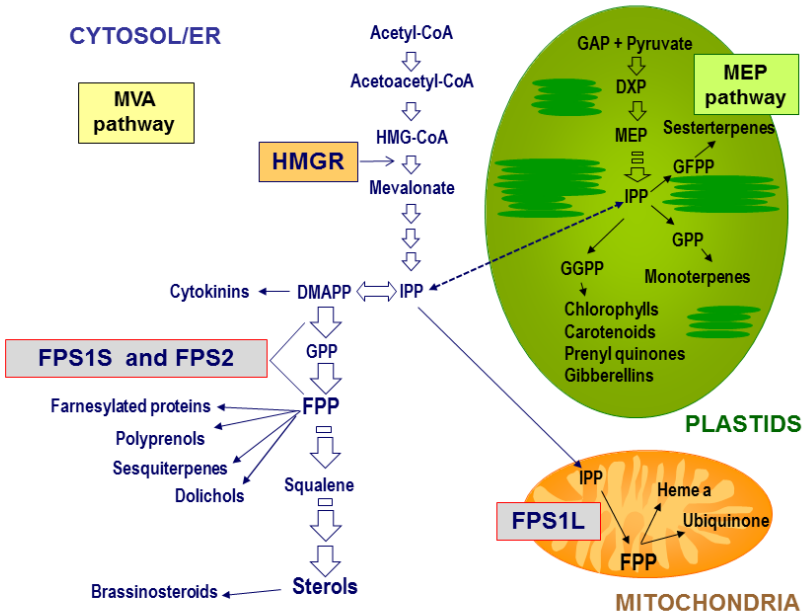
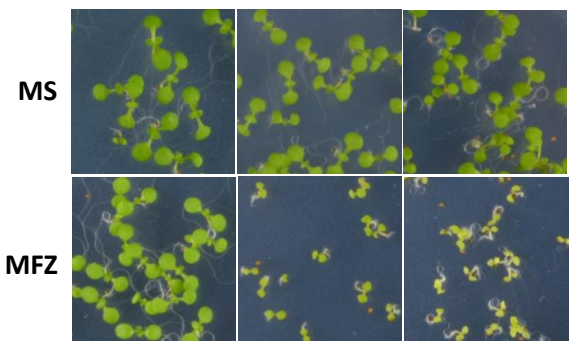


B

amiRNA	Sequence	Target gene	Position on mRNA	Hybridization energy	Mismatches
amiFPSa	5'-TATTGCGAAGTAGAATCGCGT-3'	*FPS1*	612 - 632	-33,44	4 (1,18,20,21)
		FPS2	409 - 429	-33,44	4 (1,18,20,21)
amiFPSb	5'-TAGGTCAACATAGTAAGCCTT-3'	*FPS1*	668 - 688	-32,31	2 (1,18)
		FPS2	465 - 485	-30,73	3 (1,18,20)

C



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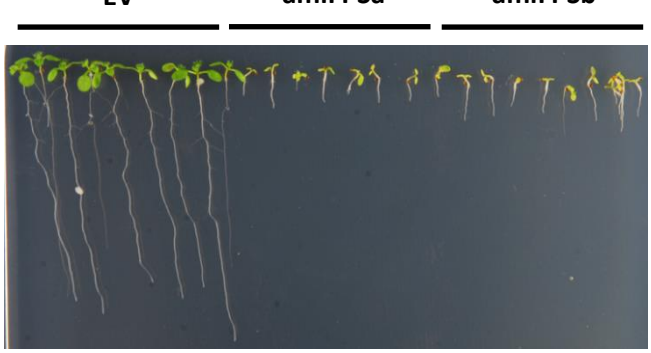


Figure 1: Silencing of Arabidopsis *FPS* gene expression using amiRNA technology alters plant shoot and root phenotypes. (A) Simplified scheme of the isoprenoid biosynthetic pathways. The reactions catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and farnesyl diphosphate synthase (FPS) are indicated. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GFPP, geranylfarnesyl diphosphate; GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate. (B) Main features of the amiRNAs designed for *FPS1* and *FPS2* gene silencing. (C) Shoot phenotype of 8-day-old EV, amiFPSa and amiFPSb seedlings grown on MS medium (top images) and MS supplemented with 30 µM MFZ (bottom images). (D) Root phenotype of 10-day-old EV, amiFPSa and amiFPSb seedlings grown on MS medium supplemented with 30 µM MFZ.

