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Plant Peroxisomes as a source of signalling molecules.

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Abstract

Peroxisomes are pleiomorphic, metabolically plastic organelles. Their essentially oxidative function led to the adoption of the name 'peroxisome'. The dynamic and diverse nature of peroxisome metabolism has led to the realisation that peroxisomes are an important source of signalling molecules that can function to integrate cellular activity and multicellular development. In plants defence against predators and a hostile environment is of necessity a metabolic and developmental response- a plant has no place to hide. Mutant screens are implicating peroxisomes in disease resistance and signalling in response to light. Characterisation of mutants disrupted in peroxisomal β -oxidation has led to a growing appreciation of the importance of this pathway in the production of jasmonic acid, conversion of indole butyric acid to indole acetic acid and possibly in the production of other signalling molecules. Likewise the role of peroxisomes in the production and detoxification of reactive oxygen, and possibly reactive nitrogen species and changes in redox status, suggests considerable scope for peroxisomes to contribute to perception and response to a wide range of biotic and abiotic stresses. Whereas the peroxisome is the sole site of β -oxidation in plants, the production and detoxification of ROS in many cell compartments makes the specific contribution of the peroxisome much more difficult to establish. However progress in identifying peroxisome specific isoforms of enzymes associated with ROS metabolism should allow a more definitive assessment of these contributions in the future.

Keywords: Peroxisome, β -oxidation, hydrogen peroxide, superoxide, reactive oxygen species, nitric oxide, stress, defence.

1. Introduction

Peroxisomes are single membrane bound subcellular organelles, ubiquitous in eukaryotic cells. The organelles are usually spherical bodies in the range of 0.1-1 μ m in diameter, however several studies indicate the ability of peroxisomes to form large reticular networks in response to changes in their cellular environment [1-3]. Peroxisomes contain coarsely granular or fibrillar matrix, occasionally dotted with crystalline inclusions containing enzymes involved in oxidative metabolism, in particular catalase [4, 5] [6].

When these organelles were first isolated in the 1960s, their sole function was deemed to be the detoxification of H₂O₂ produced by various flavo-oxidases [7] [2]. However, it is now well known that peroxisomes carry out oxidative metabolism of a variety of substrates depending on their origin and contribute to various cellular processes in virtually all eukaryotic cells, with defence against oxidative stress and β - oxidation being the most conserved functions [8] [9] [1, 10]. A remarkable property of peroxisomes is their metabolic plasticity, which enable them to remodel their size, shape and enzymatic constituents depending on the cell or tissue type, organism and prevailing environmental conditions. This adaptability is exemplified by the induction of peroxisome proliferation in response to xenobiotics, herbicides, chemical pollutants, biotic stress, abiotic stress and nutrient deprivation [7] [2, 11] [8].

Peroxisomes can be viewed as specialised organelles whose classification depends on the prevalent metabolic process at any given time [1, 12]. In plants peroxisomes differentiate into at least four different classes; glyoxysomes, leaf peroxisomes, root nodule peroxisomes and unspecialised peroxisomes [8] [13] [14]. Glyoxysomes occur in endosperms and cotyledons of germinating seeds and contain enzymes for the β -oxidation and glyoxylate cycle to convert oil seed reserves into sugars which can be used for germination before the plant is

photosynthetically active [5, 13, 15, 16]. On the other hand, leaf peroxisomes house enzymes for the oxidation of glycolate during photorespiration [5, 15, 17] [8, 18, 19]. Root nodule peroxisomes contain urate and xanthine oxidase which catalyses the oxidation of xanthine and uric acid produced during nucleotide turnover [20, 21] [6]. It should be noted that this classification is not rigid since glyoxysomes transform into photosynthetic leaf peroxisomes and vice versa, in response to changes in light intensity and developmental programme [22] [4, 8, 13, 23, 24].

The metabolic reactions occurring in peroxisomes are very complex, and in recent years much attention has been devoted to elucidating the role of peroxisomes in cellular metabolism. The introduction of post genomic approaches such as transcriptomics and proteomics and the use of bioinformatics tools is shedding some light on our understanding of peroxisome function with new functions being discovered [14, 25, 26] [27, 28].

There is a considerable body of evidence linking peroxisomal metabolism to production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and β oxidation derived signalling molecules particularly in plants (reviewed in: [29] [30] [31] [32]). In this regard the current state of knowledge in this area will be reviewed with particular reference to plants. The metabolic pathways involved in the synthesis of signalling molecules, evidence for the existence of such pathways in peroxisomes and the mechanisms involved in balancing the subcellular levels of the signalling molecules will be discussed. The gap existing in our current understanding of the involvement of peroxisomes in signalling and future challenges that need to be addressed to fully understand the role of peroxisomes in generation of signalling molecules in eukaryotic cells will also be highlighted.

2. The Role of Peroxisomes in Generation of Signalling Molecules: An Overview

The last decade has seen progress in our understanding of signal transduction pathways and the role played by various cellular compartments is beginning to emerge. Various experimental evidence indicate the role of peroxisomes in the metabolism of ROS, RNS and in β -oxidation with concomitant production of intra- and inter- cellular signalling molecules **(Figure 1)** [33] [30] [34] [35] [36]. Since these molecules are produced during normal cellular metabolism, their role in signalling largely depends on the balance between synthesis utilisation and degradation. [37] [38]. Under optimal conditions a dynamic equilibrium exists between the rate of synthesis and the rate of utilisation or breakdown of the potential signalling molecules, resulting in maintenance such molecules at levels compatible with the metabolic requirements of a specific cellular compartment [39, 40] [38] [41, 42]. However, environmental stimuli such as desiccation, salt, chilling, heat shock, heavy metals, UV radiation, ozone, mechanical stress, nutrient deprivation and biotic stress are known to perturb this balance leading to an overproduction of the signalling molecule, which may initiate a signalling cascade or cause cellular damage [36] [35] [39, 43].

A typical example of such a scenario is the increased production of ROS in response to biotic or abiotic stress (oxidative burst). This may initiate lipid peroxidation yielding products that react with DNA and proteins to cause oxidative modifications, or initiate a signalling cascade leading to acclamatory stress tolerance [44], hypersensitivity response (HR) or programmed cell death (PCD) [45, 46] [47] depending on the nature of the stimuli. This emphasises the need for the cytotoxic effects of the signalling molecules to be tightly regulated in-order for the signalling effects to be exerted without deleterious effects on the organism.

In *A. thaliana* over 152 genes belonging to the ROS gene network are thought to regulate ROS levels and signals [39]. It is possible that such gene networks may be involved in controlling the steady state levels of RNS, hormonal levels and the redox state of the peroxisomes to ensure the signalling role of potentially cytotoxic molecules [48] [49] [41]. However, the role of such gene networks in maintaining the steady state levels of signalling molecules in peroxisomes has not been investigated.

3. 0. The role of Peroxisomal β - Oxidation in generation of plant signalling molecules

β -Oxidation is the major pathway for the degradation of straight and branched chain fatty acids as well as some branched chain amino acids in a range of organisms including plants, yeast and mammals [25, 50], [30], Hiltunen and Poirier, this volume). Apart from its catabolic role, compounds derived from β -oxidation are involved in controlling a variety of cellular processes in both plants and animals [51] [52]. These include the eicosanoid family of lipid mediators such as 2, 3-Dinor-5, 6-dihydro-15-F-2t-isoprostane, which plays an important role in vasoconstriction [52], plant aromatic and cyclic oxylipins such as Jasmonic acid (JA), and other β -oxidation derivatives such as indole acetic acid (IAA) and salicylic acid (SA) which are important in signalling [51] [30] [25] [53] [54] [55, 56]. Peroxisomes, as the only site for β -oxidation in plants, should have a central role in the biosynthesis of these β - oxidation derived signalling molecules [30].

3. 1. The role of β - oxidation in the production of Jasmonates

The role of JA as a signalling molecule in plants is well established, with documented functions ranging from defence against biotic and abiotic stress, male and female fertility,

fruit ripening, root growth and tendril coiling to vitamin C synthesis [57, 58] [59-61] [53] [62].

