Review

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Understanding the evolution of endosymbiotic organelles based on the targeting sequences of organellar proteins

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Summary

Organellogenesis, a key aspect of eukaryotic cell evolution, critically depends on the successful establishment of organellar protein import mechanisms. Phylogenetic analysis revealed that the evolution of the two endosymbiotic organelles, the mitochondrion and the chloroplast, is thought to have occurred at time periods far from each other. Despite this, chloroplasts and mitochondria have highly similar protein import mechanisms. This raises intriguing questions such as what underlies such similarity in the import mechanisms and how these similar mechanisms have evolved. In this review, we summarise the recent findings regarding sorting and specific targeting of these organellar proteins. Based on these findings, we propose possible evolutionary scenarios regarding how the signal sequences of chloroplasts and mitochondrial proteins ended up having such relationship.

I. Introduction

The endosymbiosis of the chloroplast and mitochondrion from endosymbiotic cyanobacterium and α-proteobacterium, respectively, is the most intriguing event during eukaryotic cell evolution (Gray, 2012; Zimorski *et al.*, 2014). During the conversion of endosymbiotic bacteria to organelles, one of the most crucial events is the successful set-up of the protein targeting mechanisms (Dyall *et al.*, 2004; Martin, 2010; Gross & Bhattacharya, 2011; Baudisch *et al.*, 2014). These targeting mechanisms for a new organelle can arise either *de novo* or by modifying pre-existing mechanisms. In either case, a key issue is how targeting specificity is attained, which is critically dependent on cellular conditions such as the pre-existing organelles and the nature of endosymbionts. It is likely that protein targeting mechanisms were gradually established according to these conditions over a long period of time. Moreover, once the

targeting mechanisms have been established, they may not change easily due to the complexity of the system. Thus, elucidation of protein targeting mechanisms may provide new insights into the endosymbiosis of chloroplasts and mitochondria. Numerous studies on the protein targeting mechanism of chloroplasts and mitochondria revealed many important features of signal sequences and the roles of the protein import machinery. One of the most intriguing features is that the protein-targeting mechanisms of chloroplasts and mitochondria show a high degree of similarity (Schleiff & Becker, 2011).

In this review, we present the recent progress on the nature of the targeting signals and how they can support specific protein import into chloroplasts and mitochondria in plant cells. Based on these findings, we propose the possible scenarios for the evolution of targeting signals, and also discuss the implication of the relationship between transit peptides and presequences, targeting signals of chloroplast and mitochondrial interior proteins, respectively, to the evolution of these organelles.

II. Sequence determinants that ensure specific targeting of chloroplast and mitochondrial proteins in plant cells

Bioinformatic analyses of hundreds of transit peptides of chloroplast proteins and presequences of mitochondrial proteins revealed overall similarities in amino-acid composition between these two targeting signals despite the great diversity in the primary structure (Bhushan *et al.*, 2006). Both transit peptides and presequences do not have any consensus sequences. In fact, the diversity among transit peptides is as high as that between transit peptides and presequences. Systematic sequence analysis revealed that multiple small sequence motifs that are dispersed throughout the entire region in both signal sequences are functional units that play critical roles during protein import into the target organelles (Lee *et al.*, 2012; Li & Teng, 2013; Lee & Hwang, 2018). Moreover, the processes for protein import into chloroplasts and mitochondria are mechanistically similar (Schleiff & Becker, 2011). Then, how do transit peptides and presequences ensure the specific targeting to their organelles? This question is particularly relevant to plant cells where both organelles exist in contrast with animal cells and fungi with only mitochondria. Thus, in plant cells, the transit peptide and presequence must have evolved to ensure the specific delivery of proteins to chloroplasts and mitochondria, respectively. Despite the high degree of similarity between the signal sequences, one noticeable difference is that the N-terminal regions in transit peptides and presequences have moderate hydrophobicity and multiple arginine residues (MAR), respectively (Lee *et al.*, 2006, 2019; Garg & Gould, 2016). Indeed, the average charge and amino acid composition of the N-terminal regions can be used as the main parameters to predict the targeting specificity of a signal sequence to chloroplasts, mitochondria, or both (Ge *et al.*, 2014; Garg & Gould, 2016).