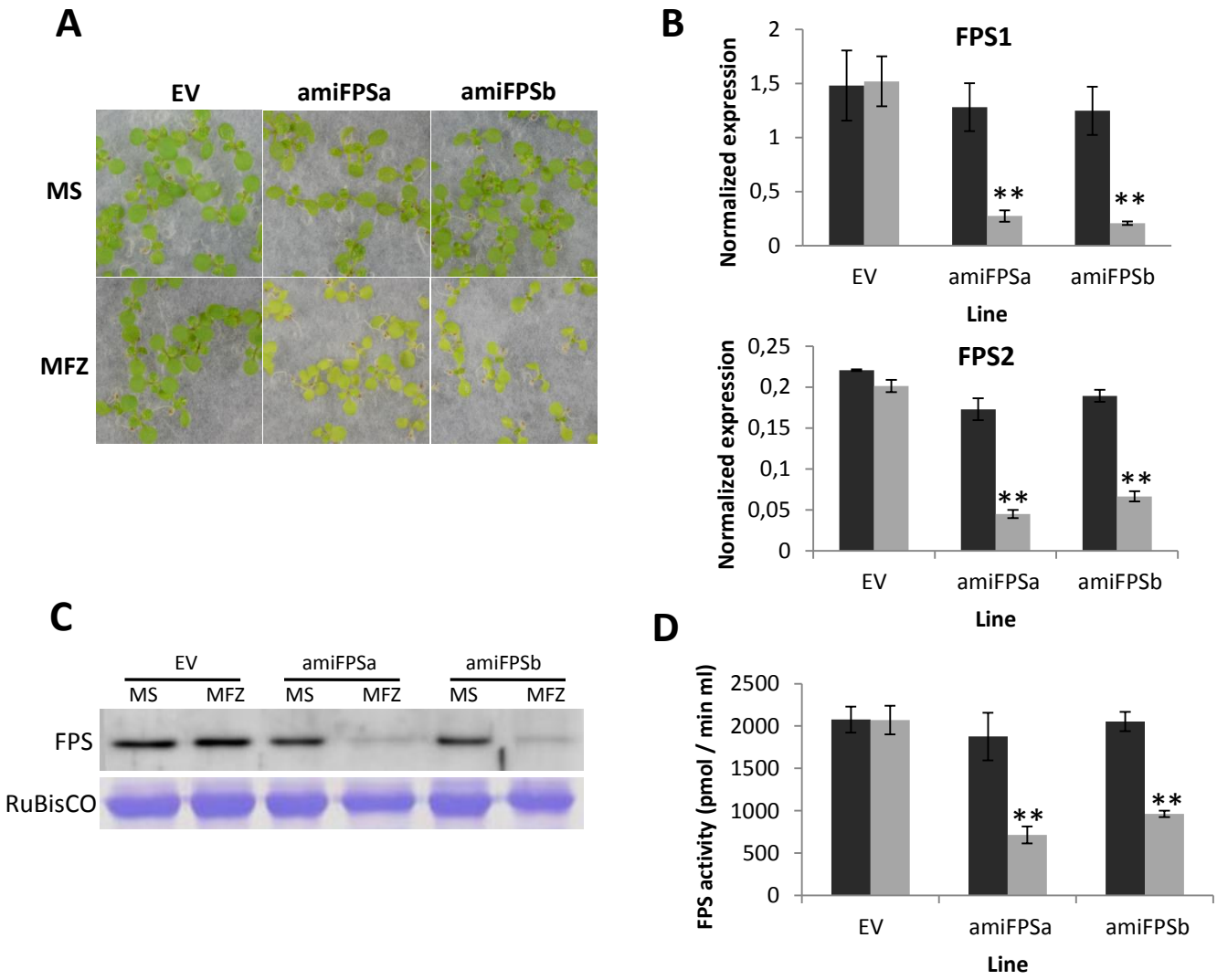


Figure 2: Expression of amiFPSa and amiFPSb leads to reduced levels of FPS mRNA, protein and enzyme activity. (A) Phenotype of EV, amiFPSa and amiFPSb seedlings grown for 8 days on MS medium (top images) or 3 days on MS medium and 5 days on MS medium supplemented with 30 µM MFZ (bottom images). (B) RT-qPCR analysis of *FPS1* and *FPS2* transcripts using RNA from EV, amiFPSa and amiFPSb seedlings grown on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (grey bars). Transcript levels were normalized relative to the mRNA levels of the *PP2AA3* gene. (C) Western blot analysis of FPS protein (top panel) and Coomassie brilliant blue stained large subunit of RuBisCO (bottom panel) in extracts of EV, amiFPSa and amiFPSb plants grown as indicated above. Image shows the result of a representative experiment. (D) FPS activity in the same extracts used for western blot analysis obtained from plants grown on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (grey bars). Values in (B) and (D) are means ± SD (n=3). Asterisks show the values that are significantly different (**$p < 0.005$) compared to those in the EV control plants.