The biosynthesis of JA occurs via the octadecanoid pathway (18:3), or hexadecanoid pathway (16:3) which are both initiated in the plastids by the oxygenation of either linolenic acid (18:3) / linoleic (18:2) or hexadecatrienoic acid (16:3) derived from the lipid bilayer. This step is followed by a series of enzyme guided dehydration and cyclisation reactions leading to the formation of 12-oxo-phytodienoic acid (OPDA), or dinor OPDA, which is exported to peroxisomes. Reduction of OPDA or dinor OPDA yields 3-oxo-2-(2'-(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC:8) or 3-oxo-2-(2'-(Z)-pentenyl)-cyclopentane-1-hexanoic acid (OPC:6), which is perceived to undergo three or two cycles of β -oxidation, to produce 3R, 7S-JA [(+)-7-iso-jasmonic acid] (**Figure 2**) [53, 57, 59, 63, 64].

The involvement of β -oxidation in this biosynthetic pathway is supported by an observation that only an even number of carboxylic acid side chains of OPC derivatives were used for JA synthesis [65]. Plant peroxisomal β -oxidation involves the sequential action of enzymes from three gene families; acyl CoA oxidases (ACX), multi functional proteins (MFP) and L-3-ketoacyl-CoA thiolases (KAT) [30, 66]. However, the exact role of the various β -oxidation enzymes in the synthesis of JA is not clearly understood. Analysis of Arabidopsis and tomato mutants isolated by forward and reverse genetics has provided some insights into the enzymes that may be involved in this pathway, although some gaps in our knowledge still exist.

Oxylipin profiling in a *cts* mutant with a defective ABC transporter (also known as PXA1 or PED3) [67] indicated a drastic reduction in the basal and wound induced levels of JA in leaves, suggesting that the import of OPDA or dnOPDA (or their CoA esters) into peroxisomes may be regulated by CTS/PXA1/PED3. A passive mechanism involving ion trapping would account for the residual levels of JA observed in the mutant [9, 30, 67].

Once imported, OPDA or dnOPDA undergoes reduction to OPC: 8 or 3-oxo-2- (2')-pentenyl)-cyclopentane-1- haxanoic acid (OPC: 6) respectively. This reaction is mediated by OPR3; a NADPH dependent OPDA –reductase, which is the only peroxisomal isoform among the three OPR isoforms of *A. thaliana* and is specific for the 9S, 13S-stereoisomer of OPDA [68, 69]. OPR3 is expressed throughout the plant and co-localises with enzymes for fatty acid β - oxidation in peroxisomes, suggesting that it may be part of the β -oxidation pathway for JA [69]. Moreover, *opr3* mutants are deficient in JA and also exhibit defective pollen production, delayed dehiscence and male sterility, suggesting that OPR3 may be the only enzyme involved in this reduction step [30, 68, 70]. The fact that OPR3 accepts free OPDA, and *opr3* mutants lack OPC: 8 suggests that reduction of OPDA to OPC:8 precedes the CoA esterification step [59, 63, 69-71]. However, the recent identification of two peroxisomal 4-coumarate: CoA ligase-like (4CL) acyl activating enzymes; At4g05160 and At5g63380 with high efficiency in activating OPDA, and OPC:6 *in vitro* [72], suggests that activation can occur at various stages in the β -oxidation pathway. Bearing in mind the large number of genes encoding acyl activating enzymes that were identified in Arabidopsis [25, 73] , and the fact that only two out of 25 4CL-like proteins of unknown biochemical function showing high sequence similarity to the 4CLs were analysed [72], the existence of other acyl activating enzymes committed to this pathway can not be ruled out. The subcellular localisation of the acyl activation step and the possible entry points of OPDA into the β -oxidation pathway is an issue that still needs to be investigated.

The subsequent oxidation step is catalysed by acyl-CoA oxidase (ACX) which oxidizes a fatty acyl-CoA to a 2-trans-enoyl-CoA. Of the five genes encoding such oxidases in *A. thaliana*, ACX1 has an important role in JA synthesis. This is supported by experimental data

whereby an *acx1-1* mutant of *Arabidopsis* accumulated acyl-CoA and had a drastic reduction in JA levels [74].

Moreover, a map based cloning approach identified an isoform of acyl-CoA oxidase, in tomato (*Lycopersicon esculentum* (*LeACX1*), which plays an essential role in the biosynthesis of JA in response to wounding. The *Leacx1A* mutant leaves had reduced basal and wound induced levels of JA. Moreover the recombinant *LeACX1A* metabolised OPC: 8: CoA and OPDA in preference to fatty acylCoA in a coupled ACS-ACX assay, suggesting a possible role of the *LeACX1A* in the oxidation of OPDA or OPC: 8 [51]. The direct oxidation of OPDA would imply that OPR3 activity may be switched on and off depending upon the metabolic needs of the cell. In the absence of OPR3 activity, OPDA may be oxidised to 4, 5-didehydro-JA [51] [63].

LeACX1A is homologous to *AtACX1* which is implicated in the wound induced biosynthesis of JA [75] and also to the peroxisomal acyl oxidase *GmACX1* from soyabean (*Glycine max*) (76). Thus; *ACX1* appears to play a pivotal role in the biosynthesis of JA. *LeACX1A*, *AtACX1* and *GmACX1-1* [76] have peroxisomal targeting sequences [25], and exhibit broad substrate specificity [51, 75, 77, 78], suggesting that these peroxisomal enzymes may catalyse equivalent reactions in subsequent rounds of β -oxidation to yield JA. However, the relative specificity of *ACX1* for OPC: 6 and OPC: 4 and the subcellular localisation of these reactions await further investigation.

Following oxidation the next step requires the activity of MFP which catalyses the hydration of 2-*trans*-enoyl-CoA to 3-hydroxyacyl-CoA and subsequent oxidation to 3-ketoacyl-CoA

A. thaliana has two peroxisomal MFPs; *AIM1* and *MFP2*, which play an important role in fatty acid beta oxidation. However the levels of JA in the *aim1* or *mfp2* mutant was not

evaluated, in this regard the role of the MFPs in relation to JA synthesis is still not known [79, 80].

Arabidopsis possesses three 3-keto acyl thiolase genes, KAT1, KAT5 and KAT2/PED1, of which the latter is the major seedling expressed thiolase. The role of *KAT2/PED1* thiolase in wound induced JA biosynthesis was demonstrated in two independent studies based on suppression of *KAT2/PED1* by antisense RNA [75] and *ped1* mutant analysis [81]. In both studies JA synthesis was not abolished indicating that another KAT isoform(s) partially compensated for the *KAT2/PED1* defect. This observation suggests the role of other KAT isoform(s) in JA synthesis, which needs further investigation [30]. Thus, the core β -oxidation pathway may be involved in JA synthesis, however due to overlapping substrate specificities for these enzymes [51, 78], the involvement of other enzymes committed to this pathway can not be ruled out. Once formed, JA should be released from its acyl-CoA ester before being exported to exert its signalling effects, the enzyme involved in mediating the hydrolysis of the acyl CoA to release JA and the export mechanism have not been investigated [25].

JA may be further oxidised, methylated or aminoacylated to produce compounds with different properties and signalling roles from JA [82, 83]. These include *Z*-jasmonate; an insect attractant produced from a further round of β oxidation of JA, amino acid conjugates particularly with isoleucine, JA derivatives such as Jasmonyl-1- β – gentiobiose, jasmonyl-1- β –glucose, hydroxyjasmonic acid, tuberonic acid and its glucoside [84] and methyl jasmonate; a volatile signal involved in intra- and inter-cellular communication as well as inter plant communication [53, 57, 85]. Although there is evidence for the role of S-adenosyl-L-methionine: Jasmonic acid carboxyl methyltransferase (JMETH)

and JAR1 in JA methylation and conjugation of isoleucine to JA respectively [85, 86], the localisation of JAR1 or JMET and the functions of these modifications remains unknown.

