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Review

The biogenesis and physiological function of chloroplast superoxide dismutases ☆

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ABSTRACT

Iron-superoxide dismutase (FeSOD) and copper/zinc-superoxide dismutase (Cu/ZnSOD) are evolutionarily conserved proteins in higher plant chloroplasts. These enzymes are responsible for the efficient removal of the superoxide formed during photosynthetic electron transport and function in reactive oxygen species metabolism. The availability of copper is a major determinant of Cu/ZnSOD and FeSOD expression. Analysis of the phenotypes of plants that over-express superoxide dismutases in chloroplasts has given support for the proposed roles of these enzymes in reactive oxygen species scavenging. However, over-production of chloroplast superoxide dismutase gives only limited protection to environmental stress and does not result in greatly improved whole plant performance. Surprisingly, plant lines that lack the most abundant Cu/ZnSOD or FeSOD activities perform as well as the wild-type under most conditions tested, indicating that these superoxide dismutases are not limiting to photoprotection or the prevention of oxidative damage. In contrast, a strong defect in chloroplast gene expression and development was seen in plants that lack the two minor FeSOD isoforms, which are expressed predominantly in seedlings and that associate closely with the chloroplast genome. These findings implicate reactive oxygen species metabolism in signaling and emphasize the critical role of sub-cellular superoxide dismutase location. This article is part of a Special Issue entitled: Regulation of Electron Transport in Chloroplasts.

1. Introduction

The reactive oxygen species superoxide (O_2^-) is formed by reduction of dioxygen by a single electron [1]. Several processes can lead to superoxide formation but respiratory and photosynthetic electron transport chains are major sources of this anionic oxygen radical; for reviews see [1] and [2]. The enzyme superoxide dismutase (SOD) catalyzes the fast dismutation of superoxide to hydrogen peroxide [3] and is thought to be ubiquitous in organisms that live in the presence of oxygen. For reviews see [4,5]. The overall dismutation reaction can be written as follows:

$$2O_2^- + 2H^+ \longleftrightarrow H_2O_2 \text{ and } O_2. \quad (1)$$

The spontaneous forward reaction is readily observed *in vitro* at high rates [6] but at lower substrate concentrations the second order kinetics cause this reaction to become much slower. In living cells however, superoxide dismutase makes the reaction effectively diffusion limited because these enzymes have extremely high turnover numbers [4]. The complete enzymatic reaction cycle involves the binding of two superoxide ions and the temporary storage of electrons and therefore requires two redox-active metal ions as cofactors in the active center for catalysis [4,5]. Indeed, superoxide dismutases function as dimers with a catalytic metal ion in each monomer [4]. We first discuss the SOD isoforms that occur in plants and then focus on the biogenesis, regulation of expression and function of SODs that play a role related to photosynthesis.

2. Isoforms of superoxide dismutase

2.1. Occurrence and cellular locations of superoxide dismutases

The four types of superoxide dismutase that are described in the literature are FeSOD (iron cofactor), MnSOD (manganese cofactor), Cu/ZnSOD (copper and zinc as cofactors where copper is the redox active catalytic metal) and NiSOD (nickel cofactor) [4,5]. NiSOD is not found in plants [7–9] and therefore it is not further discussed here.

MnSOD is found in several prokaryotes including cyanobacteria [5,7]. In eukaryotes, including plants, MnSOD is found in the matrix of the mitochondria [5,7]. In the eukaryotic alga *Chlamydomonas rheinhardtii* MnSOD is also reported to be in the chloroplast [10] but it is not found in the plastids of higher plants. This suggests that in plants the MnSOD of cyanobacterial origin was lost during evolution [7,8]. FeSOD is found in most prokaryotes and is especially abundant in cyanobacteria [7]. *C. rheinhardtii* also contains FeSOD in its chloroplast [10]. In plants, FeSOD is most frequently reported to be active in the plastids [8,9]. However recent reports have suggested a

Abbreviations: SOD, superoxide dismutase; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species

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cytosolic location for some FeSOD isoforms [11,12]. Cu/ZnSOD occurs in some bacteria and in the cytosol of eukaryotes including yeast, mammals and plants. In plants, Cu/ZnSOD also occurs in the peroxisomes [9] and plastids [9,13]. Cu/ZnSOD is not found in more primitive photosynthetic eukaryotes such as *C. rheinhardtii* [14] and the moss *Barbula unguiculata* [15].

2.2. Structure and evolution of superoxide dismutases

Protein primary structures and phylogenetic relationships of superoxide dismutases have been extensively studied [7,8,16]. Fig. 1 gives the schematic domain structures for the three FeSODs and the two most abundant Cu/ZnSOD enzymes that occur in *Arabidopsis thaliana*. FeSOD is likely the most ancient superoxide dismutase [17]. MnSOD is structurally related to FeSOD from which it probably evolved [8]. Detailed 3-dimensional structures are available which reveal that FeSOD and MnSOD enzymes have a very similar fold and use conserved residues for metal binding [4,5]. In contrast, Cu/ZnSOD, which was the first SOD to be described [3] is not related in structure to the other SOD isoforms and can be viewed as an evolutionary novelty [8]. Cu/ZnSOD probably evolved in response to the rise in oxygen in the biosphere; an event that would have caused iron to become much less bio-available and that seems to have driven the use of Cu as a cofactor in biology [18]. Phylogenetic analyses of plant sequences show that all chloroplast Cu/ZnSODs are more related to each other than to cytosolic Cu/ZnSODs, which also form their own sub-group [16]. This suggests that selective pressures, that caused divergence of these two enzymes, acted early in the evolution of higher plants.

2.3. Superoxide dismutases in the model plant Arabidopsis

The genome of the model plant *Arabidopsis* encodes for seven SODs [9]. The proposed subcellular locations and the developmental expression patterns of SOD isoforms in *Arabidopsis* are indicated in Fig. 2. The manganese SOD (MSD1) had been predicted to be mitochondrial [9] and was indeed found to be active there [19].

There are three FeSOD isoforms in *Arabidopsis* called FSD1, FSD2 and FSD3. In rosette leaves of mature plants grown on soil, *FSD1* is the most abundantly expressed FeSOD [9]. Notably, *FSD1* mRNA expression is strongly affected by Cu levels and the gene is especially expressed when plants are grown on media with low Cu content [20]. FeSOD activity is only detected in chloroplasts that were isolated from *Arabidopsis* plants grown on low Cu, which suggests that isolated chloroplasts contain FSD1 [20]. An antibody raised against FSD1 detected FeSOD in the chloroplast fraction [9]. In addition, a proteomic analysis by Zybailov et al. detected FSD1 in the chloroplast stroma [21]. Together, these observations strongly suggest that FSD1 protein is active in plastids. The location of FeSOD in plastids of higher plants would also agree with the cyanobacterial origin of higher plant