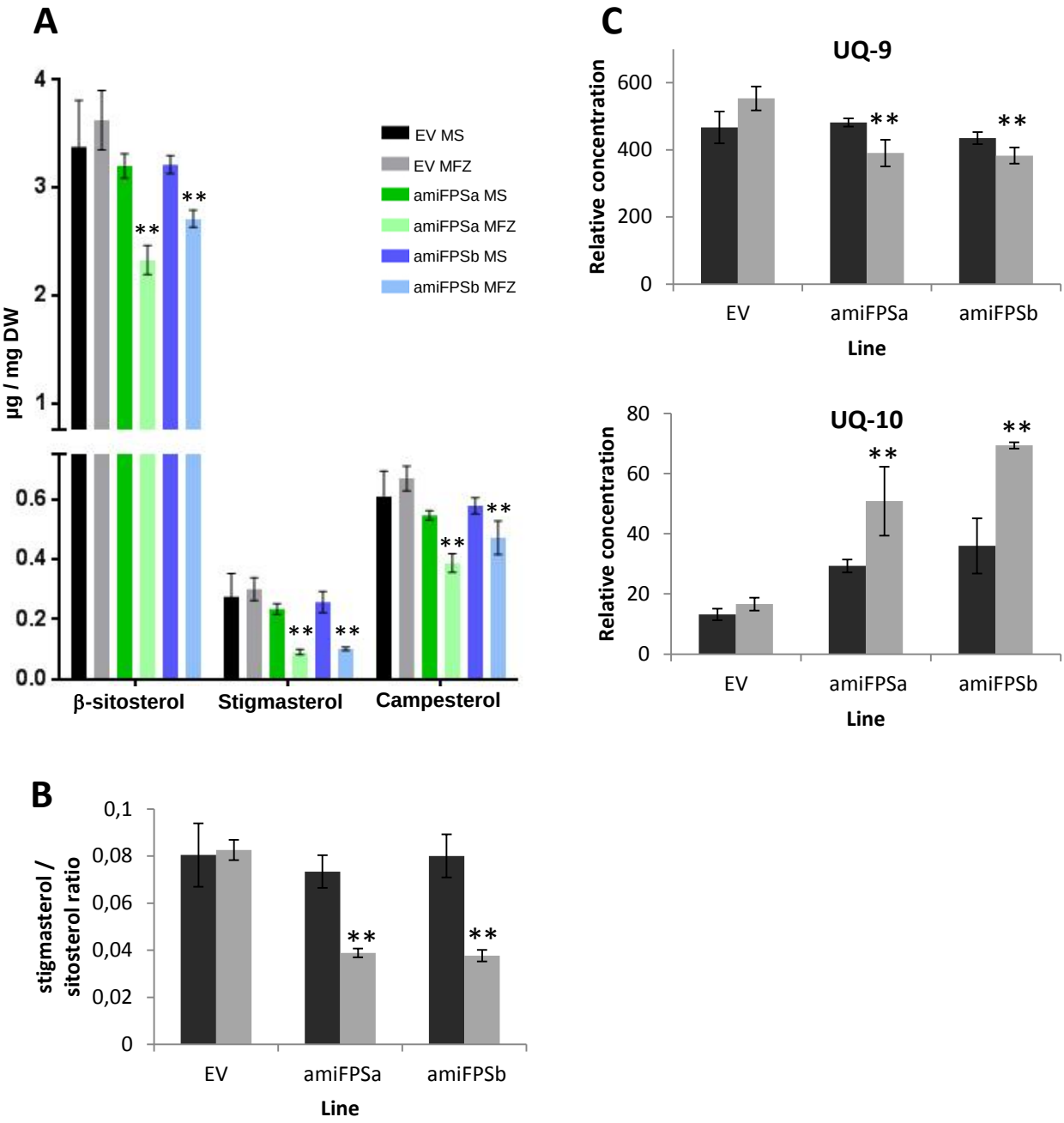


Figure 3. Down-regulation of FPS activity leads to altered profiles of sterols and ubiquinones. (A) Bulk-membrane sterols, (B) stigmasterol to β-sitosterol ratio, and (C) ubiquinones UQ-9 and UQ-10 in EV, amiFPSa and amiFPSb plants grown for 8 days on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (grey bars). Values are means ± SD (n=3). Asterisks show the values that are significantly different (**p < 0.005) compared to those in the EV control plants.