The structure and biosynthesis of JA resembles the animal eicosanoids such as hydroxyl fatty acids, leukotrienes and lipoxins, which are synthesised from arachidonic acid (20-C) by free radical-mediated peroxidation reactions via the lipoxygenase pathway [52, 87]; [88, 89]; [90, 91]. Both OPDA in plants and animal eicosanoids produced in this pathway are potent signalling molecules [55, 71]. Peroxisomal β -oxidation functions in the conversion of OPDA to JA which has different spectra of activity from OPDA [84], while in animals the pathway serves to degrade leukotrienes and other eicosanoids resulting in loss of or altered biological activity [91, 92] (**Figure 2**). The identification a bioactive β -oxidation metabolite of prostaglandins; 2, 3-Dinor-5, 6-dihydro-15-F-2t-isoprostane which plays an important role in vasoconstriction, suggests that this pathway may also be involved in generating yet uncharacterised signalling molecules in animals [52]. Whereas CTS/PXA1/PED3 is implicated in the uptake of OPDA into peroxisomes for conversion to JA, the mammalian homolog; Adrenoleukodistrophy protein (ALDP) may not be involved in the uptake of eicosanoids for degradation since X-ALD patients metabolise leukotrienes [92].

3. 2. The role of beta-oxidation in the conversion of Indole butyric acid to IBA to indole acetic acid IAA

Indole acetic acid (IAA), is an auxin that plays an important role in virtually all aspects of plant growth and development, including vascular development, lateral root initiation, apical dominance, phototropism, geotropism as well as a herbicide at higher concentrations [93-95]. The synthesis of IAA occurs through multiple pathways, including the tryptophan dependent and independent pathways, which occur in the cytoplasm and plastids. In addition hydrolysis

of IAA –conjugates and conversion of endogenous indole butyric acid (IBA) increases the intracellular pool of IAA [31, 95].

The conversion of IBA to IAA was demonstrated in a variety of plants using radiolabelling techniques [31, 95]. The proposed mechanism of IBA conversion to IAA, involves thioesterification, oxidation, hydration, dehydration, thiolysis and hydrolysis (analogous to β -oxidation of fatty acids) to release the free auxin which is then exported from the peroxisomes [25, 31, 66]. IBA or the synthetic auxin precursor 2, 4 –dichloro-phenoxy-butyric acid (2, 4 DB) are converted to IAA or 2, 4 –dichloro-phenoxy-acetic acid (2, 4 D), which inhibit root growth. The isolation and characterisation of mutants that are resistant to inhibitory concentrations of IBA or 2,4 DB but respond normally to IAA or 2,4 D has been very powerful in isolating mutants defective in IBA responses, peroxisomal β -oxidation and peroxisome biogenesis [30, 31, 93], see also article by Nishimura and colleagues in this volume). This data suggests the role of beta –oxidation in the conversion of IBA to IAA. However, some fatty acid β - oxidation enzymes such as MFP2 appear not to be involved in conversion of IBA to IAA since the *mfp2* mutant is not resistant to 2,4 DB [79]. It is probable that enzymes committed to this pathway exist, in addition to the core β -oxidation enzymes. Comparing the rate of conversion of radio labelled IBA in the IBA responsive mutants may indicate the specificity of the β - oxidation enzymes in the conversion of IBA to IAA, and possibly lead to identification of novel enzymes committed to this pathway. IAA is conjugated to amino acids and hydrolysed to release free IAA upon demand, however the subcellular localisation of these events is not known [96, 97].

3.3. Beta –oxidation; a possible pathway for the synthesis of salicylic acid

Salicylic acid plays a role in thermo-tolerance, hypersensitivity response and systemic acquired resistance [98]. SA is synthesised from the decarboxylation and side chain shortening of trans-cinnamic acid (CA) (derived from the non oxidative deamination of L – phenylalanine) followed by hydroxylation [99, 100]. The side chain can be shortened via non oxidative decarboxylation or in a manner analogous to β - oxidation resulting in the formation of benzoic acid (BA) which is hydroxylated in the meta position to yield salicylic acid [25, 101]. The role of β - oxidation in this process still needs further investigation although acetyl CoA was shown to stimulate the conversion of CA to BA [101, 102]. It is plausible that 4-coumarate CoA ligases which activate a range of hydroxyl and methoxy-substituted cinnamic acid derivatives may be involved in activating cinnamic acid to cinnamoyl CoA which enters β - oxidation to yield SA [25].

4. The role of peroxisomes in generation of reactive oxygen species

4.1. ROS as signalling molecules

The term reactive oxygen species is used to denote species with free unpaired electrons. These species include superoxide ($O_2^{\bullet -}$), hydroxyl radical ($\bullet OH$), perhydroxyl radical ($\bullet HO_2$), peroxy radicals (ROO^{\bullet} and alkoxy radicals $\bullet RO$ which are produced during normal cellular metabolism or induced by changes in the environmental conditions. However, this term is often loosely used to describe other non radical derivatives of oxygen which are highly reactive such as H_2O_2 , singlet 1O_2 , peroxyxynitrite ONOO and Hypochlorous acid HOCl [103]; [42, 43, 104]. ROS are generated when triplet oxygen undergoes sequential univalent reduction from the non reactive ground state [43]. The primary ROS formed in the cell is the superoxide which initiates a cascade of reactions that result in the formation of a variety of ROS depending on the cell type or cellular compartment [43].

H₂O₂ formed due to the enzymatic and spontaneous dismutation of superoxide, is a key signalling molecule in both plants and animals. Signalling pathways controlled by H₂O₂ include activation of the transcription factor NF-κB in mammalian cells and regulation of gene expression in bacteria [105-107]. In plants H₂O₂ is now known to be involved in programmed cell death [45, 108], peroxisome biogenesis [11], ABA-mediated guard cell closure [109], cross tolerance (resistance to a particular stress that also confers resistance to another form of stress) [110, 111], plant hormonal activity [40, 94], and gene expression in response to abiotic stress factors such as ozone, UV, high light intensity, dehydration, wounding, and temperature extremes [36, 47, 112]. A comprehensive transcript profiling by cDNA amplification fragment length polymorphism to monitor genes upregulated in response to H₂O₂ in transgenic catalase deficient tobacco plants identified thousands of genes involved in the hypersensitivity response [113]. Moreover, a microarray analysis to identify genes regulated by H₂O₂ in *A. thaliana* identified 175 non redundant ESTs, with 113 genes being activated while 62 were repressed [114]. These studies indicate the important role played by H₂O₂ in regulating gene expression, and development in plants.

Other ROS arising from the further reduction of H₂O₂ include the hydroxyl radical ([•]OH (formed when H₂O₂ is reduced in presence of metal ions via the Haber–Weiss reaction), perhydroxyl radical ([•]HO₂), peroxy radicals (ROO[•]) and alkoxy radicals ([•]RO). These molecules are powerful oxidants with a short half life, and their formation is tightly regulated by a series of antioxidant systems [43, 44, 115]. It is not surprising that our understanding of their role in signalling is still in its infancy. However such ROS, in particular [•]OH, may have a significant role during ageing, fruit ripening, drug metabolism and controlled breakdown of polymers during rearrangement of cell walls in roots, hypocotyls and coleoptiles where a

strong oxidising agent may be needed [43]. Recently singlet oxygen has also been identified as an important signalling molecule in plants [116-118].

In plants production of ROS has been demonstrated in various cellular compartments including the chloroplasts, mitochondria, peroxisomes, plasma membrane, apoplastic space and nuclei, with most of the cellular ROS originating from the first three compartments [43, 119, 120]. The mitochondria was viewed as the major contributor of ROS in cells, however this notion is worth revising as evidence are mounting for the role of other compartments, particularly peroxisomes in ROS metabolism [12, 115, 119, 121]. This is supported by the proliferation of peroxisomes observed during oxidative stress [11, 33].

