

# A newly discovered function of peroxisomes: involvement in biotin biosynthesis

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In plants, peroxisomes are the organelles involved in various metabolic processes and physiological functions including  $\beta$ -oxidation, mobilization of seed storage lipids, photorespiration, and hormone biosynthesis. We have recently shown that, in fungi and plants, peroxisomes play a vital role in biosynthesis of biotin, an essential cofactor required for various carboxylation and decarboxylation reactions. In fungi, the mutants defective in peroxisomal protein import exhibit biotin auxotrophy. The fungal BioF protein, a 7-keto-8-aminopelargonic acid (KAPA) synthase catalyzing the conversion of pimeloyl-CoA to KAPA in biotin biosynthesis, contains the peroxisomal targeting sequence 1 (PTS1), and its peroxisomal targeting is required for biotin biosynthesis. In plants, biotin biosynthesis is essential for embryo development. We have shown that the peroxisomal targeting sequences of the BioF proteins are conserved throughout the plant kingdom, and the *Arabidopsis thaliana* BioF protein is indeed localized in peroxisomes. Our findings suggest that peroxisomal localization of the BioF protein is evolutionarily conserved among eukaryotes, and required for biotin biosynthesis and plant growth and development.

## The Roles of Peroxisomes and the Molecular Mechanisms for Peroxisomal Protein Import in Plants

Peroxisomes are the eukaryotic organelles involved in various metabolic processes including fatty acid  $\beta$ -oxidation and secondary metabolisms. In plants, peroxisomes change their functions in response to developmental stages and environmental conditions. In germinating seedlings, peroxisomes act as glyoxysomes that execute glyoxylate cycle for storage lipid mobilization.<sup>1</sup> During seedling transition, peroxisomes degrade enzymes required for the glyoxylate cycle and accumulate enzymes involved in photorespiration.<sup>2</sup> In leaf, peroxisomes share metabolic processes with chloroplast and mitochondria to exhibit photorespiration.<sup>3</sup> Peroxisomes are also required for biosynthesis of plant hormones including jasmonic acid and auxin.<sup>4,5</sup> Loss of peroxisomal

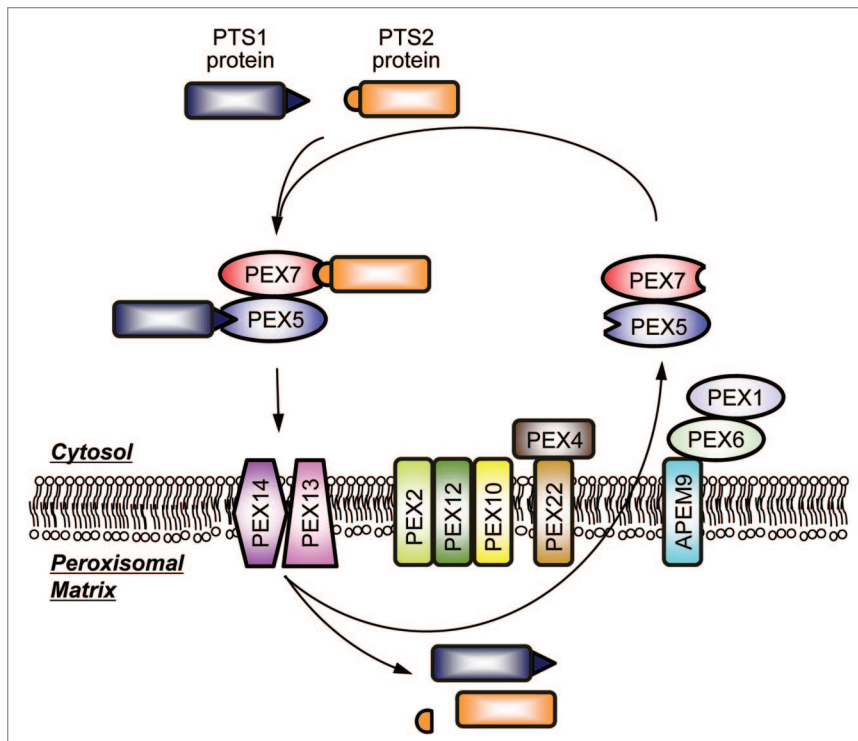
functions causes embryo lethality in plants, suggesting the importance of peroxisomes in plant development.<sup>6–10</sup>

