Plant sterol metabolism. 7-Sterol-C5-Desaturase (STE1/DWARF7), 5,7-Sterol-7-Reductase (DWARF5) and 24-Sterol-24-Reductase (DIMINUTO/DWARF1) show multiple subcellular localizations in Arabidopsis thaliana (Heynh) L

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Abstract

Sterols are crucial lipid components that regulate membrane permeability and fluidity and are the precursors of bioactive steroids. The plant sterols exist as three major forms, free sterols, steryl glycosides and steryl esters. The storage of steryl esters in lipid droplets has been shown to contribute to cellular sterol homeostasis. To further document cellular aspects of sterol biosynthesis in plants, we addressed the question of the subcellular localization of the enzymes implicated in the final steps of the post-squalene biosynthetic pathway. In order to create a clear localization map of steroidogenic enzymes in cells, the coding regions of \( \Delta^7 \)-sterol-C\(_5\)-desaturase (STE1/DWARF7), \( \Delta^5,7 \)-sterol-\( \Delta^7 \)-reductase (DWARF5) and \( \Delta^5,7 \)-sterol-\( \Delta^7 \)-reductase (DWARF5) were fused to the yellow fluorescent protein (YFP) and transformed into \textit{Arabidopsis thaliana} mutant lines deficient in the corresponding enzymes. All fusion proteins were found to localize in the endoplasmic reticulum in functionally complemented plants. The results show that both \( \Delta^5,7 \)-sterol-\( \Delta^7 \)-reductase and \( \Delta^5 \)-sterol-\( \Delta^7 \)-reductase are in addition localized to the plasma membrane, whereas \( \Delta^7 \)-sterol-\( \Delta^7 \)-desaturase was clearly detected in lipid particles. These findings raise new challenging questions about the spatial and dynamic cellular organization of sterol biosynthesis in plants.

Introduction

Sterols are well-known essential structural components that affect biophysical properties of membranes such as permeability and fluidity [1,2] and also heat-shock tolerance [3]. Their implication in the functional domain of steroidal membrane domains together with other lipid components such as sphingolipids has been discussed [4]. Sterol derivatives are also involved in many biological processes, by acting as signalling molecules in the cell cycle [5] modulating the activity of membrane bound enzymes [6] and regulating growth since they are the precursors of steroidal hormones both in plants and animals [7,8]. Sterol requirements in the case of plant cell division were recently studied with biosynthetic mutants; particularly, it was shown that sterol composition of the plasma membrane had an effect on the proper functioning of auxin transporters [9]. In the model plant \textit{Arabidopsis thaliana}, campesterol is the precursor of brassinosteroids, a group of polyoxidized steroids. Their importance in plant growth and development has been demonstrated through the study of several \textit{dwarf} mutants affected in the biosynthesis of, or in the response to, brassinosteroids. Some of these mutants are in fact deficient in the biosynthesis and accumulation of sterols which serve as brassinosteroid precursors [10–15]. Besides the structural and biological functions described above there is now an increasing number of reports that confer an ecophysiological relevance to sterols, as for instance in plant-pathogen interactions [16,17] or drought stress [18].

In contrast to animals, where cholesterol is the main sterol, plants accumulate a wide range of sterols with campesterol, sitosterol and stigmasterol being the major molecular species. Interestingly, a typical plant sterol profile contains little amounts of androst-5-ene-3beta,7beta-diol, the plant sterol precursor of brassinosteroids [19]. In addition to 3\beta-hydroxy-sterols (the so-called free sterols), plants contain sterol conjugates, in particular steryl esters (SE) and steryl glycosides (SG) [21,22]. Studies performed on a tobacco sterol overproducing mutant have shown that SE are deposited in oily
droplets herein named lipid particles (LPs) [23,24]. The low affinity of SE for membrane bilayers [25] suggested a role for these conjugates in the control of the free sterol amount in cell membranes [26]. The possible implication of the plant phospholipid sterol acyltransferase (PSAT) in this process through the enzymatically favoured esterification of sterol intermediates in the presence of high amounts of pathway end-products was recently outlined [27,29]. Despite its importance this process is not yet clearly understood in plants.

In yeast, the LPs are well described [29] and a number of enzymes involved in sterol biosynthesis has been associated with these organelles, such as ERG1p [29], ERG6p [29], ERG7p [30] and ERG27p [31] in addition to sterol acyltransferases [32] and triacylglycerol lipases [33]. These observations strengthened the idea that the LPs operate not only as a storage organelle for lipids and sterols but also as a compartment where sterol biosynthesis occurs. So far, none of the plant sterol synthesizing enzymes has been localized to LPs. This is partly due to the general acceptance that plant sterol biosynthesis occurs in the ER [7,19,20,34]. For the reasons stated above it is of great interest to investigate the spatial organization of the plant sterol pathway, by defining the subcellular localization of key enzymes.