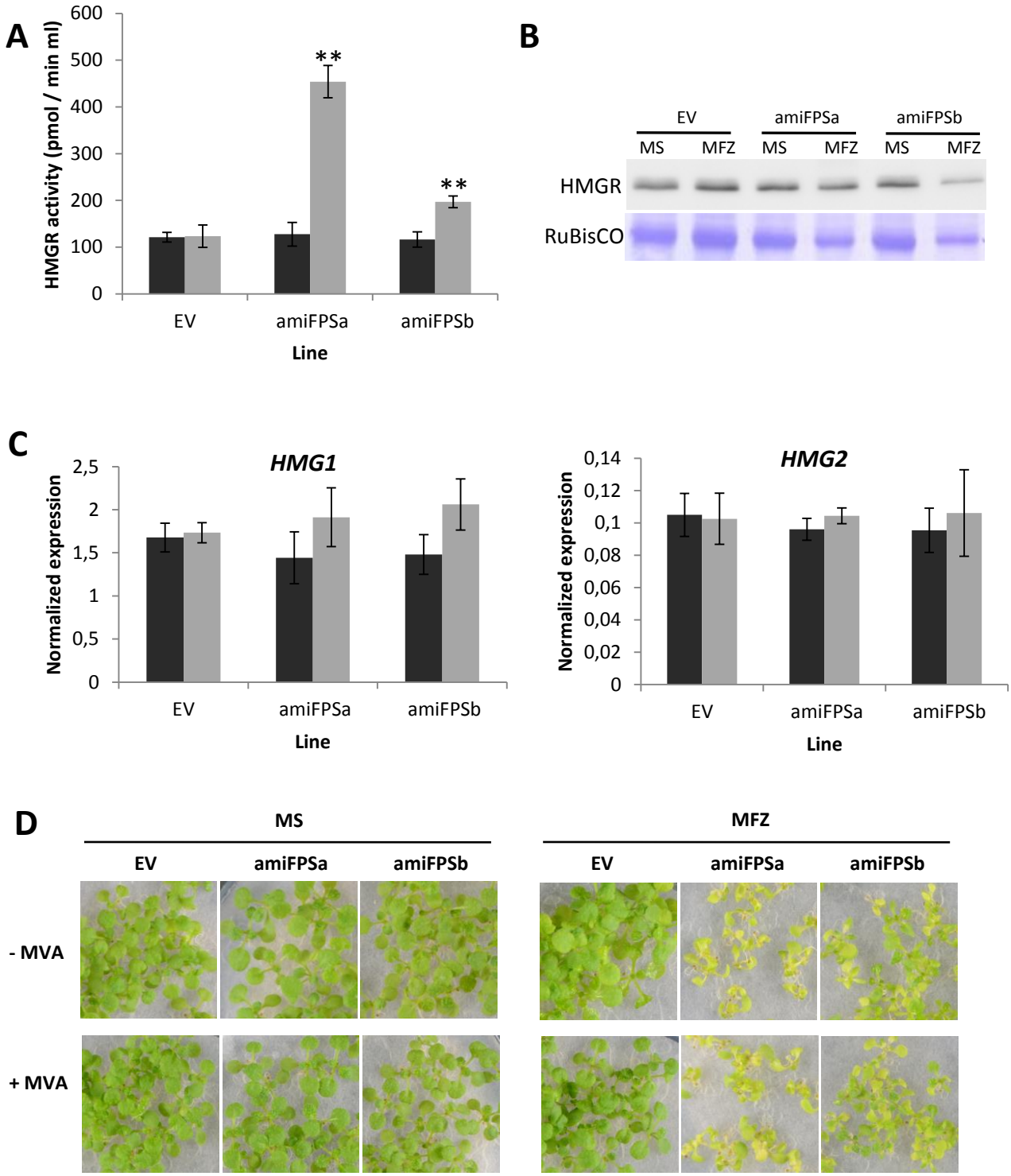
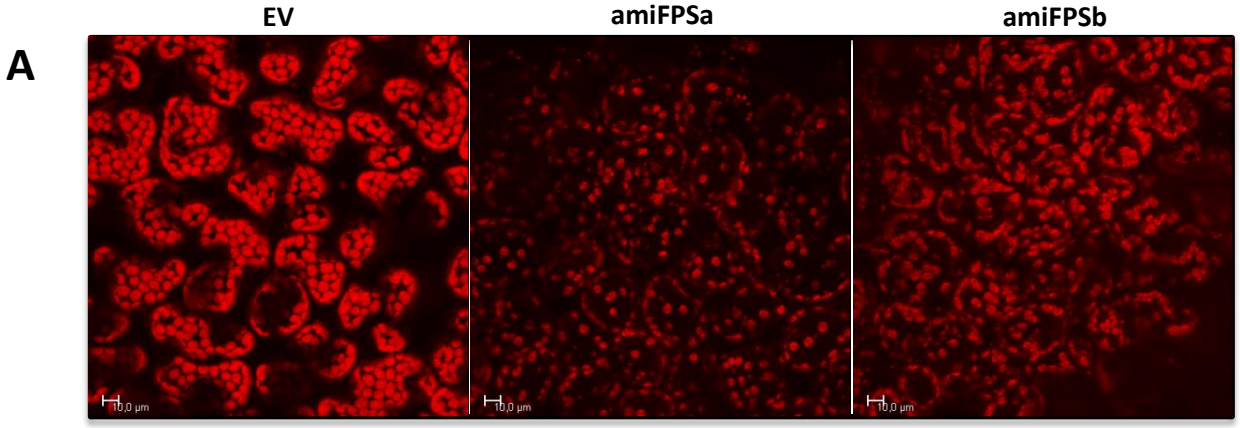
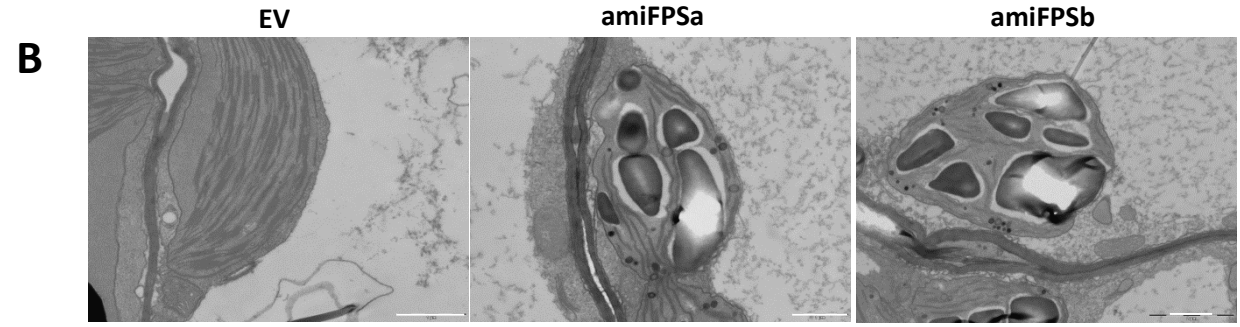


Figure 4. Down-regulation of FPS activity triggers post-translational up-regulation of HMGR activity. (A) HMGR activity measured in extracts from EV, amiFPSa and amiFPSb plants grown for 8 days on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (grey bars). Values are means ± SD (n=3). Asterisks show the values that are significantly different (**p < 0.005) compared to those in the EV control plants. (B) Western blot analysis of HMGR protein (top panel) and Coomassie brilliant blue stained large subunit of RuBisCO (bottom panel) in the same extracts used for HMGR activity determination. Image shows the result of a representative experiment. (C) Quantitative RT-PCR analysis of *HMG1* and *HMG2* mRNA levels using RNA from EV, amiFPSa and amiFPSb seedlings grown on MS (black bars) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (grey bars). Transcript levels were normalized relative to the mRNA levels of the *PP2AA3* gene. Values are means ± SD (n=3). (D) Phenotype of 8-day-old EV, amiFPSa and amiFPSb seedlings grown with or without 5 mM mevalonate (MVA) on MS (left side images) or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ (right side images).