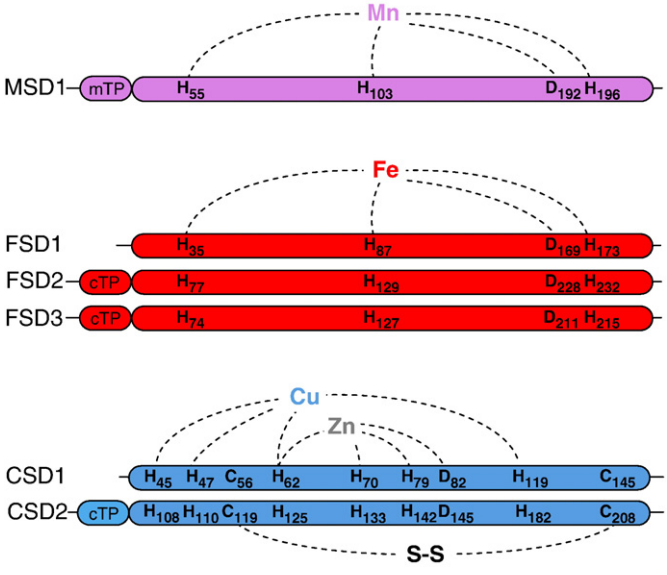

Fig. 1. Schematic primary structures of *Arabidopsis* superoxide dismutases. Canonical mitochondrial (mTP) or chloroplast transit peptides (cTP) are indicated. The position of the conserved histidine (H) and aspartic acid (D) residues involved in metal-binding as well as the cysteine (C) residues involved in intra-molecular disulfide bridging (S–S) is presented.

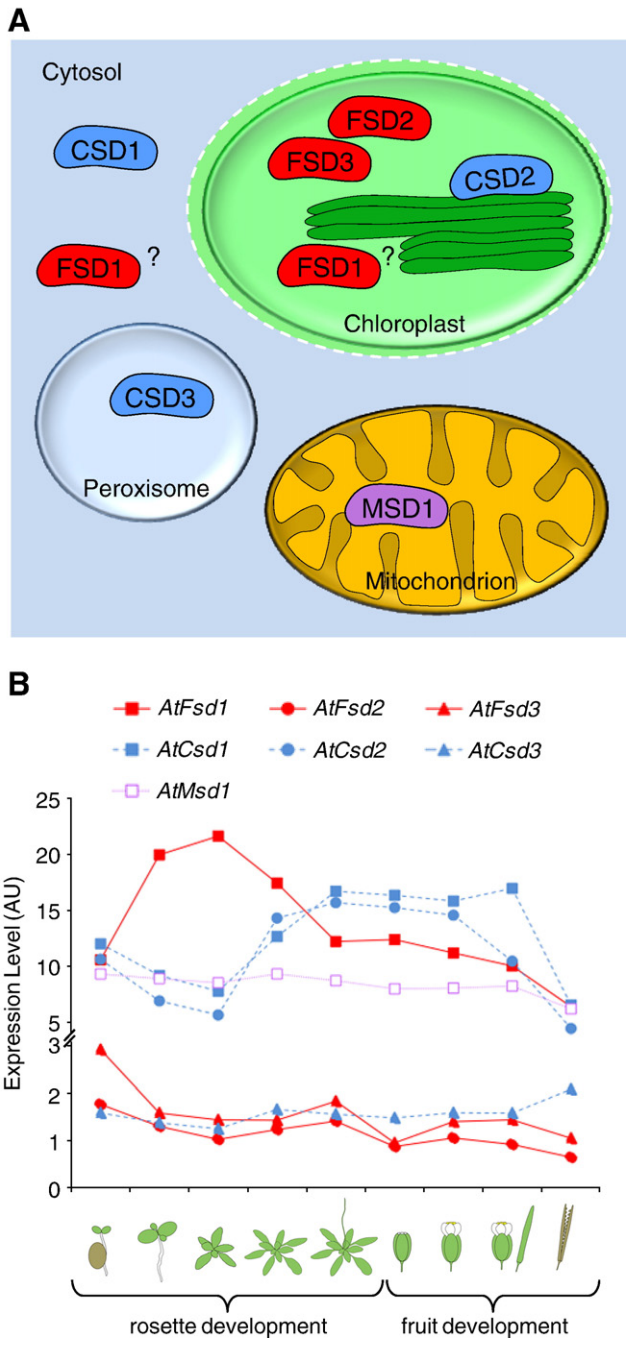

Fig. 2. Location of SODs in plants and their expression during plant development. Panel A: Subcellular location of SODs in *Arabidopsis*. Panel B: Expression of SOD genes during *Arabidopsis thaliana* development. mRNA expression levels are presented in arbitrary units (AU) during different stages of rosette and fruit development. The data set used to create the *in silico* transcriptional profiling was obtained from Genevestigator (https://www.genevestigator.com; [73]). The three Cu/ZnSOD isoforms are encoded by *CSD1–3*. The three FeSOD isoforms are encoded by *FSD1–3*. *MSD1* encodes for the MnSOD.

chloroplasts [7,8]. However, FSD1 lacks a canonical transit sequence and fusions of FSD1 to the Green Fluorescent Protein (GFP) were localized to the cytosol [11,12]. Furthermore, FSD1 that was produced by *in vitro* translation was also not imported into isolated pea chloroplasts [12]. The cellular location of FSD1, the most abundant FeSOD in *Arabidopsis*, is therefore still not fully resolved.

FSD2 and FSD3 on the other hand are synthesized with canonical chloroplast transit sequences and their plastid location has been confirmed using GFP fusions [11]. The observed lack of complementation of *fsd2/3* mutants by the abundantly expressed *FSD1* speaks for a sub-cellular location of FSD1 protein that differs from FSD2 and FSD3 [11].

The three isoforms of Cu/ZnSOD in *Arabidopsis* have different sub-cellular locations. In vegetative tissues the two abundant Cu/ZnSODs are CSD1 and CSD2. CSD1 is predicted to be cytosolic and is absent from plastids [9]. CSD2 has a chloroplast transit sequence and its location in chloroplasts has been confirmed experimentally [9,20,22]. CSD3 has a C-terminal Ala-Lys-Leu motif that is most likely a peroxisomal targeting sequence. The *CSD3* gene is abundantly expressed in flowers, which are enriched in peroxisomes [23].

3. The biogenesis and maturation of superoxide dismutase

3.1. Chloroplast uptake of SOD isoforms

All plant superoxide dismutases are encoded in the nuclear genome. The transcripts are therefore translated on 80S ribosomes and to reach the chloroplast the proteins must be imported. As mentioned, the location of FSD1 is not yet fully resolved but it is clear that it does not have a typical chloroplast transit sequence. CSD2, FSD2, and FSD3 however do have predicted cleavable chloroplast transit sequences that would allow the transport of these proteins to the stroma via the TOC/TIC protein import machinery in the envelope, followed by cleavage of the transit sequence by the stromal transit sequence peptidase. It is believed that the chloroplast import machinery has a strong unfolding capacity and precursors would be imported in an unfolded state [24]. Therefore, folding and cofactor assembly to activate the proteins must happen within the stroma.