In this report we describe the functional localization of Δ⁷-sterol-C⁵-desaturase (STE1/DWARF7), Δ⁵,7-sterol-Δ⁷-reductase (DWARF5) and Δ²⁴-sterol-Δ²⁴-reductase (DIM/DWARF1), three enzymes involved in the late steps of sitosterol biosynthesis, in Arabidopsis thaliana. These three enzymes constitute the last biosynthetic segment of the post-squalene sterol pathway where the tetracyclic moiety bearing a C⁷–C₈ double bond undergoes a Δ⁵–Δ⁷ desaturation then a Δ⁷-reduction, to yield Δ⁷-sterols, the pathway end-products in wild-type Arabidopsis thaliana [35]. To this mandatory biosynthetic sequence Δ⁵→Δ⁷→Δ⁸ (Figure 1) is associated the reduction of the Δ⁴ double bond in the side chain of eg 24-methylene cholesterol and isofucosterol [13,20,35]. Plants and other eukaryotic organisms share the same biosynthetic segment, with the notable exception of yeast which does not have a sterol-Δ⁷-reductase and therefore accumulate ergosterol, a Δ⁵,7-sterol, as the major pathway end-product. We fused STE1/DWARF7, DWARF5 and DIM/DWARF1 with the yellow fluorescent protein (YFP), used genetic complementation of the corresponding yeast deficient mutants (erg3, deficient in the yeast Δ⁷-sterol-C⁵-desaturase; erg4, deficient in the yeast Δ⁵,7-sterol-Δ⁷-reductase) or expression of DWARF5 in wild-type yeast to demonstrate that the fusion proteins were fully functional. We then genetically complemented Arabidopsis mutant lines lacking the corresponding enzyme activity due to induced genomic mutations (ste1-1 and dwarf5-2) or to a T-DNA insertion (dim). This clearly demonstrated that the fusion enzymes were active in planta and allowed detailed localization studies.

Figure 1. Simplified sterol biosynthetic pathway in Arabidopsis and yeast. Sterol biosynthesis starts preferentially with cycloartenol in plants and lanosterol in animals and fungi. Main biosynthetic steps and sterols accumulating in the mutant lines considered in this study are indicated in colors: red, ste1; green, dwarf5; blue, dim1. The major observed enzymatic activity of the YFP-fused enzymes generated is also indicated by the same color code. LAS1 = lanosterol synthase, CAS1 = cycloartenol synthase. The dashed arrows are indicating more than one enzymatic step. Accurate sterol nomenclature can be found at IUPAC http://www.iupac.org/doi:10.1371/journal.pone.0056429.g001
Results and Discussion

Yeast functional complementation

The coding regions of **STE1**, **DWARF5** and **DIM** from *Arabidopsis* were cloned as C-terminal translational fusions with the YFP reporter. In order to test whether the engineered enzymes were catalytically active these constructs were transformed into the appropriate yeast strains: STE1-YFP in *erg3*Δ, DWARF5-YFP in a wild-type yeast, and DIM-YFP in *erg4*. It is well established that the enzymes involved in the late steps of the post-squalene sterol biosynthetic segment are highly conserved among plants, animals and fungi [20]; it has previously been shown that this allowed efficient reciprocal genetic complementation[35–37]. For instance Gachotte *et al.* [12] and Husestein *et al.* [38] reported the characterization of the **ste1-1 Arabidopsis thaliana** mutant line that carries a T114I mutation in the **D**α7-sterol-C5-desaturase enzyme, using the expression of STE1 in the *erg3* yeast mutant (Figure 1).

We show here using the same *erg3* yeast strain that the fusion protein produced from of the **GAL1-10::STE1-YFP** construct is catalytically active. In fact, the *erg3* strain is unable to complete the ergosterol biosynthesis and accumulates episterol (Figure 1; Table 1) whereas the ergosterol biosynthetic pathway is partially restored after transformation of *erg3* with the **GAL1-10::STE1-YFP** fusion construct (Figure 1; Table 1)

Similarly, the **erg4** mutant was complemented with the fusion protein produced from the **GAL1-10::DIM-YFP** construct, since **DIM** encodes the corresponding plant Dα7-sterol-Dα4-reductase (Figure 1). In the **erg4** strain, ergosterol is completely replaced by ergosta-5-7-22-24(28)-tetraen-3β-ol (Figure 1; Table 1). The induction of the expression of the **GAL1-10::DIM-YFP** clearly re-established the biosynthesis of ergosterol in the mutant yeast (Table 1), demonstrating thus the catalytic activity of the engineered protein.