Figure 1 shows a schematic diagram of peroxisomal matrix protein import in plants. Peroxisomal matrix proteins are delivered from cytosol into peroxisomes by a series of the proteins called peroxins (PEX). There are two types of the peroxisomal targeting signals (PTS) for the import of peroxisomal matrix proteins: PTS1, a conserved tripeptide motif at the C-terminus, and PTS2, a nonapeptide with the motif R-L-X<sub>5</sub>-H-L typically located within 30 amino acids from the N-terminus. PTS1 and PTS2-containing proteins are recognized by the receptors PEX5 and PEX7, respectively, which interact with each other and import the proteins into peroxisomal matrix.<sup>11–14</sup> The PEX5/7-PTS protein complexes are docked on the peroxisomal membrane via binding with the PEX13 and PEX14.<sup>11,15</sup> In the peroxisomal matrix, PTS2 is removed by the activities of the proteases DEG15<sup>16,17</sup> and LON2.<sup>18</sup> After the translocation into peroxisomal matrix, the receptors appear to be relocated to cytosol for another round of the import process.<sup>19</sup> It is hypothesized that PEX4, a cytosolic ubiquitin-conjugating enzyme, and the ubiquitin ligases PEX2, PEX10, and PEX12 cooperatively promote the receptor recycling by monoubiquitination of PEX5.<sup>20</sup> PEX4 is anchored to the peroxisomal membrane by interaction with PEX22.<sup>21,22</sup> Two AAA ATPases PEX1 and PEX6, which are tethered to peroxisome via interaction with ABERRANT PEROXISOME MORPHOLOGY9 (APEM9),<sup>23</sup> are also likely to mediate relocation of the receptors to cytosol.<sup>24</sup> PEX4, PEX5, PEX6, and PEX22 are also known to be involved in selective degradation of the glyoxylate cycle enzymes.<sup>25</sup>

## Involvement of Peroxisomes in Biotin Biosynthesis in Fungi

In fungi, peroxisomes are essential for various metabolic processes and physiological functions. Their primary role is  $\beta$ -oxidation of fatty acids: the mutants lacking peroxisomes fail to grow on the minimal medium containing fatty acids as carbon source in the budding yeast *Saccharomyces cerevisiae*.<sup>26</sup> In the filamentous fungus *Aspergillus nidulans*, similar observations were reported in the studies of the mutants lacking the peroxin genes.<sup>27</sup> Peroxisomes are also required for methanol metabolism in the methylotrophic yeasts including *Pichia pastoris*.<sup>28</sup> In filamentous fungi, peroxisomes are involved in secondary metabolism including penicillin biosynthesis,<sup>29</sup> plant pathogenicity,<sup>30,31</sup> sexual development,<sup>32,33</sup>

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**Figure 1.** Schematic diagrams of the peroxisomal matrix protein import.