C

Treatment	EV	amiFPSa	amiFPSb
MS	30,14 ± 4,61	34,82 ± 5,31	33,01 ± 5,15
MFZ	34,66 ± 5,29	15,75 ± 4,55 (**)	20,58 ± 4,31 (**)

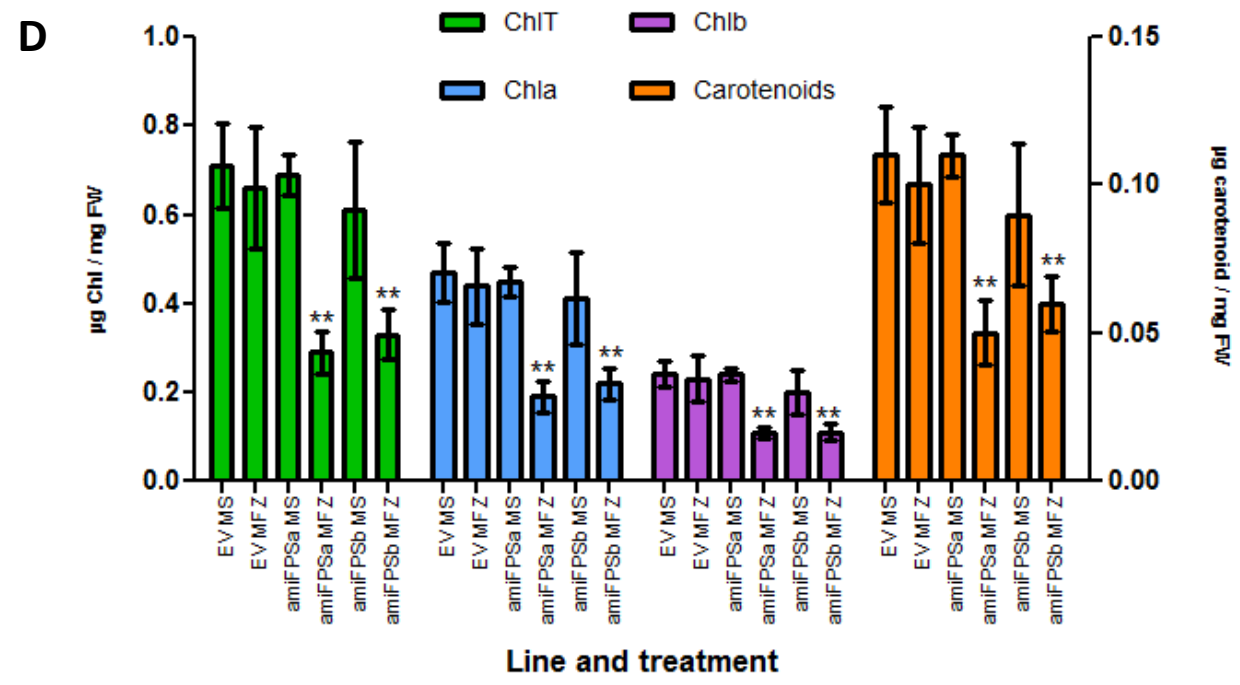


Figure 5: Down-regulation of FPS activity alters chloroplast development as well as chlorophylls and carotenoid levels. (a) Laser confocal microscopy, (b) transmission electron microscopy (bars indicate 10 µm and 1 µm, respectively), and (c) area (µm^2) of chloroplasts in leaves of plants grown for 3 days on MS and 5 days on MS supplemented with 30 µM MFZ. Chloroplast area values are expressed as means ± SD (n=36) (d) Chlorophylls (total, a and b) and carotenoid contents in EV, amiFPSa and amiFPSb plants grown for 8 days on MS or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ. Values are means ± SD (n=3). Asterisks show the values that are significantly different (** $p < 0.005$) compared to those in the EV control plants.