The wild-type W303-B1 yeast strain was used to assess the functionality of the DWARF5-YFP fusion protein encoding a Dα7,7-sterol-Dα7-reductase. Ergosterol as a Dα7,7-diene may be a substrate for the engineered Dα5,7-sterol-Dα7-reductase [40]. Our results show the reduction of the C7β(8) double bond of Dα5,7-sterols in yeast; the accumulation of brassicasterol and campesterol upon expression of the **GAL1-10::DWARF5-YFP** verifies DWARF5-YFP as a functional enzyme.

In conclusion, the expression of the fusion constructs in yeast and the detection of the expected sterol profiles confirmed that all three fusion enzymes were catalytically active.

<table>
<thead>
<tr>
<th>sterol (% of total)</th>
<th>W303</th>
<th>W303::DWARF5-YFP</th>
<th>erg3</th>
<th>erg3::STE1-YFP</th>
<th>erg4</th>
<th>erg4::DIM-YFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>lanosterol (A)</td>
<td>5</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>4,4-dimethylzymosterol</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>episterol (B)</td>
<td>1</td>
<td>—</td>
<td>77</td>
<td>62</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ergosta-7,22-dien-3β-ol (D)</td>
<td>—</td>
<td>—</td>
<td>10</td>
<td>8</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>ergosta-7,22,24(28)-dien-3β-ol (C)</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>1</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>ergosta-5,7-22-24(28)-tetroen-3β-ol (E)</td>
<td>3</td>
<td>2</td>
<td>—</td>
<td>3</td>
<td>91</td>
<td>82</td>
</tr>
<tr>
<td>ergosterol (F)</td>
<td>88</td>
<td>68</td>
<td>—</td>
<td>15</td>
<td>—</td>
<td>6</td>
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<td>brassicasterol (G)</td>
<td>—</td>
<td>11</td>
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<tr>
<td>campesterol (H)</td>
<td>—</td>
<td>14</td>
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One representative analysis out of three independent experiments is shown. The letters in parentheses refers to Figure 1.- = compound not detected.

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### Sterol Biosynthesis Enzymes Localization

**Figure 2. Genetic complementation of ste1-1, dwarf5-2 and dim sterol biosynthetic mutants expressing STE1-YFP, DWARF5-YFP, and DIM-YFP cDNAs, respectively.** GC-FID chromatograms of steryl acetates are shown. (A) dwarf5-2 mutant; (B) dwarf5-2/DWARF5-YFP partially complemented mutant; (C) dwarf5-2/DWARF5-YFP fully complemented mutant. (D) dim mutant; (E), dim/DIM-YFP partially complemented mutant; (F) dim/DIM-YFP fully complemented mutant. (G) ste1-1 mutant; (H), ste1-1/STE1-YFP partially complemented mutant; (I) ste1-1/STE1-YFP fully complemented mutant. Sterol peaks identified by their retention time and confirmed by GC-MS (prominent mass fragments not shown here) are: 1, cholesterol; 2, Δ9-cholesterol; 3, Δ7-cholesterol; 4, campesterol; 5, Δ5-campesterol; 6, Δ5,7-campesterol; 7, Δ5,7-stigmasterol; 8, Δ7-sitosterol; 9, Δ7-sitosterol; 10, sitosterol; 11, isofucosterol; 12, Δ7-sitosterol; 13, 24-methylene cholesterol; 14, stigmasterol; 15, Δ7-avenasterol. Full complementation of dwarf5-2::DWARF5-YFP and ste1-1 results in the accumulation of sitosterol (10) instead of Δ5,7-sitosterol (9), isofucosterol (11) and Δ7-sitosterol (12), respectively. The relevant peaks in each complementation are labelled in bold in the relevant panels.

dim::DIM-YFP transgenic lines fall clearly into two categories according to their semi-dwarf phenotype indicating partial complementation, or to their wild-type phenotype indicating full complementation, when compared to the parental lines (Figure 3).

The integrity of the three YFP-fusion proteins in the transgenic plants was assessed by immunoblot analysis using an antibody directed towards the YFP-protein. This showed the accumulation of products with the expected molecular size for the DIM-YFP and DWARF5-YFP fusions. The STE1-YFP fusion protein was not detected nor was a degradation product (Supplemental Figure 1). Probably the abundance of the STE1-YFP protein was below the detection limit of the antibody.