In order to understand how peroxisomes contribute to the production of ROS based signalling molecules, it is necessary to examine the mechanism by which the important ROS such as H_2O_2 and superoxide are produced, and the antioxidant systems available in the peroxisomes to prevent cytotoxicity associated with the production of such signalling molecules.

4.2. Plant peroxisomes as generators of the superoxide radical

The production of $\text{O}_2^{\cdot-}$ may be viewed as inevitable, considering the aerobic conditions under which most reactions occur. Reports on the production of $\text{O}_2^{\cdot-}$ in mammalian tissues such as neutrophils, monocytes and phagocytes exist [122-124]. In plants, chloroplasts generate $\text{O}_2^{\cdot-}$ during photoreduction of oxygen in the Mehler reaction, occurring at photosystem I (PSI) and also during the electron transport chain, whereas in the mitochondria direct reduction of oxygen to superoxide by the NADH dependent dehydrogenase occurs. A detailed account on how each of these processes leads to generation of ($\text{O}_2^{\cdot-}$) can be found elsewhere [12, 43, 115, 119].

The production of $O_2^{\bullet-}$ in peroxisomes was first demonstrated using a subglyoxysomal fraction from watermelon (*Citrullus vulgaris Schrad*) [125] and castor bean endosperm [126] and later in pea (*Pisum sativum*) leaf peroxisomes [127]. This was ascribed to a matrix localised enzyme, xanthine oxidase (XOD) which was detected in the supernatants and confirmed by electron spin resonance (ESR), biochemical and immunological data [125-128]. XOD catalyses the oxidation of xanthine or hypoxanthine to uric acid, with production of $O_2^{\bullet-}$ uric acid is further converted to allantoin via the action of urate oxidase [7, 129]. HPLC analysis detected the presence of all the metabolites for xanthine and urate oxidase in leaf peroxisomes [33, 130], reinforcing the role of peroxisomes in the metabolism of xanthine or hypoxanthine produced during turnover of nucleic acids. Xanthine oxidase and xanthine dehydrogenase are interconvertible forms of the same protein [131, 132]. Two Xanthine dehydrogenase genes are annotated in the Arabidopsis genome, but neither has an obvious peroxisome targeting signal.

Biochemical and electron spin resonance spectroscopy ESR demonstrated the generation of $O_2^{\bullet-}$ in peroxisomes via a second pathway involving a short electron transport chain in the peroxisomal membranes of castor bean (*Ricinus communis*) seeds [133-135] and potato tuber peroxisomes [136]. This electron transport chain represents a mechanism for the regeneration of NAD^+ and $NADP^+$ to sustain peroxisomal oxidative metabolism [33]. Three PMPs involved in the production of $O_2^{\bullet-}$ were purified from the membranes of pea leaf peroxisomes (**Table 1**). These include two NADH dependent proteins; PMP32, bearing biochemical resemblance to mono-dehydroascorbate reductase (MDAR) and PMP18, a putative cyt b_5 . A third protein PMP29 is NADPH dependent, and can transfer electrons to cytochrome *c* and O_2 *in vitro* [137, 138]. The $O_2^{\bullet-}$ produced has a half life of 2-4ms, before it spontaneously or

enzymatically disproportionate into H₂O₂ [42]. The signalling effects of O₂^{•-} have not been studied much, possibly due to its instability and impermeability to cell membranes [42]. However evidence suggests that O₂^{•-} may act directly as a second messenger in regulating the expression of oxidative stress response genes such as glutathione peroxidases, ascorbate peroxidase and glutathione-S-transferases and in regulating enzyme activity through oxidation of Fe-S clusters in enzymes [42].

4.3. The role of Peroxisomes in Production of Hydrogen Peroxide

The production of H₂O₂ in peroxisomes has been known for decades, however details of the biochemical pathways leading to the production of H₂O₂ (**Table 1**) are beginning to be established. The dismutation of O₂^{•-} by superoxide dismutase (SOD) (**Table 1**) constitutes the first line of defence by converting the charged (O₂^{•-}) radical species into H₂O₂ which can be metabolised by the cell's antioxidant machinery. Three types of SODs which differ in their metal cofactor are distributed in various cellular compartments [33, 139, 140].

In peroxisomes immuno-electron microscopy and density –gradient centrifugation detected the presence of two types of SODs, a 33 kDa Mn- SOD which uses manganese as a cofactor was identified in peroxisomes from pea cotyledons [139, 141, 142], while copper- zinc (Cu-Zn) SODs were detected in cotyledons of water melon [143-146]. To date the presence of SODs in plant peroxisomes has been demonstrated in at least nine species and confirmed in five species. SODs have also been identified in peroxisomes of human hepatoma cells and fibroblasts, rat liver, fish and yeast [6]. This localisation of SODs in peroxisomes, suggest a role in dismutation of (O₂^{•-}) to produce H₂O₂. *Arabidopsis thaliana* has 3 genes encoding Cu-Zn SODs of which one *CSD3* encodes a protein with the putative PTS1 peptide AKL

(**Table 1**). Neither of the two Mn SODs; (At3g10920) and *MSD1* (At3g56350) have obvious PTS's and are known or predicted to be mitochondrial.

H₂O₂ is also produced during the metabolism of ureides [5, 6, 20, 21] and also via the action of acylCoA oxidase during β -oxidation [30]. During photorespiration glycolate enters the peroxisomes and is oxidised to glyoxylate by a flavin mononucleotide dependent, glycolate oxidase with production of H₂O₂ [15, 17, 18] [147], see also the article by Reumann and Weber in this volume). Arabidopsis has five glycolate oxidase-like genes; from expression patterns it was suggested that *GOX1* and *GOX2* are the principal photorespiratory enzymes (**Table 1**). *GOX3* is expressed predominantly in non-photosynthetic tissue and the more divergent *HAOX1* and *HAOX2* (which also contain potential PTSs) are suggested by analogy to homologous mammalian enzymes to be involved in metabolism of 2-hydroxy acids [25].

Recently a peroxisomal sulfite oxidase (SO); a molybdenum containing protein (MCP), from *A. thaliana* was identified and characterised, as a molybdenum dependent, non-heme containing enzyme; an observation that is atypical of other eukaryotic sulfite oxidases [148, 149]. Experimental data indicated that AtSO is localised in plant peroxisomes [150] and catalyses the oxidation of sulfite with oxygen acting as a terminal electron acceptor with concomitant production of H₂O₂ (**Table 1**) [151]. This provides an additional pathway for the generation of H₂O₂ in plant peroxisomes which may not be present in other eukaryotic cells. It is proposed that the above reaction is followed by a non enzymatic step, where by H₂O₂ can oxidise a second molecule of sulfite to sulphate. Thus this enzyme may have a dual role in the detoxification of sulfite and balancing the level of H₂O₂ particularly under conditions of high sulfite concentration when catalase is inhibited [151].

OsMCP a homolog of the *Arabidopsis thaliana* AtSO was also isolated from rice and its localisation was confirmed to be peroxisomal. In addition seventeen putative MCP genes with a peroxisomal targeting sequence were identified in other plant species, suggesting SO may be a conserved enzyme with a dual role in plants [148]. This view is supported by the localisation of the tobacco NtSO (a protein with a SNL peroxisomal targeting motif) to peroxisomes as confirmed by biochemical and immunogold labelling techniques [149].

Recently an additional enzyme Sarcosine oxidase which catalyses the demethylation of sarcosine, with production of H₂O₂ (**Table 1**) in mammals and soil bacteria was identified in plants [152]. Localisation of this enzyme indicates that the Arabidopsis sarcosine oxidase is a peroxisomal enzyme with sarcosine-oxidising and pipecolate activity [153] (**Table 1**).