3.2. Maturation

How FeSOD acquires its Fe cofactor is not yet clear. However, the maturation of Cu/ZnSOD is extensively studied; for a review see [38]. The PAA1 Cu transporter functions in the delivery of the Cu cofactor to the chloroplast stroma and is required for CSD2 activity [20,25]. In the stroma Cu is inserted into CSD2 with the aid of a metallo-chaperone. In yeast a metallo-chaperone called CCS (for the Chaperone for Cu/Zn Superoxide dismutase) was found to be required for the activation of Cu/ZnSOD in the cytoplasm [26]. CCS seems to have at least two functions: it delivers the Cu cofactor and it assists in the formation of an intra-molecular disulfide bridge required for the full activation of Cu/ZnSOD [26]. CCS has three functional domains. The protein has an amino-terminal ATX-like metallo-chaperone domain containing a Cys-X-X-Cys motif that likely is a Cu binding site. The second domain is a central region with similarity to its target SOD that is thought to mediate interaction with the target apo-protein. Finally, a C-terminal domain containing possible metal binding Cys residues is present [26]. All three regions seem to be required for full CCS function [26].

Homologues of yeast CCS are found in plants [27–30]. The *Arabidopsis CCS* can functionally complement a yeast loss of function mutant, which indicates that the protein function is conserved [93]. In *Arabidopsis* there are three Cu/ZnSOD enzymes at three different locations in the cell but only one gene for *CCS* [30]. The one gene copy of *CCS* is however required for the efficient maturation of all three Cu/ZnSOD isoforms [23]. The *Arabidopsis CCS* gene encodes a possible precursor with a predicted and functional N-terminal chloroplast targeting sequence [23,93]. In *Arabidopsis*, *CCS* transcripts of different lengths can be detected; a longer transcript allows translation initiation at an AUG start codon that produces a chloroplast-targeted precursor. A slightly shorter transcript version lacks this first initiation codon and as a consequence translation would start at a downstream AUG initiation site. This produces a mature enzyme lacking the transit sequence which therefore remains cytosolic [22]. Expression of CCS without the transit sequence in a *CCS* T-DNA insertion line complements only the maturation of CSD1 (cytosol) and CSD3 (peroxisome) [23]. Expression of a full-length *CCS* transcript allowed for the maturation of all Cu/ZnSOD isoforms [23]. It is likely that cofactor assembly and maturation of CSD3 occurs in the cytosol because unlike chloroplasts, peroxisomes can take up fully folded enzymes. In addition, CSD3 has a canonical C-terminal peroxisomal targeting sequence whereas this is not evident for plant CCS.

4. Regulation of SOD expression

4.1. Factors that affect SOD expression

The expression pattern of superoxide dismutases and an examination of the factors that affect the expression of these enzymes can give clues about SOD function and are therefore worth examining. We will first consider the developmental regulation and then look at the effects of various environmental factors such as abiotic stress and nutrition.

4.1.1. The patterns of SOD isoform expression during development

Publicly available mRNA expression data allow a comparison of the expression patterns of all superoxide dismutases in *Arabidopsis* grown on soil over the entire life cycle from seed to seed (see Fig. 2B). The data show that *FSD1*, *CSD1* and *CSD2* encode the major SOD isoforms, which agrees with the report by Kliebenstein et al. [9]. *FSD1* is highly expressed in younger plants; then at the onset of flowering the expression of *FSD1* is reduced (Fig. 2B). The expression of cytosolic *CSD1* and chloroplast *CSD2* seem to be co-regulated and are initially expressed at somewhat lower levels but their expression is increased after the onset of flowering (Fig. 2B). At that point, vegetative development would have peaked. Thus, expression of *FSD1* on one hand and *CSD1*/*CSD2* on the other hand seems reciprocal. Among the FeSODs with a confirmed chloroplast location, *FSD2* and *FSD3* have a low basal expression level. However, *FSD2* and *FSD3* expression is slightly elevated in young developing seedlings (Fig. 2B). Similar expression data were reported by Myouga et al. [11]. It is clear that a significant expression level of chloroplast SOD activity is maintained at all stages, which suggests an important role for these enzymes.

The mitochondrial *MSD1* is constitutively expressed at a stable level (Fig. 2B). This gene was also not affected by treatments of plants reported to affect the expression of Cu/ZnSOD or FeSOD isoforms [9]. Finally, *CSD3* is expressed at much lower levels and only increases during flowering (Fig. 2B), consistent with the observation that the peroxisomal CSD3 is the dominant SOD activity in flowers [23].

4.1.2. Effects of various stress treatments on expression of major SOD isoforms

The effects of various treatments on Cu/ZnSOD and FeSOD expression in plants reported in the literature are summarized in Table 1. SODs are regulated on the transcriptional and translational level in multiple ways [9,20,36,74–92]. Most treatments affect the expression of cytosolic and chloroplastic Cu/ZnSOD in the same way. The exceptions are exposure to ozone and UV-B which up-regulate cytosolic Cu/ZnSOD and down-regulate chloroplast Cu/ZnSOD (Table 1). Most stress treatments lead to simultaneous up-regulation of both Cu/ZnSODs (Table 1). Drought, osmotic stress, and abscisic acid (ABA) all induce stromal closure, which causes a lack of CO_2 to feed into the Calvin cycle. This could result in over-reduction of the stroma in the light, which in turn may cause increased superoxide production. High light and the herbicide paraquat (methyl viologen, MV) also promote increased superoxide formation in the chloroplast; see also

[31]. The reported up-regulation of Cu/ZnSOD under these conditions in most plants (Table 1) makes sense in the context of a superoxide scavenging role. However, it was found in *Arabidopsis* that the effects of high light and MV on the expression of *CSD1* and *CSD2* were small in comparison with the much more evident up-regulation of ferritin and the ascorbate peroxidase *APX1* [32]. The effects of abiotic stresses on FeSOD expression are not always as clear. For instance, in *Nicotiana plumbaginifolia* treatment with MV was reported to cause reduced FeSOD expression in one case and up-regulation in another case (see Table 1). In a recent study, Myouga et al. [11] reported that in *Arabidopsis* the transcripts for the minor FeSOD isoforms *FSD2* and *FSD3* are up-regulated by MV treatment.

4.1.3. The effect of copper and sucrose on SOD expression

The availability of copper is by far the most important factor that controls *CSD1*, *CSD2* and *FSD1* expression. Copper is absolutely required for expression of *CSD1*, *CSD2* [20] and for the Cu chaperone *CCS* [22], and at elevated levels it suppresses *FSD1* expression almost completely [20]. We consider it possible that the increased expression of Cu/ZnSOD and decreased expression of *FSD1* after the onset of flowering in *Arabidopsis* (Fig. 2B) are also attributable to Cu. At that point vegetative development and thus consumption of Cu by cytochrome-c oxidase and plastocyanin in developing mitochondria and chloroplasts has gone past its peak, allowing more Cu to become available for Cu/ZnSOD. The mechanism for the regulation by Cu is now well understood and involves the conserved transcription factor SPL7 (see Fig. 3). SPL7 is a Cu-responsive transcription factor in plants [33] that is homologous to *Chlamydomonas* Crr1 [34]. In *Chlamydomonas*, Crr1 regulates the expression of genes such as cytochrome-c_6 which is up-regulated in response to low Cu availability [34]. The Crr1 targets have a cis-acting Cu-responsive element (CuRE) that contains the DNA sequence motif GTAC [34]. SPL7 has a number of targets in *Arabidopsis* that all display the GTAC motif and one of these is *FSD1* [33]. *FeSOD* is also a target of an SPL7 homologue in the moss *B. unguiculata* [15]. Several microRNAs, which are collectively called the Cu microRNAs, because they all negatively regulate transcripts that encode for Cu enzymes [38], are up-regulated via SPL7 on low Cu [32,33,35]. Among the Cu microRNAs is *miR398*, which targets the mRNAs of *CSD1*, *CSD2* and *CCS* [22,36,37]. Thus if Cu is limiting, SPL7 mediates up-regulation of *FSD1* and down-regulation of *CSD1* and *CSD2* (see Fig. 3). Additional targets of SPL7 include Cu transporters and putative Cu chaperones that are thought to be involved in the assimilation of Cu [33]. An *spl7* loss-of-function mutant is sensitive to low Cu [33]. Because SPL7 has so many targets it is not yet clear which critical function is lost to explain this phenotype, but we anticipate that the lack of Cu assimilation capacity is a major problem in *spl7* mutants.