The sterol profile established by GC-FID and confirmed by GC-MS for several individual plants from the T2 and T3 generations demonstrated the correct in planta enzyme activity for all fusion proteins: indeed, partial (Figure 2B, 2E, and 2H; Table 2) or full (Figure 2C, 2F, and 2I; Table 2) reversion of the sterol composition to wild-type was detected in all cases (Figure 2; Supplemental Figure 2). In addition, the level of morphogenetic complementation and metabolic complementation were completely coincident. The fact that both partial and fully complemented plants were observed for each mutant line shows that the expression of the YFP fusion proteins has been performed in the correct mutant backgrounds.

The partial restoration of sitosterol biosynthesis observed in ste1-1 mutants expressing the STE1-YFP fusion protein is in good agreement with the results reported by Husselstein et al. [38] who found the ste1-1 plants showing a sitosterol recovery of about 50% when compared to wild-type, after transformation with a 35S::STE1 construct. In conclusion, the observations of fully or partially complemented plants shows that all three C-terminal YFP fused enzymes are catalytically active in planta. The full complementation result in a sterol profile and content closely identical to that of the wild-type.

### Subcellular localization

The enzymes investigated in this work were chosen due to their implication in the late steps of sitosterol biosynthesis. Sterol metabolism and transport mechanisms have been investigated and documented in animals [43,44] and to some extent in fungi with respect to cellular transport [45]. In plants, still little is known about the regulation of sterol biosynthesis and transports. Moreover, a detailed subcellular localization analysis of the late sterol biosynthetic enzymes is lacking. In order to establish the localization of STE1, DWARF5 and DIM, an in silico investigation was performed on their peptide sequences followed by extensive experimental investigations using confocal microscopy.

The in silico analysis made with the Predotar software based on the presence of signal peptides at N-termini of the protein sequences, predicted DWARF5 to be localized to the ER with a probability of 90% (Supplemental Table 1). In contrast DIM and STE1 were predicted to be non-ER proteins with a probability of 94% and 98%, respectively. These results are in agreement with the predictions obtained with Signal P, a protein sequence evaluation software, which found a significant probability of the presence of a signal peptide only in DWARF5 (Supplemental Table 1).

To experimentally assess whether STE1, DWARF5 and DIM were localized to the ER or to other compartments, we used confocal microscopy to analyse cells from the lower epidermal layer of leaves detached from functionally complemented dwarf5-2::DWARF5-YFP, dim::DIM-YFP and ste1-1/STE1-YFP plants.

In accordance with the in silico prediction (Supplemental Table 1), the DWARF5-YFP fusion protein showed a clear reticulate
Table 2. Sterol composition of wild type and dwarf5-2 (d5), dim (d1) and ste1-1 (s1) Arabidopsis mutant plants.

<table>
<thead>
<tr>
<th>sterol (% of total)</th>
<th>wild type</th>
<th>d5</th>
<th>d5::DWARF5-YFP part. compl.</th>
<th>d5::DWARF5-YFP fully compl.</th>
<th>d1</th>
<th>d1::DIM-YFP part. compl.</th>
<th>d1::DIM-YFP fully compl.</th>
<th>s1</th>
<th>s1::STE1-YFP part. compl.</th>
<th>s1::STE1-YFP fully compl.</th>
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<tbody>
<tr>
<td>Δ8-sitosterol (8)</td>
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<td>8.2</td>
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<td>Δ7-cholesterol (3)</td>
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<td>2.5</td>
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<td>Δ7-campesterol (5)</td>
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<td>Δ7-avenasterol (15)</td>
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<td>Δ7-sitosterol (12)</td>
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<td>1.5</td>
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<tr>
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<tr>
<td>cholesterol (1)</td>
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<td>tr</td>
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<td>campesterol (4)</td>
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<td>12.9</td>
<td>2.0</td>
<td>11.7</td>
<td>16.3</td>
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<td>3.8</td>
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<tr>
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<td>18.3</td>
<td>49.9</td>
<td>76.8</td>
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<td>63.0</td>
<td>77.0</td>
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<td></td>
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<td>1.0</td>
<td>0.5</td>
<td>2.5</td>
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</table>

The values refer to the partially and fully complemented plants compared to the not complemented. The numbers in parentheses refers to Figure 1 and Figure 2. tr = trace amount; - = compound not detected. Accurate sterol nomenclature can be found at IUPAC http://www.iupac.org.