The exact determinants of the targeting signals and the mechanisms underlying the specific delivery of proteins to chloroplasts or mitochondria have been elusive for a long period of time. A recent study revealed the sequence determinants for the specific delivery of proteins to chloroplasts and mitochondria (Lee *et al.*, 2019). Intriguingly, transit peptides and presequences have an intimately close relationship as follows: first, both targeting signals can be divided into two functional domains, the N-terminal specificity domain (NSD) and C-terminal translocation domain (CTD) (Fig. 1). Second, the NSD of both targeting signals function as sequence determinants to confer targeting specificity to either chloroplasts or mitochondria (Fig. 1) (Garg & Gould, 2016; Lee *et al.*, 2019). For transit peptides, moderate hydrophobicity at the N-terminal region is sufficient to specify protein import into chloroplasts. By contrast, the NDS of presequences contain two critical sequence motifs, MAR and a moderately hydrophobic sequence motif (MHSM). Indeed, MAR at the N-terminal region is a prominent feature of presequences (Bhushan *et al.*, 2006; Garg & Gould, 2016) (Fig. 1). Surprisingly, the removal of MAR from presequences was sufficient to switch the targeting specificity from mitochondria to chloroplasts (Fig. 2) (Lee *et al.*, 2019). This is analogous to trafficking by the default pathway in the endomembrane system; removal of a vacuolar sorting motif from vacuolar proteins causes secretion out of the cell because of the hierarchical relationship between secretion and vacuolar trafficking (Matsuoka & Nakamura, 1991). Conversely, incorporating MAR into the N-terminal region of transit peptides prevented chloroplast targeting, and caused cytosolic localisation of client proteins. Thus, MAR in presequences behave as a chloroplast-evading signal. Additional incorporation of the MHSM into transit peptides changes the targeting specificity from chloroplasts to mitochondria (Fig. 2) (Lee *et al.*, 2019; McKinnon & Theg, 2019). However, the MHSM alone cannot affect the targeting specificity of transit peptides. Thus, the presence and absence of MAR function as the main determinant in specific protein targeting to mitochondria and chloroplasts, respectively. Third, the CTD of both targeting signals is remarkably interchangeable for targeting to chloroplasts or mitochondria. For instance, the CTD of FA[N77] (N-terminal 77 amino acids of Arabidopsis F1-ATPase γ-subunit) functioned as a competent transit peptide if the NSD was replaced with that of a transit peptide. Also, the CTD of transit peptides was competent for translocation across mitochondrial membranes. Moreover, the sequence motifs required for chloroplast import (Lee *et al.*, 2006) were also critical for mitochondrial import (Lee *et al.*, 2019), indicating that sequences motifs in the transit peptides are recognised by mitochondrial import machinery, and vice versa. These results suggest that critical sequence motifs are functionally conserved between the transit peptides and presequences. However, it is not known whether the sequence motifs are conserved at the amino acid sequence level, and this needs to be studied further in the future. Indeed, mitochondrial proteins can be imported into chloroplasts in the *in vitro* import system (Hurt *et al.*, 1986). These results strongly suggest that, although the molecular machineries for protein import into chloroplasts and mitochondria differ from each other (Schleiff & Becker, 2011), the mitochondrial machinery can recognise the sequence motifs in transit peptides to translocate proteins across mitochondria envelopes and vice versa. Thus, this study (Lee *et al.*, 2019) provides a clear answer to the long-lasting question of how