Thus peroxisomes are endowed with pathways for the synthesis of H₂O₂, although it is not yet clear which of these pathways may have an important role in generating H₂O₂ for signalling. Bearing in mind the metabolic plasticity of the peroxisomes, it may be that each different pathway may be of importance in a particular tissue, stage of development or type of peroxisome. However the possibility of these being complementary pathways can not be ruled out. Another question worth addressing in this regard is the contribution of peroxisomes in the generation of these signalling molecules in comparison to other cellular compartments. Foyer, *et al.*, 2003 described peroxisomes as a major site of H₂O₂ production in C3 plants during photorespiration. Under such conditions the rate of H₂O₂ production in peroxisomes is estimated to be about twice that in chloroplasts and even 50 fold higher than that in the mitochondria [119]. This needs to be evaluated for other pathways and the relative contribution of peroxisomes assessed in context of the cell, tissue type, stage of development and the organism involved.

5. ROS scavenging systems and redox signalling

Apart from their signalling role, ROS are capable of causing oxidative damage, implying the need to regulate their intracellular concentrations [34, 44, 154]. Plants possess non-enzymatic antioxidant molecules such as ascorbate (AA), α -tocopherol, carotenoids and glutathione (GSH) and an array of antioxidant enzymes including catalase (CAT), ascorbate peroxidase (APX), dihydroascorbate reductase (DHAR), monohydroascorbate (MDAR), glutathione reductase (GR), glutathione peroxidase (GPX) and thioredoxin dependent peroxidases, in various cellular compartments [34, 44, 154] [155]. Peroxisomes as a source of RO signalling molecules must have an efficient ROS scavenging system to ensure a balance is maintained between the two opposing effects of ROS [38].

The SOD provides the first line of defence by converting the $O_2^{\cdot-}$ to H_2O_2 , thereby limiting the $O_2^{\cdot-}$ available to react with nitric oxide resulting in the formation of the peroxynitrite radical (a powerful oxidising agent) [33, 46]. The existence of catalase in peroxisomes was unequivocally established years ago [2, 7, 156, 157]. Three isoforms of this enzyme; CAT1, CAT2 and CAT3 exist in the peroxisomal matrix of *A. thaliana* (**Table 1**). Analysis of the expression pattern of the three catalase isoforms of pumpkin indicate that the three isoforms are differential expressed in glyoxysomes and leaf peroxisomes and at different stages of development; with CAT1 expression being correlated to senescence [158]. In Arabidopsis CAT2 is the predominant leaf isoform and plants with reduced CAT2 levels showed increased sensitivity to ozone and photorespiratory induced cell death [159]. Similarly, catalase deficient tobacco plants developed leaf necrosis in response to high light, showed perturbed redox balance and were more susceptible to paraquat, salt and ozone stress [160]. These plants exhibited an activation of defence responses in response to excess hydrogen peroxide

and were more resistant to pathogens due to the triggering of programmed cell death in response to lower titres of pathogen compared to control plants [161-163]. The transcriptome of CAT2 deficient plants showed large changes in gene expression compared to controls, emphasising the role of photorespiratory H₂O₂ in cell signalling [159].

The peroxisomal concentration of catalase is estimated to be in the range of 10-25% of the total peroxisomal proteins [25], and may play an important role in protecting other enzymes or their products from oxidative damage possibly by association [164]. This view is supported by experimental evidence in which isocitrate lyase (ICL) retained its function after H₂O₂ challenge when cross linked to catalase [164].

Although catalase is an important enzyme in the metabolism of H₂O₂ its location in the matrix coupled to its low affinity for H₂O₂ reduces its efficiency in mopping up H₂O₂, implying that H₂O₂ may still diffuse into the cytosol particularly during oxidative burst [33, 34, 43]. This inefficiency is further compounded by the inhibition of CAT exerted by nitric oxide, and peroxynitrite, which is inevitable following increased production of ROS in response to particular stimuli [165, 166]. Additional defence is provided by the ascorbate –glutathione cycle (AA-GSH) which makes use of ascorbate and glutathione and four enzymes; APX, MDAR, DHAR and GR to inactivate H₂O₂ via a series of coupled redox reactions (**Table 2**) [29, 34, 154]. This cycle has a dual role as it also re-oxidises NAD(P)H to supply NAD(P)⁺ for the continuity of oxidative reactions occurring in peroxisomes such as β - oxidation which have been pointed out as key players in the generation of H₂O₂ [33]. Jimenez *et al.*, 1997 demonstrated the presence of this cycle in both the mitochondria and peroxisomes from pea leaves. The four enzymes of this cycle were present in peroxisomes isolated from pea leaves and also from tomato leaves and roots [167-169]. In addition the reduced and oxidised forms

of ascorbate and glutathione were detected in peroxisomes from pea leaves by HPLC analysis [169].

APX is localised on the peroxisomal membrane with its active facing the cytosol [33, 170-172]. However, contrary to the view that the active site for MDAR also faces the cytosol, recent work by Lisenbee *et al.*, suggests that the active site of the 54 kDa MDAR faces the peroxisomal matrix as the protein was completely protected from added protease [173]. This strategic arrangement ensures H₂O₂ would be degraded by the coordinated action of the two enzymes as it leaks from the peroxisomal matrix into the cytosol [33].

The characterisation of APX from cytosol and chloroplast is now at an advanced stage, with crystal structures solved for some of the isoforms [174-176]. However, progress in characterisation of the enzymes for the peroxisomal ASC-GSH cycle is still in its infancy with APX taking the lead. APX was identified in various plant species including pumpkin leaves (*Cucurbita pepo*) [177] [170], glyoxysomes from cotton seeds (*Gossypium hirsutum* L [171], pea leaf peroxisomes [137, 169], spinach (*Spinacia oleracea* L) glyoxysomes [172] and recently in glyoxysomes from castor bean [178], and the mechanism of targeting of this enzyme to peroxisomes has been studied in detail (see article by Mullen and Trelease, this volume).

A. thaliana has six APX genes; *APX1* to *APX6*. However, experimental data suggests that only *APX3* may have an important role in the peroxisomal ASC-GSH cycle. Overexpression of *AtAPX3* in tobacco transgenic plants increased protection against oxidative stress caused by aminotriazole, an inhibitor of catalase, suggesting *At APX3* is peroxisomal [179].

A. thaliana has four *MDAR* genes *MDAR1* to *MDAR4*, which have recently been characterised. Two isoforms; a 47 kDa protein (AtMDAR1) localised in the matrix targeted by a PTS1 and a 54kDa protein (AtMDAR4) localised in the peroxisomal membranes were identified [173]. This observation is in agreement with the analysis carried out by Leterrier *et al.*, 2005 in which a genomic clone of *MDAR1* from peas encoding a matrix targeted MDAR with a predicted MW of 47kDa and a presumptive PTS1 was localised in peroxisomes as indicated by confocal microscopy [180]. Previously MDAR was variously described as a 32kDa [135, 137, 169] or 47 kDa integral membrane protein in pea and castor bean [178]. Cloning of the genes responsible for these other MDAR activities should resolve the question as to whether these are different isoforms or simply reflect interspecies variation in the size of the protein.

The peroxisomal location of GR demonstrated by Jimenez *et al.*, 1997 [169], was recently confirmed by IEM and the protein of 56 kDa was purified [181]; indicating the role of peroxisomal GR in the AA-glutathione cycle). However of the three putative GR genes, none of these has been cloned and the protein demonstrated to be targeted to peroxisomes.

DHAR was also identified in peroxisomes from peas and tomato [167-169]; however, no candidate genes for the peroxisomal isoforms of these enzymes are yet known.

Glutathione- and thioredoxin-dependent peroxidases are found in multiple cellular compartments. GPX was purified from peroxisomes of rat hepatocytes [182] [183]. A glutathione peroxidase with activity towards alkyl hydroperoxides and H₂O₂ is found in the peroxisome of *Candida boidini* (CbPMP20) and is required for growth of this yeast on methanol [184]. A peroxidase (TcGPX1) that can be reduced by glutathione or trypanothione is found in glycosomes (specialised peroxisomes of trypanosomes that contain most of the

glycolytic pathway) [185] (see also article by Michels in this volume). The human and *Saccharomyces* homologues of CbPMP20 are thioredoxin dependent peroxidases. HsPMP20 bound the PTS1 import receptor via its non-canonical PTS –SQL and was partially located in peroxisomes [186]. There are two PMP20 homologues in the *Arabidopsis* genome, *AtTPX1* (At1g65980) and *AtTPX2* (At1g65970), and the protein encoded by *AtTPX2* was shown to possess thioredoxin-dependent peroxidase activity *in vitro* [187]. Whether these enzymes are also targeted to peroxisomes in *Arabidopsis* does not appear to have been tested experimentally. Whilst the *Candida* and *Saccharomyces* homologues have a PTS1 signal -AKL and -AHL respectively, the two *Arabidopsis* proteins and a rice homologue end with the sequence -KAL, which would not be expected to function as a PTS1.