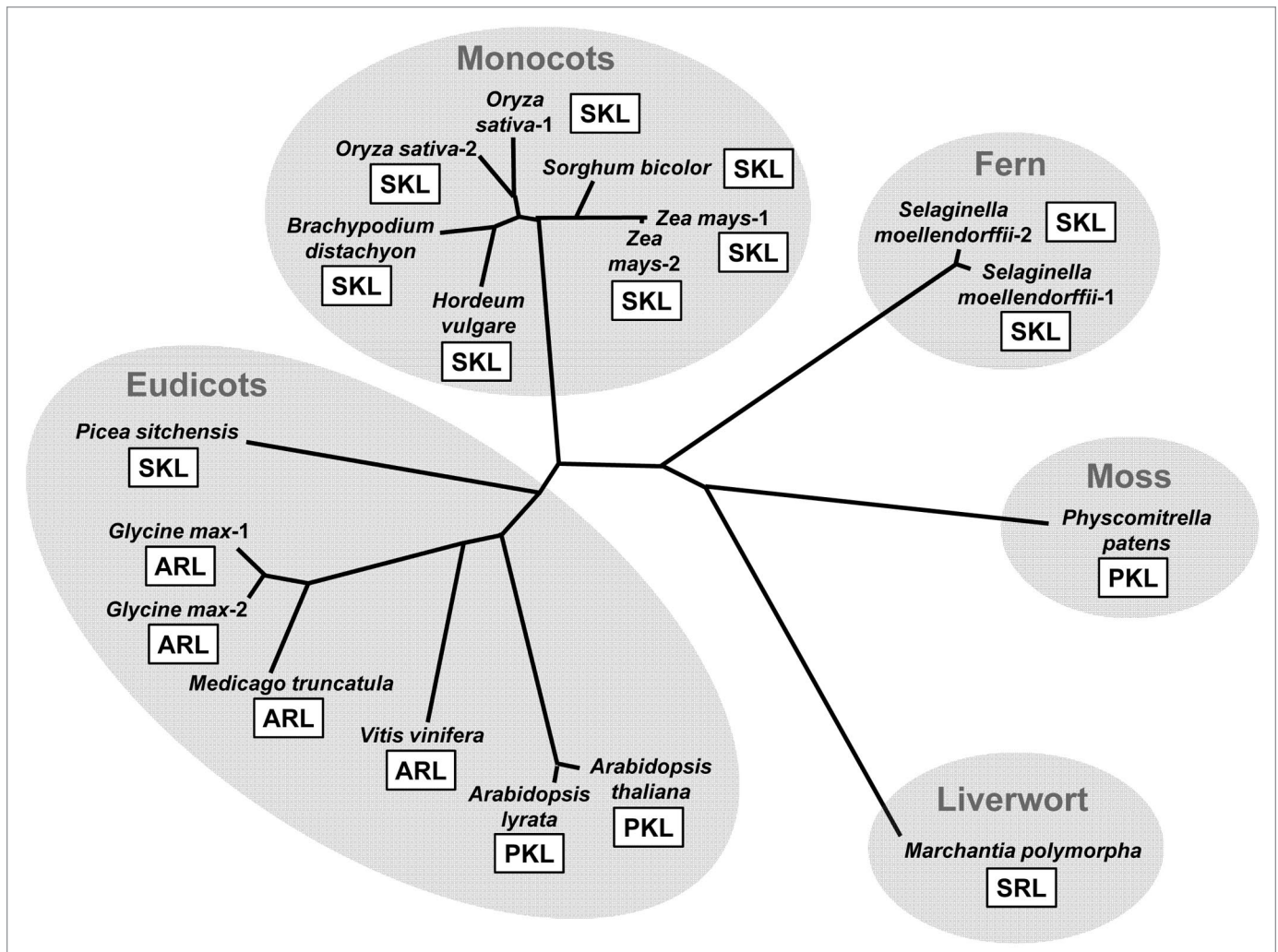
and formation of the Woronin body, an organelle for wound healing.<sup>34,35</sup> We have reported that the Pex11 protein, which is required for peroxisomal division, is involved in the formation of Woronin body from peroxisomes in the filamentous fungus *Aspergillus oryzae*.<sup>35</sup> To further clarify the role of peroxisomal proteins in the Woronin body formation, we generated *Aspergillus* mutants of which the *Aopex5* and *Aopex7* genes are disrupted. They are defective in protein import into the peroxisomal matrix due to the lack of the PTS1 and PTS2 receptors, respectively.<sup>36</sup> These gene disruptants failed to grow on the minimal medium containing oleic acid as the sole carbon source due to the defects in peroxisomal  $\beta$ -oxidation. Unexpectedly, we found that they also exhibit growth deficiency on the minimal medium containing glucose as the sole carbon source, which is restored by addition of biotin and its biosynthetic precursors.<sup>36</sup> It has been suggested that, in fungi, biotin is synthesized through the sequential activities of the Bio proteins: BioF, a 7-keto-8-aminopelargonic acid (KAPA) synthase catalyzing the conversion of pimeloyl-CoA to KAPA, BioD/A, a chimeric protein of a 7,8-diaminopelargonic acid (DAPA) synthase and a desthiobiotin (DTB) synthase which converts KAPA to DTB, and BioB, a biotin synthase catalyzing the conversion of DTB to biotin.<sup>36-38</sup> We have shown mitochondrial localization of the *Aspergillus* BioD/A protein, suggesting that mitochondria are the site for the conversion of KAPA to biotin.<sup>36</sup> The *Aspergillus* BioF protein possesses the PTS1 sequence at the C terminus, and its peroxisomal targeting is required for biotin biosynthesis.<sup>36</sup> Our study suggests that peroxisomes are functionally coupled with mitochondria and involved in biotin biosynthesis.