The presence of sucrose was reported to affect *miR398* and thus *CSD1* and *CSD2* expression independently of Cu [37]. Dugas and Bartel [37] report that the addition of sucrose to plant agar culture media promotes *miR398* expression and therefore causes down-regulation of *CSD1* and *CSD2* in *Arabidopsis*. Interestingly, FeSOD protein levels were hardly affected [37] suggesting that sucrose does not assert its effect on *miR398* via SPL7. Sunkar et al. [36] and Dugas and Bartel [37] also investigated the effect of altering the miR398 recognition sites in *CSD1* and *CSD2* mRNA. These mutated mRNAs did accumulate as expected, even in conditions when *miR398* expression was high. However, the protein levels of CSD1 and CSD2 were still affected by low Cu. This

Table 1
Differential regulation of SODs in response to various treatments.

Treatment	SOD	Regulation	Organism	Effect on	Reference
Transition metals					
+Cu	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*	mRNA	[20,36]
	$Cu/ZnSOD_{Pl}$	Up	*N. tabacum*, *A. thaliana*	mRNA, activity	[20,36,74]
	FeSOD	Down	*N. tabacum*, *A. thaliana*, *M. struthiopteris*	mRNA, activity	[20,74,75]
+Fe	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*	mRNA	[36]
	$Cu/ZnSOD_{Pl}$	Up	*A. thaliana*	mRNA	[36]
	FeSOD		*N. plumbaginifolia*	mRNA	[76]
+As(V)	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*	mRNA	[77]
	$Cu/ZnSOD_{Pl}$	Up	*A. thaliana*	mRNA	[77]
	FeSOD	Down	*A. thaliana*	mRNA	[77]
+Zn	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*	mRNA	[78]
	$Cu/ZnSOD_{Pl}$		*A. thaliana*	mRNA	[78]
	FeSOD	*			
Radiation					
Light	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*	mRNA	[36]
	$Cu/ZnSOD_{Pl}$	Up	*A. thaliana*, *N. plumbaginifolia*	mRNA	[36,74]
	FeSOD	Up	*N. tabacum*, *A. thaliana*, *O. sativa*, *M. paleac*	mRNA, protein	[9,74,79,80,81]
UV-B	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*	mRNA	[9]
	$Cu/ZnSOD_{Pl}$	Down	*A. thaliana*, *N. plumbaginifolia*	mRNA, protein	[9,82]
	FeSOD	Up	*A. thaliana*	mRNA, protein	[9]
Circadian	$Cu/ZnSOD_{Cyt}$	?			
	$Cu/ZnSOD_{Pl}$	?			
	FeSOD		*A. thaliana*	mRNA	[9]
Pro-oxidant					
Paraquat	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*, *L. esculentum*	mRNA	[36,83]
	$Cu/ZnSOD_{Pl}$	Up	*A. thaliana*, *L. esculentum*	mRNA	[36,83]
		Down	*N. tabacum*	mRNA	[84]
	FeSOD	Up	*N. plumbaginifolia*	mRNA	[79]
		Down	*N. tabacum*	mRNA	[84]
Norflurazon	$Cu/ZnSOD_{Cyt}$	?			
	$Cu/ZnSOD_{Pl}$	Up	*N. tabacum*	mRNA	[84]
	FeSOD	Up	*N. tabacum*	mRNA	[84]
Ozone	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*	mRNA	[9,85]
	$Cu/ZnSOD_{Pl}$	Down	*A. thaliana*	mRNA	[9,85]
	FeSOD	Down	*A. thaliana*	mRNA	[9]
Temperature					
Cold	$Cu/ZnSOD_{Cyt}$		*N. plumbaginifolia*	mRNA	[79]
	$Cu/ZnSOD_{Pl}$	Up	*T. aestivum*	mRNA	[86]
	FeSOD	Up	*N. plumbaginifolia*, *A. thaliana*	mRNA, protein	[79,87]
Heat	$Cu/ZnSOD_{Cyt}$	Up	*N. plumbaginifolia*	mRNA	[79]
	$Cu/ZnSOD_{Pl}$				
	FeSOD				
Drought/osmotic					
Drought	$Cu/ZnSOD_{Cyt}$	Up	*L. esculentum*	Activity	[83]
	$Cu/ZnSOD_{Pl}$	Up	*L. esculentum*	Activity	[83]
	FeSOD	?			
Rehydration	$Cu/ZnSOD_{Cyt}$	?			
	$Cu/ZnSOD_{Pl}$	Up	*T. aestivum*	mRNA	[86]
	FeSOD	?			
PEG	$Cu/ZnSOD_{Cyt}$				
	$Cu/ZnSOD_{Pl}$				
	FeSOD	Up	*S. salsa L.*	Activity	[88]
NaCl	$Cu/ZnSOD_{Cyt}$	Up	*A. thaliana*, *S. italica* (cv. Prasad)	mRNA	[85,89,90]
	$Cu/ZnSOD_{Pl}$	Up	*A. thaliana*, *S. italica* (cv. Prasad)	mRNA, activity	[85,89,90]
	FeSOD	Up	*S. salsa L.*	Activity	[88]
		Down	*S. salsa L.*	Activity	[88]
Phytohormones					
ABA	$Cu/ZnSOD_{Cyt}$	Up	*Z. mays*, *A. thaliana*	mRNA	[89,91]
		Down	*P. tremula*	mRNA	[89]
	$Cu/ZnSOD_{Pl}$	Down	*N. tabacum*	mRNA	[84]
		Up	*A. thaliana*	mRNA	[89]
	FeSOD	?			
Auxin	$Cu/ZnSOD_{Cyt}$	?			
	$Cu/ZnSOD_{Pl}$	Down	*N. tabacum*	mRNA	[84]
	FeSOD	Down	*N. plumbaginifolia*	mRNA	[92]
Cytokinin	$Cu/ZnSOD_{Cyt}$	?			
	$Cu/ZnSOD_{Pl}$	Down	*N. tabacum*	mRNA	[84]
	FeSOD	Up	*N. tabacum*	mRNA	[84]
		Down	*N. plumbaginifolia*	mRNA	[92]
Ethylene	$Cu/ZnSOD_{Cyt}$	?			
	$Cu/ZnSOD_{Pl}$	Down	*N. tabacum*	mRNA	[84]
	FeSOD	Up	*N. tabacum*, *L. esculentum*	mRNA	[83,84]
Gibberelins	$Cu/ZnSOD_{Cyt}$	?			
	$Cu/ZnSOD_{Pl}$	Up	*N. tabacum*	mRNA	[84]
	FeSOD	Up	*N. tabacum*	mRNA	[84]

Notes to Table 1:
Shown are significant effects of various treatments on the mRNA expression, protein abundance or enzymatic activity of the cytosolic (Cyt) and plastidial (Pl) Cu/ZnSOD as well as FeSOD in multiple plant species. The different box colors indicate up-regulation (red), down-regulation (green) or no effect on SOD expression (white). A question mark indicates that the effects of a treatment on a specific SOD were not investigated.
*The FeSOD (FSD1) mRNA increases and decreases with the rhythm of the circadian clock.