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structure in confocal observation corresponding to a typical ER localization (Figure 4A). This ER localization is consistent with observations reported in the case of the orthologous enzyme 7-dehydrocholesterol reductase (7-DHCR) in mammalian cells [46]. Moreover we observed a clear signal at the periphery of the epidermal cell (Figure 4). An ER-like pattern was seen for the DIM-YFP fusion protein (Figure 5A; Supplemental Figure 3A), which also showed a clear signal in the peripheral area (Figure 5A, 5D). In both cases the peripheral signal suggests that these proteins are synthesized in the endomembrane system for delivery to the PM [47].

To further investigate the association of these two proteins to the PM, we stained the leaves of transgenic dwarf5-2 and dim plants expressing the DWARF5-YFP (Figure 4E) and DIM-YFP (Figure 5B and 5C), respectively, with the membrane dye FM4-64. Interestingly, the human 24-DHCR (SELADIN1), as well as the yeast ERG4p, two orthologs of the plant DIM, have been both localized mainly to the ER [39,48] but also to a lesser extent to Golgi complexes [48], indicating possible dual localizations for enzymes of this type. Previously, Klahre et al. [13] found a N-terminal GFP-DIM fusion protein localized in speckled structures in the cytoplasm of pollen tubes; this was interpreted as ER. Localization to the Golgi may indicate a possible translocation of this protein to the PM in secretory vesicles. This vesicular transport is known and has been well described in the case of yeast [45]. Moreover, the DIM localization in the PM can be interpreted as necessary for the fine tuning of the membrane properties by regulating the content of sitosterol (i.e. the ratio of isofucosterol to sitosterol) and campesterol and consequently the response to environmental changes [49]. In addition, DIM was already identified as a component of detergent resistant membranes (DRM) or so-called lipid rafts isolated from tobacco PM [4].

In addition to DWARF5-YFP and DIM-YFP, the STE1-YFP expressed in transformed ste1-1 mutant plants also displayed an ER-like pattern (Figure 6A; Supplemental Figure 3D). Surprisingly, for the STE1-YFP fusion we further observed an unexpected localization in small and round structures moving across the cytoplasm (Supplemental Movie 1). As the observed structures resembled LPs (Figure 6A, 6D), the STE1-YFP fusion localization was further investigated by incubating leaves from ste1-1 mutant plants with Nile Red, a lipid specific dye staining cellular LPs. Results from Nile Red staining showed a pattern characterized by clear signals deriving from round bodies dispersed into the cytoplasm (Figure 6B, 6C, 6D, 6G, 6H). The localization of STE1-YFP was observed to overlap with Nile Red signals, indicating that STE1-YFP is localized to LPs as well as to ER (Figure 6D).

LPs consist of a phospholipid monolayer which sequesters the hydrophobic core of LPs from the cytosol. This has been well described in yeast. The core of the particles consists of triacylglycerols (TAG) surrounded by several layers of SE [50,51]. The LPs represent a storage compartment for fatty acids and sterols in the form of conjugates, and appear to be involved in their translocation from ER to all membranes of the cells. The mechanism of sterol translocation between ER and LPs is not completely clear [52], although it has been recently demonstrated that the lipid droplets could originate from the ER [53] and are probably involved into a non-vesicular sterol transport to the PM [45].

Figure 4. Subcellular localization of DWARF5-YFP protein in Arabidopsis dwarf5-2::DWARF5-YFP plants. (A) Confocal images of leaves showing protein distribution in the ER and (D) to the periphery of the cell (yellow arrow). (B) Chlorophyll autofluorescence. (E) In red is shown the chlorophyll autofluorescence combined with the FM4-64 fluorescence localized to the PM. The overlay images show (C) the complete separation of red and yellow signal and (F) the co-localization of DWARF5-YFP and FM4-64 indicating the PM association of DWARF5. Scale bars = 25 µm.

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Studies performed on a tobacco mutant overproducing sterols showed that the excess of sterols was converted into steryl esters (steryl palmitate, oleate, linoleate and linolenate) which were accumulated into LPs [24]. The storage and translocation mechanism would then be impossible without enzymes that mobilize these conjugates. In yeast, although the sterol synthesizing enzymes are mainly localized in the ER, ERG1, ERG27 and ERG6 enzymes show a dual localization on ER and LPs while ERG7 has been localized only on LPs [52]. In addition, four of the seven acyltransferases [32], one of the three lipases [33] and two of the three SE hydrolases identified until now [50] have been localized to LPs in yeast. Recently, a phospholipid-sterol acyltransferase (PSAT1) and a sterol-O-acyltransferase (ASAT1) have been identified in Arabidopsis where the enzyme activities were associated with microsomal membranes [27,28,54]. The esterification reaction and the major enzyme responsible for this process, namely, the PSAT1, was described but so far plant SE hydrolases have not been identified.