It should be noted that the AA-GSH cycle is dependent on NAD(P)H for continuity and peroxisomes to be an efficient scavenger of ROS, should have a means of regenerating NAD(P)H [33]. This may be mediated by Glucose- 6- phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate-dehydrogenase (EC 1.1.1.44) and NADP⁺-dependent isocitrate dehydrogenase (EC 1:1.1.42) (**Table 2**) which were detected in peroxisomes of young and senescent pea leaves by immunoblotting and immunoelectron microscopy [188, 189]. *In silico* predictions [25], identified likely candidates for peroxisomal 6-phosphogluconate dehydrogenase and NADP⁺-dependent isocitrate dehydrogenase in *Arabidopsis*. Of the two Glucose-6-phosphate dehydrogenase enzymes in *Arabidopsis* one is plastidial and the other cytosolic. Whether one of these enzymes can also be targeted to peroxisomes remains to be established. However a putative 6-phosphogluconolactonase with a potential PTS1 has been identified (**Table 2**) and could provide 6-phosphogluconate for 6-phosphogluconate dehydrogenase, if 6-phosphogluconolactone can enter peroxisomes from the cytosol [25].

The scavenging activity of the AA-GSH cycle means that under normal circumstances the H_2O_2 produced is detoxified, as APX activity is twice as high as required to deal with the measured rate of H_2O_2 production in castor bean seeds [178]. However in conditions of stress more H_2O_2 is produced than is being degraded. This may escape into the cytosol via porin-like channels which were identified on peroxisomal membranes [111, 190]. The expression of genes for the AA-GSH is also induced by H_2O_2 as an up regulation of enzyme activity is noted in situations where plants experience stress [167, 168, 191, 192]. The overall residual amount of H_2O_2 available for signalling therefore depends on the activity of SOD, APX and CAT in the peroxisomes at any given time [178, 193].

During senescence peroxisomes may have a more protective role than mitochondria, as the AA-GSH cycle was sustained for a longer time in the peroxisomal matrix compared to mitochondrial matrix [121]. In addition there was a marked increase in the reduced and oxidised GSH pools in peroxisomes [121], an indication of an alteration in the redox state of the peroxisomes. This forms an important cellular signal arising from the AA-GSH cycle that is worth considering [41, 119, 154, 194]. The interaction of ROS and the AA-GSH cycle bring about changes in the ROS concentration as well as compartment specific differences in the redox status [41, 119, 194].

The level of AA, GSH as well as the ratios of GSSG/GSH, DHA/AA and NAD (P)⁺/NAD (P)H has a signalling and regulatory role [154]. Gene expression and signalling are usually altered by changes in the ratios of the redox couples which are usually stable under normal physiological conditions [44, 154]. The GSSG/GSH couple influences a number of physiological processes ranging from meristem formation, phytochelatin synthesis, flowering, and somatic embryogenesis to the transport of a variety of compounds including xenobiotics

and cellular signals such as NO [41, 154, 195]. Regulation of enzyme activity occurs via the oxidation of thiol groups and / or S-glutathionylation which is also important in signalling [154, 196, 197].

Changes in the NAD(P)H/ NAD(P)⁺ ratio determines the processing of ROS, as synthesis of signalling molecules such as nicotinamide, and calcium channel agonist cADPR as well as protein import into peroxisomes respond to changes in this ratio [154, 198, 199]. Alteration in the steady state ratio of AA/DHA affects the cell cycle, shoot growth in *A. thaliana*, enzyme activity, the expression of defence genes and the of phytohormones, gibberellins, abscisic acid and salicylic acid [44, 154, 194].

Although a change in the levels of enzymes for the AA-GSH was demonstrated during stress [121, 180], there is very limited evidence to link the changes in the redox potential to peroxisomes due to the communication existing between cellular compartments. Measuring such ratios is a complex process requiring rapid non aqueous fractionation techniques to avoid metabolic exchange between compartments. However, the availability of redox sensitive GFP and enzymes linked fluorescent probes may facilitate the estimation of peroxisomal thiol redox potential [154].

6. The role of Peroxisomes in generation of Reactive Nitrogen Species

NO is known to be an important signalling molecule in animals [200], and more recently in plants (reviewed in [201, 202]). Processes reported to be influenced by NO include root growth, photomorphogenesis, the hypersensitive response and programmed cell death, stomatal closure, flowering, pollen tube guidance and germination. The generation of NO in animals is catalysed by NOS (EC 1.14.13.39) which mediates the oxidation of arginine with

production of NO and citrulline in a reaction dependent on FAD, FMN, tetrahydrobiopterin (BH₄), calcium and calmodulin [202].

Plants have (at least) two pathways to produce NO; from nitrate via nitrate reductase and nitrite reductase or from arginine [203]. An additional pathway for NO production involving xanthine oxidase has also been identified, in which the peroxynitrite radical activates the conversion of xanthine dehydrogenase to xanthine oxidase [132, 204]. It has also been proposed that plant peroxisomes contain a NOS activity related to mammalian iNOS [205, 206]. This is based on measurement of NOS activity in highly purified peroxisome fractions from pea leaf by conversion of arginine to citrulline and also by direct chemiluminescent detection of NO. By these assays the NOS activity was strictly dependent on the presence of NADPH, calmodulin and tetrahydrobiopterin, as for the mammalian NOS, and sensitive to a range of inhibitors that also inhibit mammalian NOS. A characteristic EPR signal for NO was detected in purified peroxisomes using the spin trap Fe (MGD)₂. Furthermore NOS was localised to peroxisomes by immunogold electron microscopy and immunofluorescence using antibodies to mammalian iNOS [206, 207]. Interpretation of results obtained with anti-mammalian NOS antibodies need to be cautious as these antibodies have been demonstrated to cross react with a number of unrelated plant proteins [208]. However, independent support for the generation of NO by peroxisomes comes from a study of the role of NO in pollen tube guidance [209] where peroxisomes in living pollen tubes were shown to stain intensely with the NO specific probe 4,5-diaminofluorescein diacetate.

While genes or proteins with sequence similarity to animal NOS have not been identified in plants, *AtNOS1* a homolog of a *Helix pomatia* (snail) gene implicated in NO production was identified. Analysis of insertion mutants of *AtNOS1* which had impaired root growth, fertility

and germination had reduced NO production when compared with wild type. The protein showed similarity to GTPase domains and arginine-dependent production of NO was not dependent on FAD, FMNA, heme or BH₄ but was calcium, calmodulin and NADPH dependent, and was also inhibited by L-NAME an inhibitor of mammalian NOS activity [210]. However, AtNOS1 has been shown to be targeted to mitochondria [211].

The detection of NO in peroxisomes suggests that these organelles are an important source of NO and may play a role in NO signal transduction mechanisms [206, 209]. However it is currently unclear which of several possible candidate proteins are responsible for the production of peroxisomal NO [207, 212]. This will require biochemical and molecular characterisation of the enzyme(s) responsible for this activity and demonstration that the candidate protein is located in peroxisomes.

Once NO is formed it can freely diffuse into the cytosol to effect gene regulation, it can also conjugate to glutathione to form GSNO which serves as a carrier of the NO signal between cells [213, 214]. In addition the production of superoxide and the nitrite : nitrate ratio controls the level of NO available for signalling [215].