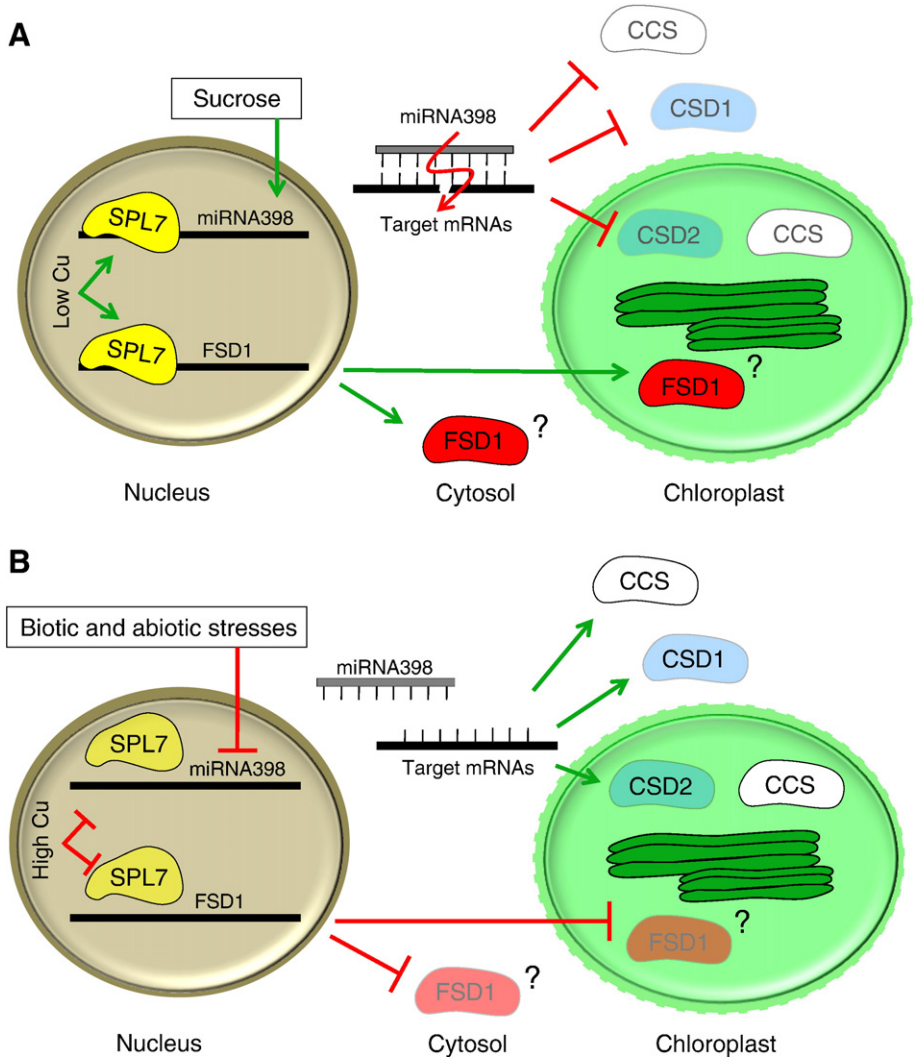

Fig. 3. SPL7-mediated regulation of SOD expression. A. In low-Cu conditions and in response to sucrose, SPL7 initiates transcription of *miRNA398* which targets and down-regulates *CCS*, *CSD1* and *CSD2*. In the same conditions, *FSD1* is up-regulated by SPL7. B. At high Cu concentrations and other biotic or abiotic stresses, FSD1 expression is repressed and CCS, CSD1 and CSD2 abundance increases. Abbreviations: CCS, copper chaperone for superoxide dismutase; CSD1, cytosolic Cu/Zn SOD; CSD2, chloroplastic Cu/Zn SOD; FSD1, FeSOD; SPL7, SQUAMOSA promoter binding protein-like7.

observation led to the suggestion that *miRNA398* not only affects transcript stability but that it can also down-regulate *CSD1* and *CSD2* expression by inhibiting translation [37]. However, an alternative explanation could be that CSD1 and CSD2 protein accumulation requires the stabilizing Cu cofactor, which is delivered by the CCS protein that is also controlled by Cu levels via *miR398*.

4.2. Implications of regulation of Cu/ZnSOD and FeSOD

It was proposed that a major function of the Cu microRNA-mediated regulation is to economize the use of Cu so that critical Cu proteins such as plastocyanin and cytochrome-c oxidase can remain active during impending deficiency [38]. Indeed, Cu/ZnSOD is abundant in plant chloroplasts [13] and there is evidence that PC and CSD1 compete for the same Cu pool in *Arabidopsis* [39]. In the context of Cu nutrition we can interpret the up-regulation of *FSD1* expression as a sign of stress or impending stress. Ultimately, a lack of Cu could lead to a defect in plastocyanin function and thus impaired photosynthesis. Why sucrose would cause down-regulation of *CSD1* and *CSD2* is harder to rationalize.

Because Cu seems to have such a large effect on the expression of SODs, its concentration in media and availability to plants needs to be very tightly controlled in experiments that investigate other factors. This can be challenging, especially on soil. Therefore it cannot be excluded that some of the observations on SOD expression (see Table 1) could be explained by differences in soil composition or fertilizer treatments that affected Cu levels or Cu availability. This idea would certainly explain some of the seemingly contradicting reports.

Another possible problem with the expression analysis approach is that sometimes the treatments have been extremely harsh on the plants, which may have caused unspecific secondary effects. It would be of interest to investigate if the various treatments that affect SOD mRNA levels exert their effect via the same or via different pathways. In this respect it is interesting that some of the effects of treatments such as high light and MV on Cu/ZnSOD expression have been ascribed to a reduction of *miR398* expression [36].

It is remarkable that FeSOD and Cu/ZnSOD expression is found to be reciprocal in response to Cu as well as in response to a large number of stress treatments (Table 1). *FSD1* compared to *CSD1*/*CSD2* expression was also reciprocal when examining development in *Arabidopsis* (Fig. 2B). If FSD1 is indeed chloroplastic, then a basal SOD activity level would always be maintained in the chloroplast. Such a regulatory mechanism would underscore the importance of SOD in the chloroplast. The function of SOD is examined in the next section.

5. Possible FeSOD and Cu/ZnSOD functions in plastids

5.1. Overview of possible functions

Mehler first described that PSI can donate electrons to molecular oxygen forming superoxide [2,40]. Superoxide formation may also be possible through reduction of oxygen by PSII or plastoquinone [31]. The SOD substrate superoxide and hydrogen peroxide, the product of SOD, are both reactive oxygen species (ROS) [1]. Several functions have been postulated for SOD in chloroplasts: protection to oxidative stress, photoprotection, regulation of electron transport and signaling. We will first discuss these possible functions in the context of chloroplast biochemistry and the properties of SODs and associated enzyme systems. We will then scrutinize these models by examination of the phenotypes that result from the manipulation of SOD activity levels *in planta*.