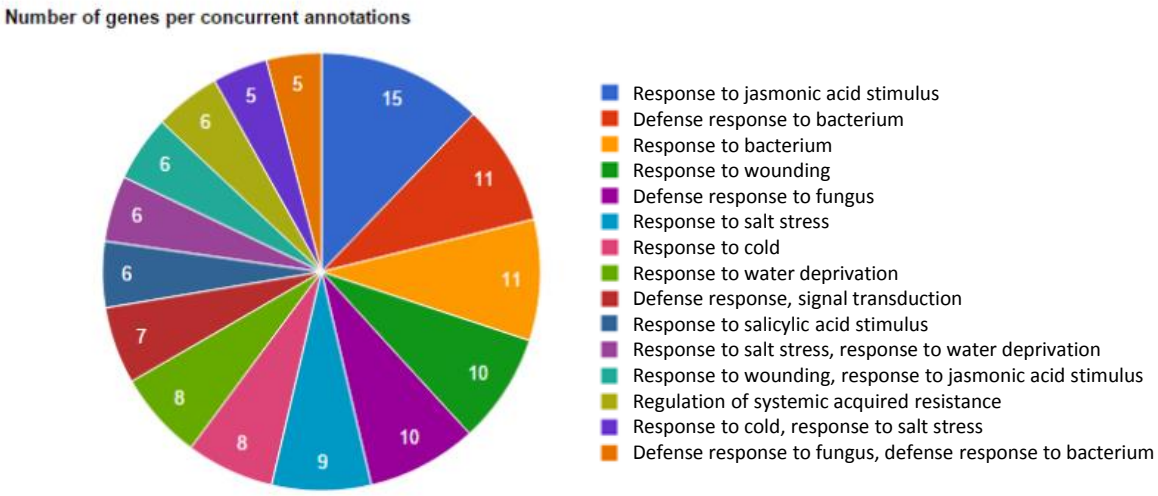


Figure 6. Gene ontology (GO) classification of the differentially expressed genes in FPS silenced plants. The 168 genes showing at least a $\log_2$ (fold_change) of ≥ 2 or ≤ −2 and q-value <0,05 (Supplemental Table 1) were classified by "Biological Process" category using GeneCodis tool (http://genecodis.cnb.csic.es/) (Carmona-Saez et al., 2007)**.**