7. Peroxisomes and light signalling

Recent experimental evidence suggests that peroxisomes may have an important role in photomorphogenesis. *DET1*, *COP*, and *FUS* proteins act as global repressors of an array of genes involved in photomorphogenesis [216]. Mutants in *DET1*, a 62kDa nuclear protein, exhibit a phenotype typical of light grown plants when grown in the dark and vice versa. However, *ted3* a gain of function mutant of *PEX2* was isolated as a suppressor of the *det1-1*

mutant. While *det1-1* plants had defective peroxisomes, depended on sucrose for germination, and were IBA resistant; *ted3* rescued such mutants. *PEX2/TED3* is expressed in all tissues particularly cotyledons, pollen, ovules and seeds suggesting it has an important function in reproduction and development. This is further supported by the fact that null mutants were not isolated (presumed embryo lethal) and expression of antisense *PEX2/TED3* mRNA lead to sterility. The *ted3* mutant, also partially suppressed a de-etiolated *cop1* mutant suggesting that *PEX2/TED3* may have a central role in the photomorphogenesis pathway [28], although the mechanism by which it does so remains to be determined.

8. Peroxisomes and disease processes

Recent studies point towards an important role for peroxisomes in the process of infection by fungal pathogens. Conidia of powdery mildews germinate on the surface of leaves and within 24 hours develop appressoria, penetrate the cell cuticle and cell wall and form haustorial complexes (feeding structures) within the epidermal cells. At early stages of infection preceding and immediately following penetration, cytoplasm and organelles accumulate at sites of infection. Measurements using plants where the peroxisomes are tagged with a fluorescent protein suggest these organelles preferentially accumulate at such sites [217]. Clearly entry of the parasite is a critical stage in the establishment of infection. Normally, parasitic fungi only attack specific (host) species. However, Arabidopsis mutants have been isolated that permit penetration by fungal pathogens that are not normally invasive on Arabidopsis. *AtPEN1* encodes a SNARE protein, supporting a role in vesicular trafficking in non-host resistance. *AtPEN2* encodes a glycosyl transferase that is located in peroxisomes [27]. Peroxisomes containing PEN2 accumulate at infection sites and catalytic activity of PEN2 is required for resistance. It is postulated that PEN2 activity directly or indirectly

produces a product with broad range toxicity for normally non pathogenic fungal species [27]. Changes have also been reported in the activities of anti-oxidant enzymes and in the redox ratios of peroxisomes in tomato plants infected with the pathogen *Botrytis cinerea* [194].

Peroxisomes are also important for the ability of the fungal pathogen to mount a successful invasion. A non-pathogenic strain of *Colletotrichum lagenarium* was found to be deficient in the peroxisome biogenesis gene *PEX6* [218]. These mutants cannot form penetration hyphae and infect the plant. This may be due in part at least to inability to mobilise stored triacyl glycerol which is required to generate high turgor pressure to drive insertion of the penetration peg. In filamentous ascomycete fungi Woronin bodies are specialised forms of peroxisome [219]. Mutants of the rice blast fungus *Magnaporthe grisea* lacking the major Woronin body protein HEX1 were compromised in infectivity due to inability to survive nutritional stress [220]. Thus the metabolic and signalling capacities of both pathogen and host peroxisomes are likely to be important in establishing the outcome of infection.

9. Conclusions

Through the study of mutants involved in β -oxidation, clear evidence has recently emerged for an important role of this pathway and hence peroxisomes in shaping diverse processes in plant development [30] although much still has to be learned about the specific contribution of nutritional status and the roles of known and potentially yet unknown signalling molecules that might be the product of this pathway. Evidence is also beginning to emerge for a role of peroxisomes in establishing the outcome of infection by plant pathogens, and this will surely be an active area of future research, driven by the possibility of manipulating and improving plant defences to pathogens. The isolation of the gain of function mutation in *PEX2* as a suppressor of mutants in the photomorphogenesis pathway emphasises that peroxisomes talk

to the nucleus and other cell compartments, probably by a range of signals. The role of reactive oxygen and nitrogen species in signalling events underlying responses to both biotic and abiotic stress is well documented [36] but the specific contribution of peroxisomal enzymes and redox ratios are not currently well understood with the possible exception of catalase. This is because most of the enzymes involved are also present in other compartments and identifying the genes encoding the peroxisomal isoforms is still in progress [173, 179]. Although peroxisomal location can sometimes be inferred from the presence of putative PTS sequences, the occurrence of non-canonical PTS, the difficulty of predicting mPTS sequences accurately and the ability of proteins lacking a PTS to be piggybacked into peroxisomes by virtue of association with another protein that has a PTS [221], implying that correspondence between an enzyme activity or immunoreactive protein and its gene must be established experimentally. Once this has been achieved the individual contribution of these components can be tested via mutagenesis approaches.

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FIGURES AND LEGENDS

Figure 1: Signalling molecules generated in peroxisomes: Normal peroxisomal metabolism results in generation of signalling molecules which fall into four broad categories; β -oxidation derived signalling molecules which include jasmonates (jasmonic acid and its derivatives; methyl jasmonate, Z-jasmonate and tuberonic acid), reactive oxygen species, reactive nitrogen species and changes in peroxisomal redox state ([30, 33, 154, 202]. * The peroxynitrite radical is formed via a reaction of nitric oxide and superoxide radical [165].

Figure 2: A comparison of the jasmonic acid (JA) biosynthesis and eicosanoid catabolism as exemplified by leukotriene B₄.

JA synthesis (a) is initiated in the chloroplasts from either linoleic acid (18:3 (octadecanoid pathway) or hexadecatrienoic acid (16:3). The reactions occurring in the chloroplast terminate with the production of (9S, 13S)-12-oxo-phytodienoic acid (cis- (+) - OPDA) or dn OPDA, which are imported into peroxisomes via the action of an ABC transporter protein (CTS/PED3/PXA1) or by passive means. Once in the peroxisomes oxophytodienoate reductase (OPR3) catalyses the reduction of OPDA or dn OPDA (not shown) to yield 3-oxo-2-(2'-penenyl)-cyclopentane-1-octanoic acid (OPC: 8) or OPC:6 [30]. OPC: 8 or OPDA is then esterified to an acyl CoA probably via the action of by 4 coumarate CoAligase (4CL)-like acyl activating enzymes [25, 72]. This is followed by the sequential action of beta oxidation enzymes acyl CoA oxidase 1 (ACX1), and possibly multifunctional proteins and 2-keto acyl CoA thiolase (KAT2) in three rounds of beta oxidation. The release of JA from its co-ester is thought to be mediated by a thioesterase [30].

On the other hand the catabolism of eicosanoids (b) such as leukotriene B₄ (LTB₄) is initiated in the microsomes where ω -oxidation results in the formation of 20 -carboxy-LTB₄ [91]. The mechanism of import for the ω -carboxy acids into peroxisomes is still unknown however recent findings suggest that ALDP is not involved [92]. Once inside peroxisomes 20 carboxy-LTB₄- is presumably activated to its CoA ester. The metabolite 20 carboxy-LTB₄-CoA also undergoes beta oxidation via the sequential action of either straight chain acyl oxidase (SCOX) or branched chain acyl oxidase (BCOX), the D-bifunctional protein (D-BFP) which has hydratase and dehydrogenase activity. Finally keto acyl CoA thiolases or sterol carrier protein X (SCPx) catalyses the thiolitic cleavage to yield 18-carboxy-19,20-dinor LTB₄ CoA which either enters a second round of beta oxidation to yield 16 carboxy-14,15-dihydro-17,18,19,20 tetranor LTB₄. Thioesterase are also thought to catalyse the release of these metabolites from their co-esters [91, 92, 222]. While the biosynthesis of JA converts one biologically active signalling molecule to another one with different signalling roles [55, 71, 84],

the catabolism of eicosanoids produces compounds of altered biological activity which are excreted in urine or bile [91]. Biologically active molecules with signalling roles are highlighted in blue.