5.2. ROS scavenging

5.2.1. Superoxide and ROS production

The function of SOD is frequently associated with tolerance to abiotic stress [8]. This view is supported by the observation that microorganisms such as yeast and cyanobacteria become more sensitive to ROS when they lack SOD [41,42]. The phenotype of yeast mutants deficient in cytosolic SOD function is informative. These mutants are lysine and leucine auxotrophs during aerobic growth because they lack sufficient activity of the enzymes homoaconitase (Lys4p) and isopropylmalate dehydratase (Leu1p) which are 4Fe–4S cluster proteins with similarity to aconitase [41]. The genes are expressed but the proteins are not active most likely because the Fe–S clusters are highly sensitive to superoxide. This situation suggests a scavenging function for SOD in yeast, necessary to protect ROS-sensitive cellular enzymes that would otherwise become limiting to growth. Chloroplasts have over twenty Fe–S proteins. Several of these have a function in the electron transport chain but there are also 4Fe–4S cluster proteins that are active in the stroma [43].

Superoxide can be damaging to Fe cofactors of proteins but compared to other reactive oxygen species it is not considered to be very reactive by itself [1]. Nevertheless, the presence of superoxide can lead to the formation of hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•). The latter is a reactive oxygen species that cannot be removed enzymatically. It is highly reactive and considered to be especially damaging to membrane lipids and macromolecules including nucleic acids [1]. Free redox-active metal ions such as Fe^{2+} promote the formation of the very reactive hydroxyl radical in the Fenton reaction [1]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}.$$

The presence of an electron donor such as ascorbate leads to a cycle which further increases the damage to biological molecules. Superoxide can also serve as a reductant that can drive the so-called Haber–Weiss cycle, which consists of the following two reactions:

$$H_2O_2 + OH^{\bullet} \rightarrow H_2O + O_2^{-} + H^{+} \text{ and } H_2O_2 + O_2^{-} \rightarrow O_2 + OH^{-} + OH^{\bullet}$$

The second reaction has a negligible rate constant but it is believed that Fe^{3+} complexes catalyze this reaction [1]. In this case Fe^{3+} is first reduced by superoxide, followed by the oxidation by hydrogen peroxide. There are several reasons why organisms need to control these cycles. Reactive oxygen species can cause damage to electron transport chains which also could lead to the release of redox-active iron atoms. Iron in turn promotes the Haber–Weiss cycle and Fenton reaction which promote the production of the highly toxic hydroxyl radical [1]. Defects in electron transport chains could again lead to over-reduction of upstream components possibly causing more superoxide formation. If ROS and free metal ions are "partners in crime" then there are two obvious remedies required to control such a destructive negative spiral: prevent ROS accumulation and avoid accumulation of free metal ions.

5.2.2. Control of superoxide and hydrogen peroxide in the water–water cycle

In the context of oxidative stress protection chloroplast SOD can be viewed as part of a reactive oxygen species scavenging system, working in synergy with either peroxidase or peroxiredoxin (see Fig. 4). Peroxidase and peroxiredoxin can eliminate the peroxide produced by SOD in the so called water–water cycle [2]. In the water–water cycle electrons that originate from water at PSII and that are lost from the electron transport chain react with molecular oxygen, forming superoxide. This process may be promoted by high light intensities, low temperature, drought or inhibition of the Calvin cycle. Superoxide is then converted to hydrogen peroxide by SOD. Hydrogen peroxide is removed and water is again formed by ascorbate peroxidase at the expense of ascorbate, which becomes oxidized. Reduced ascorbate is regenerated with electrons coming from ferredoxin. Alternative pathways for the removal of H_2O_2 in the stroma are described in Fig. 4.

Scavenging would be most efficient if SOD is close to the site where it is produced and close to an enzyme that can consume H_2O_2 because hydroxyl radical formation must be prevented. The arrangement of SOD in the stroma close to the thylakoid membrane surface, where it can work together with ascorbate peroxidase, would contribute to the efficiency of the water–water cycle (see Fig. 4). Within the chloroplast, FeSOD and Cu/ZnSOD are both found in the stroma [9,20], but within this aqueous compartment the enzymes may have specific locations. It was found using immuno-gold labeling electron microscopy that the majority of plastid Cu/ZnSOD is closely associated with the outer surface of the thylakoids and it would therefore be close to PSI, which is thought to be a major source of superoxide production. [13]. What would keep Cu/ZnSOD in this membrane-associated location is not clear but perhaps weak ionic interactions play a role. The membrane association is apparently easily lost since Cu/ZnSOD behaves like a soluble enzyme which is recovered in stromal fractions after lysis and fractionation of *Arabidopsis* chloroplasts [22]. Similarly, Van Camp et al. concluded that FeSOD co-fractionated preferentially with the chloroplast membrane fraction in centrifugation experiments [44]. Thus, the biochemical properties of SODs which are highly efficient in the removal of superoxide and the suggested location of chloroplast SOD close to PSI and to thylakoid-bound ascorbate peroxidase suggests that these enzymes would work as ROS scavengers.

FSD1 and CSD2 expression are reciprocal in *Arabidopsis* as discussed earlier. However, it is not yet clear if FeSOD and Cu/ZnSOD would interact with the same proteins on the thylakoids. Although both types of enzymes are highly efficient catalysts, it is not yet clear if FeSOD and Cu/ZnSOD are truly interchangeable because they may have different locations and therefore different interacting partners. Not all chloroplast SOD isoforms are preferentially membrane associated. *Arabidopsis* FSD2 and FSD3 were shown to form a functional hetero-dimer that was co-localized with the plastid nucleoid, the area of the stroma that holds the plastid DNA genome [11].

5.2.3. Control of free redox-active metals

A second important measure to prevent hydroxyl radical formation is the sequestration of redox-active metal ions especially Fe and Cu. Uptake and distribution of these metal ions is tightly controlled. The task is delicate because the chloroplast has a high demand for Cu, Fe and Mn. In this context it makes sense that expression of ferritin, which forms an Fe sequestering protein complex, is up-regulated in the presence of H_2O_2 or elevated Fe [45,46]. It had been postulated that ferritin could serve to provide a storage pool of Fe to be used for cofactor assembly. However, based on the study of ferritin knock-out mutants in