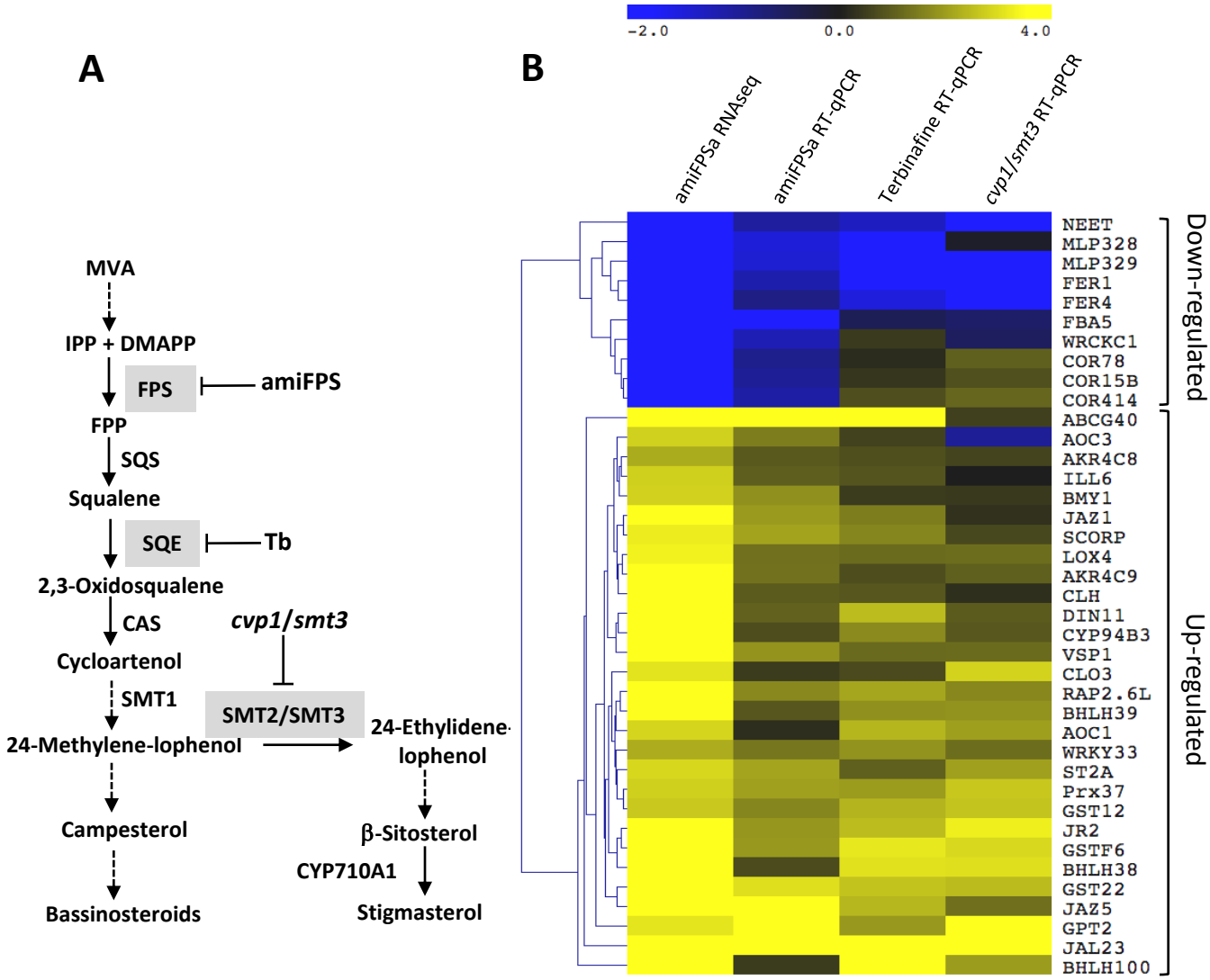


Figure 7. Inhibition of sterol biosynthetic pathway mimics the transcriptional response of FPS silenced plants. (A) Simplified scheme of the post-MVA sterol biosynthesis pathway. The position of reactions catalyzed by farnesyl diphosphate synthase (FPS), squalene synthase (SQS), squalene epoxidase (SQE), cycloartenol synthase (CAS), sterol methyltransferases (SMT1, SMT2 and SMT3), and sterol C22-desaturase (CYP710A1) is shown. Dashed arrows represent multiple enzymatic steps. (B) Heatmap showing the expression changes of a selection of 39 representative genes (Table 1) in seedlings silenced for FPS (amiFPSa) and seedlings where the sterol pathway was inhibited chemically with 150 µM Tb (Col-0 Tb) or genetically (*cvp1*/*smt3*). Color scale indicates the level of gene expression change with values ranging from $\log_2$ fold change (FC) -2.0 (lower expression-blue color) to 4.0 (higher expression-yellow color). Hierarchical clustering was made using the Euclidean distance. Rows represent genes and columns the FC of mRNA levels for each gene under each experimental condition compared to its corresponding control: amiFPSa RNAseq: amiFPSa seedlings grown for 3 days on MS and 5 days on MS supplemented with 30 µM MFZ *vs* amiFPSa seedlings grown for 8 days on MS; amiFPSa RT-qPCR: amiFPSa *vs* EV seedlings grown both for 3 days on MS and 5 days on MS supplemented with 30 µM MFZ; Terbinafine RT-qPCR; Col-0 seedlings grown for 8 days on MS supplemented with 150 µM Tb *vs* Col-0 seedlings grown on MS; *cvp1*/*smt3* RT-qPCR: *cvp1*/*smt3* seedlings *vs* Col-0 seedlings grown for 8 days on MS.