Table 1 Enzymes involved in the production of hydrogen peroxide and superoxide in plant peroxisomes.

| Enzyme (<i>gene</i>) | Reaction | Pathway | Method of localisation | Arabidopsis gene and (putative) PTS | Ref |
|---|---|-----------------------------------|---|-------------------------------------|-------------------------|
| Medium-long chain Acyl CoA oxidase (<i>ACX1</i>)* | Acyl CoA + FAD → Enoyl CoA + FADH ₂ FADH ₂ + O ₂ → FAD + H ₂ O ₂ | β-oxidation | Presumed based on presence of PTS and demonstration of β-oxidation of palmitoyl CoA by Ricinus glyoxysomes. | At4g16760 ARL> | [25, 77, 223], |
| Long chain Acyl CoA oxidase (<i>ACX2</i>)* | Same as for ACX1 | β-oxidation | Cell fractionation (pumpkin homologue) | At5g65110 RIX ₅ HL | [25, 223, 224] |
| Medium chain Acyl CoA oxidase (<i>ACX3</i>)* | Same as for ACX1 | β-oxidation | Presumed based on presence of PTS | At1g06290 RAX ₅ HI | [25, 223, 225, 226] |
| Short chain Acyl CoA oxidase (<i>ACX4</i>)* | Same as for ACX1 | β-oxidation | Cell fractionation and immunolocalisation | At3g51840 SRL> | [25, 223, 227] |
| Glycolate oxidase (<i>GOX1</i>) | 2 Glycolate + O ₂ → 2 Glyoxylate + H ₂ O ₂ | Photorespiration | Cell fractionation, various species | At3g14420 ARL> | [4, 15, 25, 147] |
| Glycolate oxidase (<i>GOX2</i>) | 2 Glycolate + O ₂ → 2 Glyoxylate + H ₂ O ₂ | Photorespiration | Cell fractionation, various species | At3g14415 PRL> | [4, 15, 25, 147] |
| Sulfite oxidase (<i>SO</i>)* | SO ₃ ²⁻ + O ₂ + H ₂ O → SO ₄ ²⁻ + H ₂ O ₂ | Sulfur assimilation | IEM & GFP fusion, | At3g01910* SNL> | [149, 150] |
| Sarcosine oxidase (<i>SOX</i>)* | Sarcosine + H ₂ O + O ₂ → Glycine + HCHO + H ₂ O ₂ | Sarcosine & piperolate metabolism | In vitro import | At2g24580 *? | [153] |
| Xanthine oxidase | Xanthine + O ₂ → Uric acid + H ₂ O ₂ + O ₂ ⁻ | Purine metabolism | Biochemical measurement in purified peroxisomes ESR | ND | [125-128, 130] |
| Urate oxidase (Uricase) | Uric acid + O ₂ → Allantoin + H ₂ O ₂ + CO ₂ + O ₂ ⁻ | Purine metabolism | Biochemical measurement in purified peroxisomes | At2g26230 SKL> | [125-128, 130] |
| Cu-Zn Superoxide dismutase (<i>CSD3</i>) | O ₂ ⁻ → H ₂ O ₂ | Dismutation of superoxide | Biochemical measurement in purified peroxisomes IEM | At5g18100 AKL> | [144-146] [6, 143, 228] |
| Mn Superoxide dismutase | O ₂ ⁻ → H ₂ O ₂ | Dismutation of superoxide | Biochemical measurement in purified peroxisomes IEM | ND | [139, 141, 142, 229] |

| | | | | | |
|--|--|-----------------------|---|----|-----------------|
| PMP32 (presumptive monodehydroascorbate reductase) | Transfer of electrons from NADH to O ₂ directly or via PMP18 with formation of O ₂ ^{•-} | Re-oxidation of NADH | Biochemical measurement in purified peroxisomes | ND | [134, 137, 138] |
| PMP18 (<i>b</i> -type cytochrome?) | Transfer electrons from MDAR to O ₂ with formation of O ₂ ^{•-} | Re-oxidation of NADH | Biochemical measurement in purified peroxisomes | ND | [134, 137, 138] |
| PMP29 | Transfer electrons from NADPH to O ₂ with formation of O ₂ ^{•-} | Re-oxidation of NADPH | Biochemical measurement in purified peroxisomes | ND | [134, 137, 138] |

Abbreviations: PMP, peroxisomal membrane protein; ND Not determined ; GFP; green fluorescent protein ; IEM, immunogold labelling electron microscopy; ESR; Electro spin resonance spectroscopy. * indicates gene cloned and characterised at the molecular level.

Table 2. Peroxisomal enzymes involved in scavenging ROS.

| Enzyme (<i>gene</i>) | Reaction | Pathway | Method of localisation to plant peroxisomes | Arabidopsis gene and (putative) PTS | References |
|---|---|---|--|--|----------------------|
| Catalase (<i>CAT1</i>)* | $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ | decomposition of H_2O_2 | In vivo immunofluorescence Immunocytochemical analysis. | At1g20630 | [156, 158, 230-232] |
| Catalase (<i>CAT2</i>)* | $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ | decomposition of H_2O_2 | Immunocytochemical analysis and immunolocalisation, | At4g35090 | [156, 158, 230] |
| Catalase (<i>CAT3</i>) | $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ | decomposition of H_2O_2 | Immunocytochemical analysis and immunolocalisation | At1g20620 | [156, 158, 230] |
| Ascorbate peroxidase (<i>APX3</i>)* | $\text{H}_2\text{O}_2 + \text{ASC} \rightarrow \text{MDA} + \text{H}_2\text{O}$ | Ascorbate glutathione cycle | Cell fractionation and immunofluorescence | At4g35000 mPTS TMD + basic cluster | [169, 171, 173, 178] |
| Mono-dehydroascorbate reductase (<i>MDAR1</i>)* | $\text{MDA} + \text{NAD(P)H} \rightarrow \text{ASC} + \text{NAD(P)}^+$ | Ascorbate glutathione cycle | Western blot on purified peroxisomes & in vivo immunofluorescence | At3g52880* AKI> | [173, 180] |
| Mono-dehydroascorbate reductase (<i>MDAR4</i>)* | $\text{MDA} + \text{NAD(P)H} \rightarrow \text{ASC} + \text{NAD(P)}^+$ | Ascorbate glutathione cycle | Western blot on purified peroxisomes & in vivo immunofluorescence | At3g27820* mPTS TMD + basic cluster | [173] |
| Glutathione Reductase | $\text{GSSG} + \text{NAD(P)H} \rightarrow \text{GSH} + \text{NAD(P)}^+$ | Ascorbate glutathione cycle | Biochemical measurement in purified peroxisomes IEM | ND | [181] |
| Glucose 6 phosphate dehydrogenase | $\text{Glucose-6-P} + \text{NAD(P)}^+ \rightarrow \text{6-P-gluconolactone} + \text{NADPH}$ | Oxidative Pentose Phosphate pathway | immunoblotting and immunoelectron microscopy , biochemical measurement in purified peroxisomes | ND | [25, 189] |
| 6-Phosphogluconate dehydrogenase | $6\text{-P-Gluconate} + \text{NAD(P)}^+ \rightarrow \text{Rubulose - 5-phosphate} + \text{NADPH} + \text{CO}_2$ | Oxidative Pentose Phosphate pathway | immunoblotting and immunoelectron microscopy, biochemical measurement in purified peroxisomes | At3g02360 SKI> | [25, 189] |
| NADP-Isocitrate dehydrogenase | $\text{Isocitrate} + \text{NAD(P)}^+ \rightarrow 2 \text{oxoglutarate} + \text{NAD(P)H} + \text{CO}_2$ | Regeneration of NADP^+ | immunoblotting and immunoelectron microscopy biochemical measurement in purified peroxisomes | At1g54340 SRL> | [25, 188] |
| 6-Phosphogluconolactonase | $6\text{-P-Gluconolactone} + \text{H}_2\text{O} \rightarrow 6\text{-P-gluconate} + \text{H}^+$ | Oxidative Pentose Phosphate pathway | ND | At5g24400 SKL> | [25] |

Abbreviations: ND, not determined; IEM, immunogold labelling electron microscopy; * represent genes which have been cloned and characterised at a molecular level