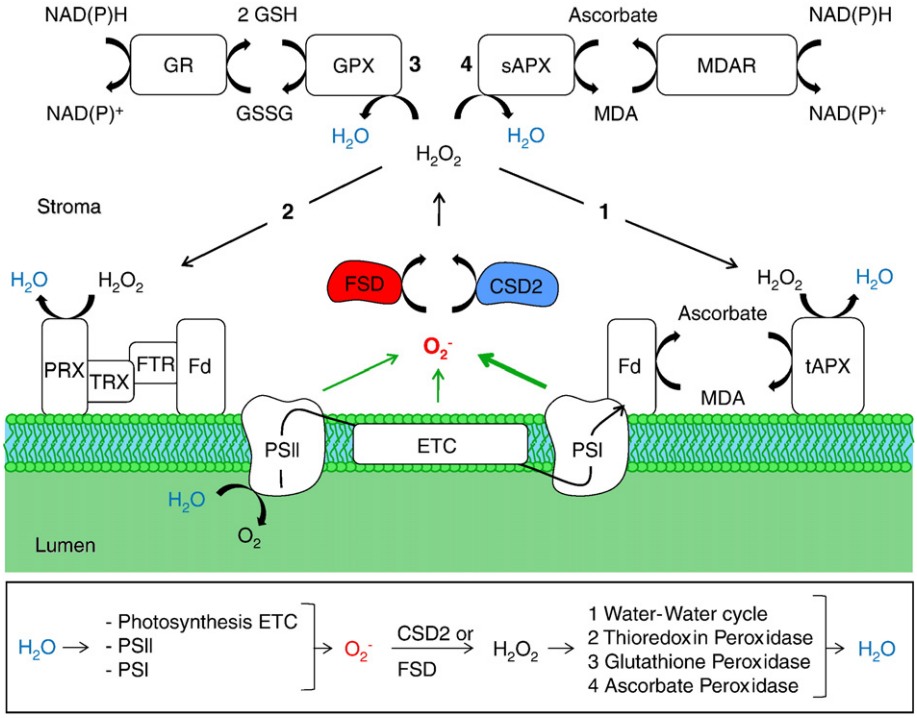

Fig. 4. The water–water cycle and detoxification of superoxide in the chloroplast stroma. Hydrogen peroxide produced by SOD can be removed via four possible pathways labeled 1–4. Abbreviations: sAPX, stromal ascorbate peroxidase; tAPX, thylakoid-bound ascorbate peroxidase; ETC, electron transport chain; Fd, ferredoxin; FTR, Fd-Trx-reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, monodehydroascorbate; MDAR, MDA reductase; PRX, peroxiredoxin; PSI, photosystem I; PSII, photosystem II; TRX, thioredoxin.

Arabidopsis it is now clear that the main function of ferritin is in protection against oxidative stress [47]. Ferritin sequesters Fe and as such prevents ROS metabolism from spinning out of control. It is noticeable that FeSOD and Cu/ZnSOD also bind and therefore sequester redox-active metal ions. The up-regulation of Cu/ZnSOD expression in response to Cu seems to make sense in view of the need to bind excess Cu in order to prevent Fenton chemistry.

5.3. Photo-protection and regulation of electron transport

Plants are sessile and must acclimate to ambient light conditions in the short term. PSII can be damaged by excess excitation, which causes photoinhibition. Several mechanisms give photoprotection by venting off excess excitation energy [48].

However, the water–water cycle is quantitatively not a significant pathway to vent off excess excitation energy during high light or low CO_2 availability; see also Foyer [31]. In comparison, non-photochemical quenching and photorespiration are probably much more effective shunts [48–50].

Non-photochemical quenching (NPQ) involves the loss of excitation energy at PSII in the form of heat and requires a low pH in the thylakoid lumen [48]. As such, NPQ is only active after a steep ΔpH is established due to electron transport activity. Alternative electron pathways such as the water–water cycle and cyclic electron transport could contribute to formation of a steeper pH gradient compared to what is possible by linear flow. However, whether the water–water cycle contributes enough electron transport to make a significant difference for the induction of NPQ is highly debated because it is difficult to measure flow of electrons to oxygen separately from other electron transport pathways. The water–water cycle is generally estimated to contribute less than 10% of total electron transport in steady state conditions in the light [31]. Ruusza et al. [49] found the rate of the water–water cycle to be even lower. In contrast, Makino et al. [50] suggested that oxygen-dependent electron transport in the water–water cycle can be higher. Both reports used plants with reduced RuBisCO expression to eliminate contributions of the Calvin cycle and photorespiration [49,50]. It is not impossible that the water–water cycle could have significant contributions to electron flow under fluctuating light conditions and dark–light transitions [50]. The water–water cycle can also be a mechanism that helps tune the correct ratio of ATP/NADPH coenzyme charging. However, Foyer and Noctor [31] argued that any electron transport activity will be highly restricted at the acceptor site of the cytochrome-b_6/f complex if ATP production is not consuming the trans-thylakoid proton gradient. Therefore, all electron flow will be very low when the Calvin cycle is inactive. SOD probably does not limit the water–water cycle [31] and compared to mutants defective in cyclic electron flow, which show drastic phenotypes [51], the phenotypes of plants with reduced CSD2 or FSD1 protein accumulation are very mild (see later).

6. Plants with altered SOD levels

6.1. Studies that employ over-expression and loss of function mutants

SOD function can be studied by altering its expression level and examination of phenotypes. A very large number of studies have been done that examine the effects of SOD over-expression in chloroplasts in transgenic plants [11,36,37,44,52–66]. MnSOD was fused to a chloroplast targeting sequence to obtain expression in plastids [55,56]. In other studies FeSOD or Cu/ZnSOD was over-expressed. Although in most cases benefits were reported under stress conditions, these studies did not indicate greatly improved whole-plant performance as a consequence of just SOD over-expression. However, careful analyses have allowed interesting conclusions about the role of SOD in the water–water cycle and the level of protection that SODs may offer. An FeSOD cDNA from *Arabidopsis* which lacked a part of the 5′ region was fused to a chloroplast transit sequence and expressed in tobacco [44], maize [62] and poplar [58]. Over-expression of this chloroplast targeted FeSOD resulted in increased flux through the water–water cycle that was especially apparent at high light and low

temperature [58]. In tobacco and maize, FeSOD over-expression in plastids resulted in improved protection from photoinhibition and decreased leakiness of the plasmalemma in response to MV treatment [44,62]. The latter effect was also seen when MnSOD was expressed in chloroplasts but this did not protect from photoinhibition [44]. This observation suggests that the location of FeSOD, which could be close to the thylakoids [44] is required to protect the photosynthetic machinery in these membranes. In contrast, a stromal location of SOD may only suffice to protect against secondary effects of superoxide production. Over-expression of *FSD2*/*FSD3* in *Arabidopsis* resulted in improved resistance to MV [11].

One study has indicated noticeably enhanced photo-protective benefits from chloroplast Cu/ZnSOD based on analyses of Cu/ZnSOD over-expression [66]. In a very elegant approach Sunkar et al. [36] expressed a *CSD2* mRNA that was modified to become *microRNA398* resistant which causes elevated expression of *CSD2*. These authors did observe improved stress tolerance from enhanced *CSD2* expression in response to high light, excess metal ions and MV. However in some of the treatments the differences between wild-type lines and transgenics were seen only with very harsh treatments [36]. If stress is so severe that all plants suffer dramatically one may question the physiological relevance. Other studies reported that over-expression of SOD in the chloroplast does not by itself result in increased oxidative stress tolerance [37,52,53,56]. Although FeSOD expression did not improve photosynthetic performance under oxidative stress, it did result in improved winter survival in alfalfa [61].

In conclusion, SOD over-expression has resulted in only limited benefits to plant performance under stress. However, increased tolerance to MV or photooxidative stress was observed by over-expressing Cu/ZnSOD together with ascorbate peroxidase in various plant species [67–69].