## Involvement of Biotin Biosynthesis in Plant Embryo Development

Plants are the other eukaryotic organisms that synthesize biotin. Molecular genetic studies using the *Arabidopsis* mutants auxotrophic for biotin have revealed that biotin biosynthesis is vital for plant growth and development. A forward genetic screen isolated an embryo-lethal mutant, *bio1-1*, of which embryogenesis is arrested during globular to mature-cotyledon stages.<sup>39,40</sup> The arrested embryo of the *bio1-1* mutant plant accumulates virtually no detectable biotin,<sup>41</sup> and is rescued by the exogenous addition of biotin as well as DTB.<sup>39</sup> The *bio1-1* mutant phenotype was complemented by a transgene containing the *Escherichia coli bioA* gene, indicating that the *BIO1* gene encodes a DAPA synthase.<sup>42</sup> In the *Arabidopsis* genome, the *BIO1* gene is positioned immediately downstream of the *BIO3* gene encoding a DTB synthase.<sup>43</sup> Genetic complementation analysis revealed that the *bio1* and *bio3* mutants define a single locus which exhibits a chimeric gene transcript.<sup>43</sup> A subsequent study demonstrated that this locus produces a chimeric protein of

*BIO3* and *BIO1*, which has a bifunctional enzyme activity catalyzing both of DTB synthase and DAPA synthase reactions.<sup>44</sup> The *BIO2* gene encodes a biotin synthase, which catalyzes the final step of biotin biosynthesis, and the *bio2* mutation causes embryo lethality.<sup>40,45</sup> While the *bio1* mutant embryos abort at the torpedo or later stages, more than 50% of the *bio2* mutant embryos abort at the early globular stage, implying that the *bio2* mutation has more severe impact on embryo development than that of the *bio1* mutation.<sup>40</sup> These observations consistently suggest that biotin biosynthesis plays a vital role during embryo development in plants.

Recent studies have demonstrated that, in plants, the final three reactions of the biotin synthesis are likely to occur in mitochondria. The *BIO2* protein contains a mitochondrial targeting sequence (MTS) at its N terminus.<sup>45-49</sup> Biochemical studies showed that the recombinant *BIO2* protein produced in *E. coli* is localized in the mitochondrial fraction from pea (*Pisum sativum*) leaf extracts,<sup>48</sup> and its biotin synthase activity is detectable only in the mitochondrial fraction from pea leaf and potato (*Solanum tuberosum*) extracts.<sup>49</sup> A molecular genetic study demonstrated that a truncated version of the *BIO2* protein lacking MTS was not able to complement the *bio2* mutant phenotype, regardless of exogenously added DTB, a substrate of the biotin synthase, suggesting that mitochondrial localization, rather than substrate availability, is required for the *BIO2* activity.<sup>45</sup> Recent studies showed that the *BIO3-BIO1* fusion protein contains MTS at its N terminus,<sup>43,44</sup> and biochemical analysis and fluorescent protein-based microscopic observations demonstrated its mitochondrial localization.<sup>44</sup> These observations suggest that mitochondria are