The Δ7-sterol-C5-desaturase (STE1) displays a dual localization in the ER and the LPs in Arabidopsis, revealing an unexpected subcellular localization for this enzyme, and a possible yet unknown role of the LPs in sterol biosynthesis. Interestingly, the link between sterol esterification and the activity of the Δ7-sterol-C5-desaturase has been shown in yeast where the disruption of the sterol acyltransferase genes ARE1 and ARE2 were followed by a decrease of ERG3 expression [55]. This indicates that the absence of sterol esterification leads to a decrease in total intracellular sterols. It could assign a role for ERG3 in the storage process of sterol esters. Moreover, the examination of microarray data related to the expression of STE1 in wild-type Arabidopsis (The Bio-Array Resource for Plant Biology http://www.bar.utoronto.ca/; [56]) revealed a high level of STE1 expression in seeds. This indication also could be viewed as part of the lipid accumulation process in seeds. We further analyzed ste1-1::STE1-YFP plants by confocal microscopy and were able to detect strong signals along the vascular tissues from giant compartments which were then identified as LPs (Figure 7). Interestingly, it has been reported that the silencing of the major seed oleosin gene of Arabidopsis resulted in an aberrant phenotype of embryo cells that contain larger oil bodies than those found in the wild-type [57]. Changes in the size of oil bodies caused disruption of storage organelles, altering accumulation of lipids and proteins and causing delay in germination. The vascular localization could suggest a systemic lipid transport in plant, probably associated with lipoproteins.
for instance oleosins [58] and steroleosin [59]. A mechanism of that type is well known in the case of mammalian cholesterol homeostasis, implicating in particular an extracellular transport of cholesterol through the LDL/LDL receptor machinery [60].

In conclusion we have shown that C-terminal YFP-fusion proteins of DWARF5, DIM and STE1 were found to localize to the endoplasmic reticulum. Surprisingly, Δ^{7}-sterol-Δ^{24}-reductase (DIM) was further localized to the plasma membrane, whereas Δ^{24}-sterol-C_{2}\text{-desaturase (STE1)} was also clearly detected in lipid particles. We suggest that the partial and/or full complementation of all three biochemical mutants, that is, a partial and/or full restoration of a wild-type sterol profile and morphology upon expression of the YFP-fusions, indicate a relevant subcellular localization of the engineered enzymes. In fact, the identical confocal images observed whether the complementation was partial or full rule out a hypothetical mislocalization of the accumulated YFP-fusions. Therefore, the different localization patterns observed for the three different enzymes support their correct subcellular localization. Together these findings raise new challenging questions about the spatial organization of sterol biosynthesis in plants and the implication of this localization in cellular lipid homeostasis.

Figure 7. Subcellular localization of STE1-YFP protein in Arabidopsis ste1-1::STE1-YFP plants. (A) STE1-YFP fluorescence signal in leaf vascular tissue. (B) Chlorophyll autofluorescence. (C) Overlay image of (A) and (B). Scale bars = 250 μm.

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Methods

Plant lines and culture conditions

*Arabidopsis thaliana* plants from the ecotype Enkheim 2 (wild-type En-2 and mutant line *dwarf 5-2*; [10]), Columbia-*glabrous 1* (wild-type Col-0 and mutant line *ste1-1*; [12]) and Columbia-0 (wild-type Col-0/C24 and mutant line *dim*; [13]) were grown in greenhouse conditions with a light/dark regime of 16/8 hours and a temperature setting of 18 °C during the light period and 15 °C during the dark period. Four-week old seedlings were transferred to individual pots and grown to flowering stage. Plant transformations with the relevant constructs were performed using the *Agrobacterium tumefaciens* floral dip method [61]. Primary transformants were grown for two more generations for further analysis. One-month-old rosette leaves were sampled from 5 plants of a given genotype in three independent experiments and immediately frozen in liquid nitrogen, freeze dried (ScanVac® model CoolSafe Pro 100-4, ScanLab A/S, Denmark) and stored at −80 °C prior to sterol analysis. One-week old seedlings, germinated on Murashige and Skoog medium (0,4% MS, 2% sucrose, 1% agar) were used for confocal microscopy observations. A minimum of three transgenic lines for each type of genetic complementation experiment were considered in the present study.