6.2. Phenotypes associated with loss of SOD function

The most direct evidence about SOD function comes from the study of loss-of-function mutants in *Arabidopsis*. Loss-of-function mutants are reported for *FSD1*, *FSD2*, *FSD3* [11] and *CCS* [22]. These mutants are discussed later. In addition, a knock-down mutant for CSD2 with a T-DNA insertion in the promoter region was originally reported to show severe light sensitivity and a strong growth phenotype [70]. However, the plant line used in the latter report also carried an unrelated T-DNA insert that perhaps could account for the reported phenotype [22]. Furthermore, it was found that the T-DNA insertion at the *CSD2* promoter did not affect CSD2 protein accumulation [22].

6.2.1. Reduction of Cu/ZnSOD function

CSD2 activity requires activation by CCS [23]. A T-DNA insertion mutant in *CCS* was shown to have less than 2% of the wild-type SOD activity in its chloroplasts when grown on media with sufficient Cu [22]. Since *FSD1* expression is absent at sufficient Cu levels the *CCS-KO* line has virtually no SOD activity in the cytosol or chloroplasts in this growth condition [22]. Nevertheless, these plants show surprisingly normal phenotypes. The *CCS-KO* line grows and develops the same as wild-type plants, it has the same rate of seed production and seeds are as viable. Even high light and MV treatments did not show significant differences in performance between the wild-type and *KO* lines [22]. Only the feeding of selenate, which could interfere with sulfur assimilation and glutathione production, caused a mild growth inhibition in the *KO* lines when compared to wild-type plants [22]. These findings suggest that the chloroplast has a large redundancy in its anti-oxidant and ROS scavenging systems, at least under the growth conditions that have been tested [22]. A drawback of this study is that low levels of SOD activity could have persisted in the chloroplasts because some Cu/ZnSOD activity may be retained without CCS present [23]. Furthermore, FSD1 activity could have been present although it was not detected. In the study by Cohu et al. [22] the levels of FSD2 and FSD3 were not affected but the expression of these enzymes is very low during vegetative growth (see Fig. 2B). No SOD activities were detected in the *ccs* mutant under conditions that still allowed the detection CSD2 activity in an extract of the wild-type that was diluted 50-fold [22]. Apparently, a dramatic reduction of chloroplast SOD activity can be tolerated under the tested growth conditions.

6.2.2. Loss of FeSOD activity

FSD1 is one of the abundant SODs in *Arabidopsis* with a possible location in chloroplasts. Surprisingly, a knock-out of *FSD1* showed no change in phenotype [11]. In contrast, mutants in chloroplast localized *FSD2* and *FSD3* and especially a *fsd2/3* double mutant showed severely changed phenotypes [11]. The plants were dwarfed and showed clear developmental defects. Particularly the chloroplasts were under-developed which could be ascribed to a lack of transcription in the chloroplast. The expression of the chloroplast genes *psaA*, *psbA* and *petB*, which depend on the plastid-encoded RNA polymerase, was reduced [11]. Since FSD2/3 were localized close to the chloroplast genome it could be that FeSOD function is required for locally elevated hydrogen peroxide levels [11]. Hydrogen peroxide could therefore act as a signaling factor that affects chloroplast gene expression by the plastid-encoded RNA polymerase. The mechanism could be important to coordinate the expression of nuclear and chloroplast-encoded components of the photosynthetic machinery. In this scenario, superoxide produced by photosynthetic electron transport could be an entry point of a positive feedback loop which signals that conditions are favorable for photosynthetic electron transport, and as such promotes expression of the plastid genome. The *fsd2/3* mutant plants were also more sensitive to treatments that cause photooxidative stress, but in view of the large defects in chloroplast biogenesis and morphology such sensitivity could be a secondary effect [11].

7. Conclusions and outlook

Superoxide dismutases are conserved in evolution, which suggests important functions for these proteins. Therefore, the observation that loss of activity of FSD1, the major FeSOD, and of the cytosolic and plastidic Cu/ZnSODs in *Arabidopsis* plants causes no changes in phenotype [11,22] was a large surprise. The reciprocal pattern for the expression of *FSD1* and *CSD1*/*CSD2* in response to Cu and other treatments suggests that FeSOD and Cu/ZnSOD functions are interchangeable. One topic that clearly needs to be addressed is the location of FSD1, the major FeSOD. Is FSD1 located in the chloroplast (as we believe), or is it cytosolic after all? *CSD1* and *CSD2* can be shut off by Cu limitation [38]. If FSD1 protein is not chloroplastic, this regulation would imply that chloroplasts can lose most of their SOD activity without much consequence. If FSD1 turns out to be plastidic then it is still not clear whether FSD1 and CSD2 have comparable biochemical functions in terms of enzyme kinetics and association with other proteins at specific locations in the plastids. An interesting approach would be to analyze a *fsd1*/*ccs* double mutant in order to determine plant phenotypes after both FSD1 and CSD2 protein functions are lost.

Superoxide dismutases in chloroplasts can be involved in several processes: ROS scavenging, regulation of electron transport, photoprotection and signaling. These roles are certainly not mutually exclusive. Studies that used SOD over-expression and SOD loss of function lines have generated support for each possible role. However, SODs perhaps have only minor roles to play in ROS scavenging and photoprotection because these processes are not drastically affected by loss of the majority of SOD activity in the plastids. This situation contrasts with findings in *Synechococcus* where loss of FeSOD causes sensitivity to photooxidative stress and MV [42]. Chloroplasts probably

have other redundant mechanisms to detoxify ROS. In this context APX may play a more important role than SOD. The water–water cycle may be more significant at moments when the demand for ATP or NADPH changes, for instance during dark–light transitions. Therefore it will be interesting to investigate the phenotypes of *CCS* and *FSD1* mutants under variable light conditions. *CSD2* and *FSD1* are also expressed in roots [Pilon et al. unpublished observations]. Since roots do not have chlorophyll, this observation suggests that these SOD proteins also serve roles that are unrelated to photosynthesis.

Hydrogen peroxide and other ROS have also been implicated in signaling [71,72]. Thus, signaling or regulation of chloroplast biochemistry must be considered another possible function of SOD. The involvement in chloroplast development of FSD2 and FSD3 which are isoforms with a low expression level is inferred from the phenotypes of *fsd2*, *fsd3* and *fsd2/3* double mutants. These plant lines were defective in the expression of chloroplast genes that are transcribed by the plastid-encoded RNA polymerase [11]. These observations support a signaling role of hydrogen peroxide which is a product of SOD. Apparently, this signal needs to be generated in a very specific location. Maybe this explains why FSD1, which is more abundantly expressed than FSD2 and FSD3, cannot take over this function in the *fsd2/3* knock-out. The charged superoxide is not membrane permeable. However the uncharged product of SOD, hydrogen peroxide, can pass membranes and could therefore diffuse over the envelope. The question thus arises whether chloroplast SOD activity could also affect signaling pathways outside of the chloroplast.

Careful analysis of the phenotypes of plant lines that carry single mutations in SOD genes and combinations of such mutants will prove to be fruitful in generating better insight into the biological roles of these enzymes. This analysis should be performed for a variety of growth conditions and results should be considered with the sub-cellular location and expression patterns of these enzymes in mind. SODs are fascinating enzymes and as we proceed to further reveal the secrets of their functions in plants we may be in for a few more surprises.

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