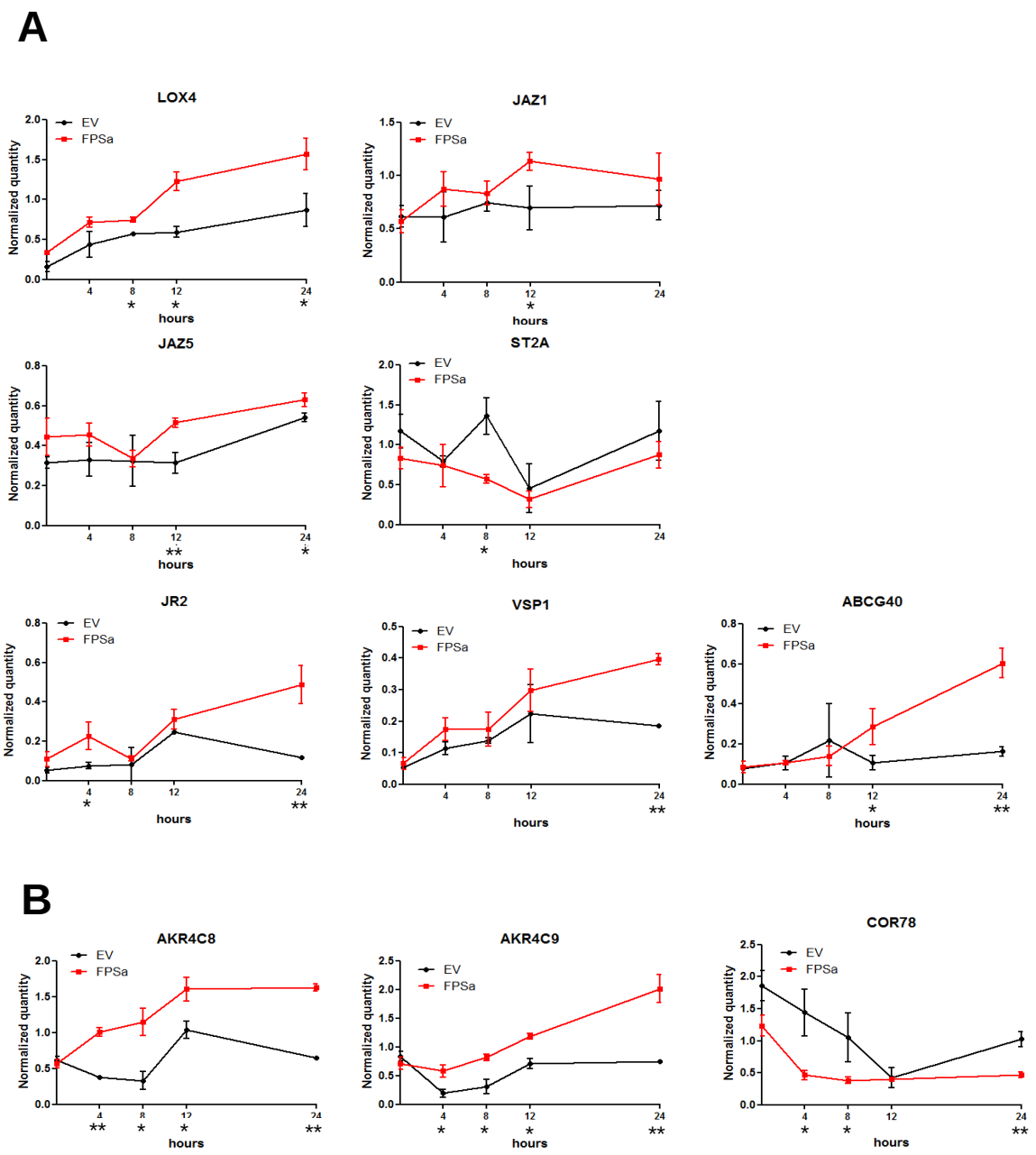


Figure 8. Plants perceive a reduction in bulk membrane sterols as a stress signal that triggers an early up-regulation of genes involved in both biotic and abiotic stress responses. Three-day-old amiFPSa and EV seedlings grown on MS were transferred to MS supplemented with 30 µM MFZ and tissue samples were collected at the indicated time points from the start of MFZ treatment. The mRNA levels of genes involved in (A) the JA pathway, including JA synthesis (*LOX4*), signaling (*JAZ1* and *JAZ5*), homeostasis (*ST2A*) and target genes (*JR2*, *VSP1* and *ABCG40*), and (B) different abiotic stress responses (*AKR4C8*, *AKR4C9* and *COR78*), were quantified by RT-qPCR using RNA samples from seedlings collected at the indicated time points. Data are expressed as the mean of normalized quantity values ± SD (n=3) calculated using three independent housekeeping genes (*UBC*, *UBC9* and *PP2A*) (Ballester et al., 2013). Asterisks show the values that are significantly different (*p < 0.05 and **p < 0.005) compared to those in the EV control plants.

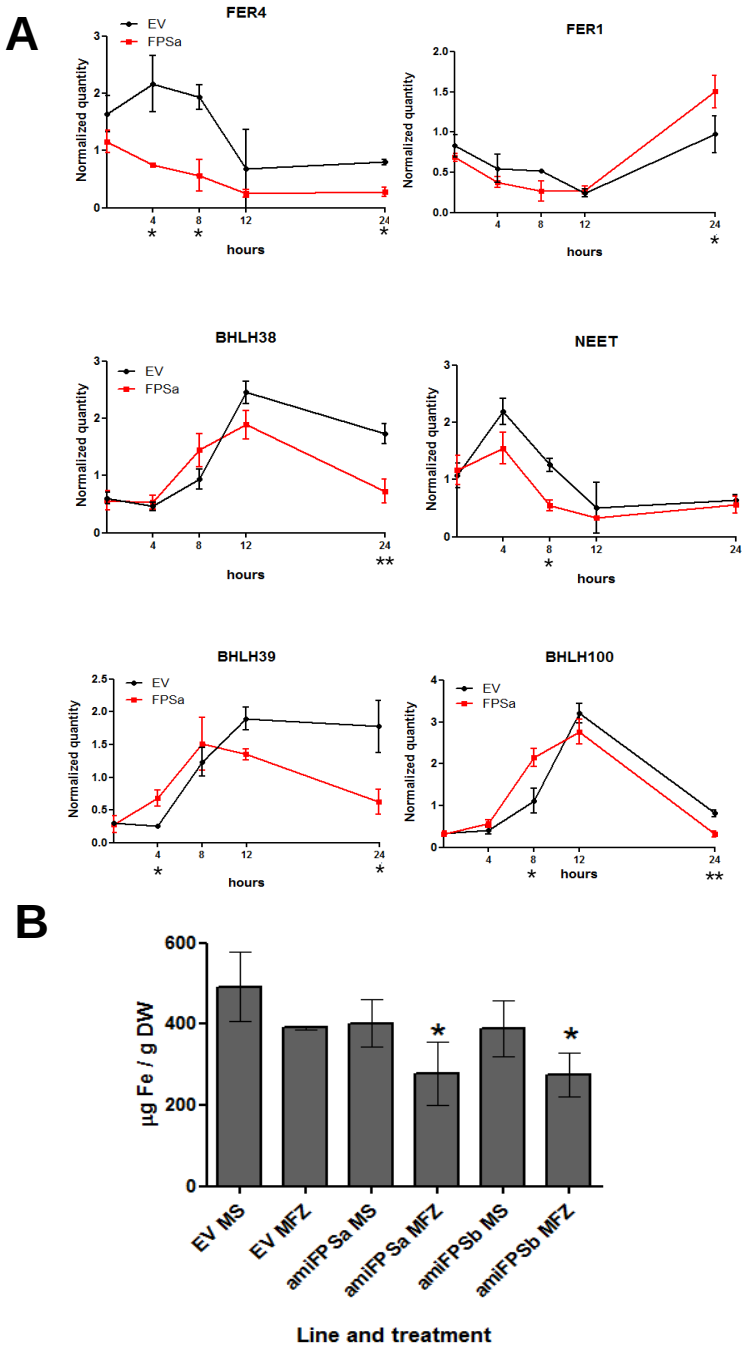


Figure 9. Down-regulation of FPS causes early misregulation of genes involved in maintaining Fe homeostasis. (A) The mRNA levels of genes encoding proteins involved in Fe storage (*FER1* and *FER4*), metabolism (*NEET*), and sensing and signaling (*BHLH038*, *BHLH039* and *BHLH100*) were quantified by RT-qPCR using RNA samples from seedlings collected at the indicated time points. Data are expressed as the mean of normalized quantity values ± SD (n=3) calculated using three independent housekeeping genes (*UBC*, *UBC9* and *PP2A*) (Ballester et al., 2013). Asterisks show the values that are significantly different (*p < 0.05 and **p < 0.005) compared to those in the EV control plants. (B) Fe levels in samples of EV, amiFPSa and amiFPSb seedlings grown for 8 days on MS or 3 days on MS and 5 days on MS supplemented with 30 µM MFZ were determined using an Inductively Coupled Plasma Optical Emission Spectrometer. Values are means ± SD (n=3). Asterisks show the values that are significantly different (*p < 0.05) compared to the EV control plants.

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