Yeast strains and growth conditions

The wild-type yeast strain *Saccharomyces cerevisiae* W303-1A (*MAT a; ura3-52; his3-112; his3-11; ade2-1; can1-100) and the mutant strains *erg3* (ORF YLR056wc: BY4741; *MATa*; *his3A1;* *leu2Δ0; met15Δ0; ura3Δ0; YLR056wc:kanMX4) and *erg4* (ORF YGL012wc: BY4742; *MATa*; *his3A1;* *leu2Δ0; lys2Δ0; ura3Δ0; YGL012wc:kanMX4) were obtained from the European *Saccharomyces Cerevisiae* Archives for Functional Analysis (EURO-SCARF). The yeast strains were grown aerobiocally at 30 °C either in YPD containing 2% glucose (Merck), 1% Yeast Extract (Oxoid) and 2% peptone (Oxoid) or in synthetic minimal (SM) media containing 0.67% Yeast Nitrogen Base w/o amino acids and w/o ammonium sulphate (Remel®), 0.2% yeast synthetic Drop-out medium supplements (Sigma-Aldrich, Steinheim, Germany) excluding uracil (W303 and *erg3*) or histidine (*erg4*) and 2% bactoagar (Difco®). The SM media contained either 2% glucose or galactose (Sigma-Aldrich®) as carbon source. Milli-Q water was used (18 MΩ, Millipore®, Billerica, USA).

The strains bearing the YFP fused gene constructs integrated into the genome were generated by the lithium acetate method [62]. Liquid cultures in selective synthetic media containing 2% galactose were performed at 30 °C in a shaking incubator (Thermo-Forma Scientific, Lytzenlab A/S, Denmark) at 220 rpm in order to activate the YFP fused gene transcription. Liquid cultures in presence of 2% glucose were produced as control. Yeast pellets were collected by centrifugation at 4000xg (Laborzentriﬁugen, Sigma®) of 50 ml of three days-old liquid culture were frozen in liquid nitrogen, freeze dried and stored at −80 °C prior to sterol analysis.

ENZYME::YFP constructs

The cDNAs encoding DWARF5 (At1g50430), STE1 (At3g19820) and DIM (At3g02580) were obtained from the Arabidopsis Biological Research Centre (ABRC, Ohio, US). Translational fusions of *DWARF5*, *STE1* and *DIM* to the *YFP* reporter were generated by cloning the corresponding ORFs into a pLIpE001 vector, a modified version of pCAMBIA 2300 plant expression vector suitable for User cloning [63]. The pLifeE001 vector contains the YFP coding sequence at the 3′ of the MCS where its expression is under the control of the CaMV 35S
Sterol Biosynthesis Enzymes Localization

Sterol extraction and analysis

Lipid extraction. The freeze-dried plant tissues and yeast pellets (50 mg ± 2 mg) were transferred into 50 ml tubes, mixed with 15 ml 6% (w/v) KOH in methanol and homogenized using an Ultra Turrax® homogenizer. Saponification was carried out at 70 °C for 2 hours. Subsequently, 5 ml Milli-Q water and 20 ml pentane were added to each tube and vortexed twice for 30 sec. The tubes were subsequently centrifuged at 4000 g for 5 minutes (Laborzentrifugen, Sigma®) at room temperature, and the organic phases were transferred to Zellenmeyer flasks. The extraction was repeated twice with 20 ml pentane and the organic phases were pooled. The combined extracts were evaporated to dryness in a rotary evaporator at 30 °C (Rotavapor® model R-215, Buchi, Switzerland). The residues were resuspended in 1 ml pentane and transferred to a 1.5 ml glass GC vials (Mikrolab A/S, Denmark). The samples were then evaporated under a stream of nitrogen gas and stored in argon atmosphere at −80 °C prior to analysis.

Pentane and methanol were GC grade (Fluka®). Potassium hydroxide pellets (for analysis) were from Merck® (Darmstadt, Germany).

Sterols purification from plant extracts

Plant or yeast sterols were purified by TLC (60 F254 silica plates, Merck, Darmstadt, Germany), with two runs in dichloromethane. 7-dehydrocholesterol (Purity 98%) was purchased from Sigma-Aldrich and used as standard. After the TLC run, the bands in the samples lanes showing the same retention factor (Rf) as the standard, resolved by UV-B light exposure, were scraped off the plate and eluted with pentane. The eluted fractions were transferred to Erlenmeyer flasks. The extraction was repeated twice with 10 ml pentane and the organic phases were pooled. The combined extracts were evaporated to dryness in a rotary evaporator at 30 °C (Rotavapor® model R-215, Buchi, Switzerland). The residues were resuspended in 1 ml pentane and transferred to a 1.5 ml glass GC vials (Mikrolab A/S, Denmark). The samples were then evaporated under a stream of nitrogen gas and stored in argon atmosphere at −80 °C prior to analysis.