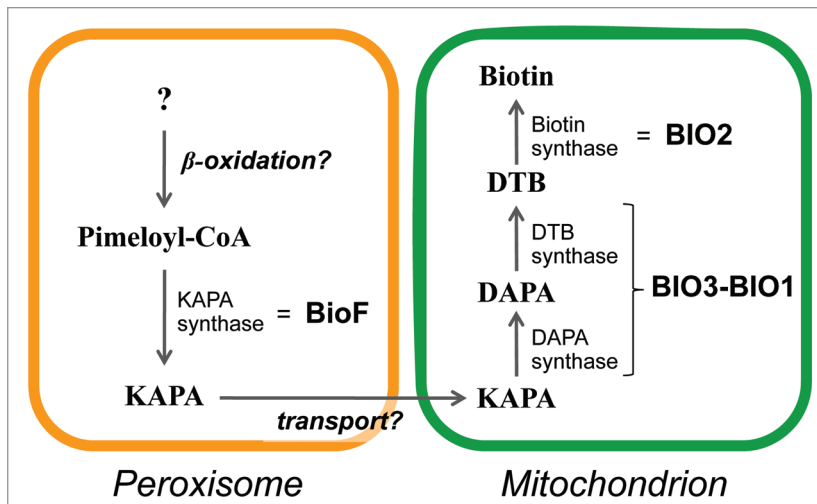


**Figure 2.** Phylogenetic relationship of the plant KAPA synthases. The amino acid residues of their C-terminal peroxisomal targeting signals (PTS1) are also shown with open squares. The full-length amino acid sequences of the plant KAPA synthases were aligned using Clustal W program (version 2.1) and the unrooted phylogenetic tree was constructed using the neighbor-joining method. The GenBank accession numbers for the sequences retrieved are as follows: *Arabidopsis thaliana*, NP\_974731.1; *Arabidopsis lyrata*, XP\_002871105.1; *Oryza sativa-1*, BAD87813.1; *Oryza sativa-2*, NP\_001065381.1; *Hordeum vulgare*, BAK03504.1; *Brachypodium distachyon*, XP\_003574335.1; *Sorghum bicolor*, XP\_002467492.1; *Zea mays-1*, ACG35792.1; *Zea mays-2*, ACG35881.1; *Selaginella moellendorffii-1*, XP\_002969752.1; *Selaginella moellendorffii-2*, XP\_002981364.1; *Physcomitrella patens*, XP\_001769874.1; *Picea sitchensis*, ABR18106.1; *Vitis vinifera*, XP\_002268950.1; *Medicago truncatula*, XP\_003598166.1; *Glycine max-1*, XP\_003527547.1; *Glycine max-2*, XP\_003522881.1. The peptide sequence of the putative *Marchantia polymorpha* KAPA synthase was confirmed by PCR amplification and sequencing of its cDNA based on the information from the *Marchantia* expression sequence tag database (S. Yamaoka, unpublished data).

the main sites for a series of the reactions converting KAPA to biotin in plants.

In contrast to the other three enzymes, the KAPA synthase has been less characterized in plants. Pinon et al. (2005) showed that the *AtBioF* gene encodes an ortholog of the bacterial KAPA synthase, which is able to complement an *E. coli* mutant deficient in KAPA synthase activity.<sup>50</sup> They also demonstrated that the recombinant AtBioF protein purified from *E. coli* extracts has the catalytic properties of KAPA synthase. Using the AtBioF protein of which C terminus is fused to GFP, they concluded AtBioF as a cytosolic enzyme.<sup>50</sup> We found that the AtBioF protein contains a PTS1 sequence at its C terminus.<sup>36</sup> While the C-terminal fusion of GFP allowed AtBioF to be

cytosolic as shown in the previous study, the N-terminal fusion of GFP showed co-localization of AtBioF with a fluorescent peroxisomal marker, and the PTS1-deleted version of AtBioF was diffused throughout the cytoplasm, suggesting that AtBioF is localized to peroxisome by the activity of its PTS1 sequence.<sup>36</sup> In addition, our phylogenetic analysis revealed that the orthologs of AtBioF from various plant species possess the PTS1 sequences at their C termini (Fig. 2), suggesting that the peroxisomal localization of the BioF protein is conserved throughout the plant kingdom. Our findings suggest that the plant BioF protein is an evolutionally conserved peroxisomal enzyme. The reaction product KAPA is likely to be transported from peroxisome to mitochondria as a substrate for the subsequent series of



**Figure 3.** A model of the biotin biosynthetic pathway in plants. KAPA, 7-keto-8-amino-pelargonic acid; DAPA, 7, 8-diaminopelargonic acid; DTB, desthiobiotin.

the biotin biosynthesis reactions. Thus, metabolic interaction between peroxisomes and mitochondria is required for biotin biosynthesis in plants, which in turn may play a vital role during plant embryo development.

### Conclusion and Perspective

We proposed a new model for the biotin biosynthesis in plants (Fig. 3). In peroxisomes, KAPA is synthesized from

synthesis and peroxisomal functions during plant growth and development.

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