Pentane and methanol were GC grade (Fluka®). Potassium hydroxide pellets (for analysis) were from Merck® (Darmstadt, Germany).

GC-MS and GC-FID characterization

The yeast and plant sterol fractions were resuspended in 100 μl toluene and derivatized for GC assays by adding 40 μl of acetic anhydride (Sigma) and 25 μl of pyridine (Fluka). Acetylation was carried out for 1 hour at 70 °C. The samples were then evaporated to dryness and resuspended in 300 μl of hexane. Sterols were separated and identified by GC-FID (gas chromatography–flame ionization detection) using a Varian 8400 gas chromatograph equipped with a DB-5 capillary column, wall coated, open, and tubular; 30 m; 0.25 mm film thickness, 32 mm i.d.; Agilent J&W, USA) using H2 as carrier gas (2 ml/min). The GC oven program included a fast increase from 60 °C to 220 °C (30 °C/min) and a slow increase from 220 °C to 300 °C (2 °C/min). Data from the detector were monitored with the VARIAN STAR computer program (Varian, Walnut Creek, CA, USA). Sterol structures were confirmed by GC-MS (Agilent 6890 gas chromatograph and 5973 mass analyzer) equipped with a HP-5MS glass capillary column (wall coated, open, and tubular; 30 m; 0.25 mm film thickness, 32 mm i.d.; Agilent J&W, USA) using hydrogen as carrier gas.

Supporting Information

Figure S1 Western blot analysis of wild type Arabidopsis and dwarf5-2, ste1-1 and dim mutant plants expressing the corresponding YFP-fused proteins. The figure shows the accumulation of DWARF5-YFP (~79 KDa) and DIM-YFP (~92 KDa). STE1-YFP was not detected probably due to the relative low abundance in the analysed tissues. Protein extracts were prepared by homogenization of frozen tissues in sample buffer. Equal amounts of total proteins were loaded on a 12% acrylamide gel and analysed by western blot using anti-GFP antibodies. A protein extract form plants carrying a 35S::eYFP construct was used as control. (TIF)

Figure S2 Sterol profile and composition of an Arabidopsis wild type plant. Sterol peaks identified by their retention time and confirmed by GC-MS (prominent mass fragments not shown here) are: 1, cholesterol; 3, Δ7-cholesterol; 4, campesterol; 10, sitosterol; 11, isofucosterol; 14, stigmasterol; 16, brassicasterol. In bold the more abundant sterol. (TIF)

Figure S3 Subcellular localization of DIM-YFP and STE1-YFP proteins in Arabidopsis. Confocal images of leaves showing localization of (A) DIM-YFP and (D) STE1-YFP in...
the cell. (B, E) Chlorophyll autofluorescence. (C, F) Overlay images of YFP and autofluorescence channels. Scale bars = 25 μm. (THF)

Table S1 In silico prediction of subcellular localization (Predotar) and signal peptide presence (SignalP) for DWARF5, STE1 and DIM based on their amino acid sequences. (DOC)

Table S2 Primers used to assemble the YFP fused constructs. (DOC)

Table S3 Primers used for cloning of the YFP-fused constructs into inducible yeast expression vectors. The YFP reverse primer is common for all the constructs being generated on the YFP 3’ sequence. (DOC)

Movie S1 Subcellular localization of STE1-YFP in Arabidopsis ste1-1::STE1-YFP plants. The movie is showing the subcellular trafficking of LPs observed while collecting the YFP signal. (MOV)

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Author Contributions
Obtained the vectors containing the coding sequences of the investigated enzymes: DS PEJ. Created the constructs and vectors for both yeasts and plant transformations: DS. Generated the transgenic yeast lines: DS HS. Generated the transgenic Arabidopsis lines: DS. Analyzed the yeast transformed lines: DS HS. Analyzed the transgenic Arabidopsis lines: DS HS. Analyzed the subcellular localization by confocal microscopy: DS TGA. Manuscript writing: DS PEJ HS TGA. Conceived and designed the experiments: DS HS. Performed the experiments: DS TGA HS. Analyzed the data: DS TGA HS. Contributed reagents/materials/analysis tools: PEJ HS. Wrote the paper: DS PEJ.

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