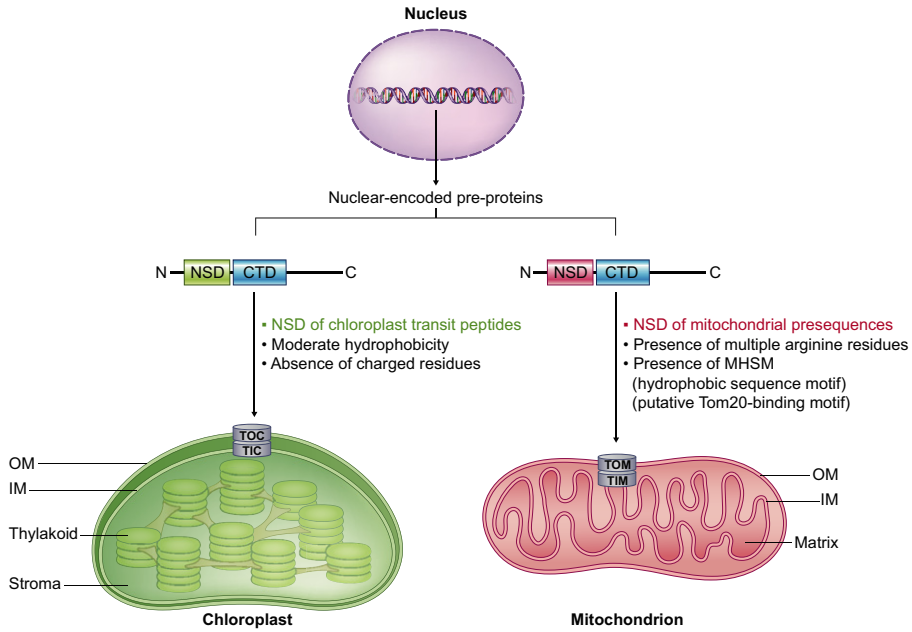

Fig. 1 A working model of how preproteins are specifically imported into chloroplasts and mitochondria in plant cells. Most chloroplast and mitochondrial proteins are encoded in the nuclear genome. After translation, chloroplast and mitochondrial interior proteins are thought to be sorted in the cytosol. The targeting signals of both organellar proteins are composed of two domains, namely, the N-terminal specificity domain (NSD) and the C-terminal translocation domain (CTD). The CTDs of both organellar proteins are interchangeable. Conversely, the NSD of both targeting signals determines targeting specificity of these organellar proteins. The NSD of chloroplast transit peptides generally avoids charged amino acids, but instead favours moderate hydrophobicity for efficient targeting. In the case of mitochondrial presequences, the existence of both multiple arginine residues (MAR) and the moderately hydrophobic sequence motif (MHSM) is crucial for specific protein import into mitochondria. IM, inner membrane; OM, outer membrane; TOC/TIC, translocon at the outer/inner envelope of chloroplasts; TOM/TIM, translocase of outer/inner membrane.

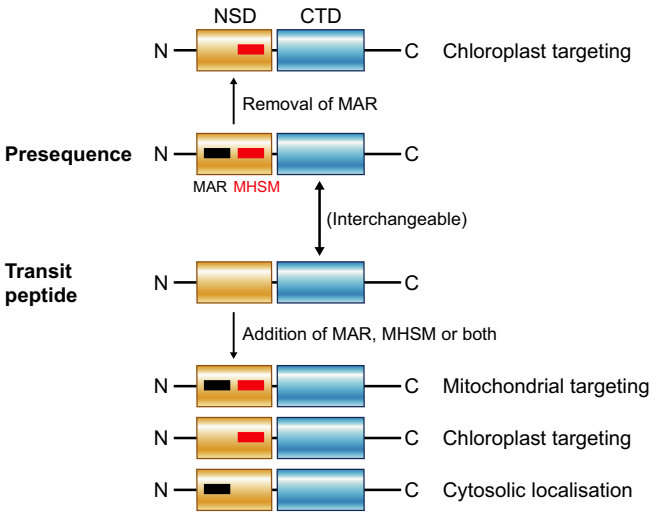

Fig. 2 The relationship between chloroplast transit peptide and the mitochondrial presequence. The N-terminal specificity domain (NSD) of mitochondrial presequences possesses multiple arginine residues (MAR) and a moderately hydrophobic sequence motif (MHSM). Removal of MAR is sufficient to switch the targeting specificity from mitochondria to chloroplasts. Conversely, incorporation of both MAR and MHSM into the NSD of the chloroplast transit peptides leads to a change in the targeting specificity from chloroplasts to mitochondria. Insertion of MAR or MHSM alone into a transit peptide results in cytosolic localisation or chloroplast targeting, respectively. C-terminal translocation domains (CTDs) are interchangeable between these targeting signals.

proteins can be specifically targeted to chloroplasts and mitochondria despite the similarity in signal sequences. Now, this brings a new question of how they ended up having such a hierarchical relationship.

III. Conservation of mitochondrial presequences throughout eukaryotic species

Mitochondria are present in all eukaryotic species. Plants and algae have additionally chloroplasts, and therefore need a mechanism(s) to discriminate these two organellar proteins. This brings the questions of whether the presequences are conserved in all eukaryotic species or presequences of plant proteins are different from those in animal and fungal proteins. In fact, the existence of MAR has been regarded as a common characteristic in presequences throughout the species (Doyle *et al.*, 2013). Moreover, a recent study showed that the presequences from nonplant species could deliver GFP to mitochondria in plant cells (Lee *et al.*, 2020). (Fig. 3). In addition, a plant presequence could deliver GFP to mitochondria in human cells. Moreover, substitution of MAR in the nonplant presequences with alanines led to chloroplast targeting in plant cells (Fig. 3) (Lee *et al.*, 2020). Furthermore, insertion of MAR and MHSM in the N-terminal region of a transit peptide led to mitochondrial targeting in animal cells. These results suggest functional conservation of presequences in all eukaryotic species.

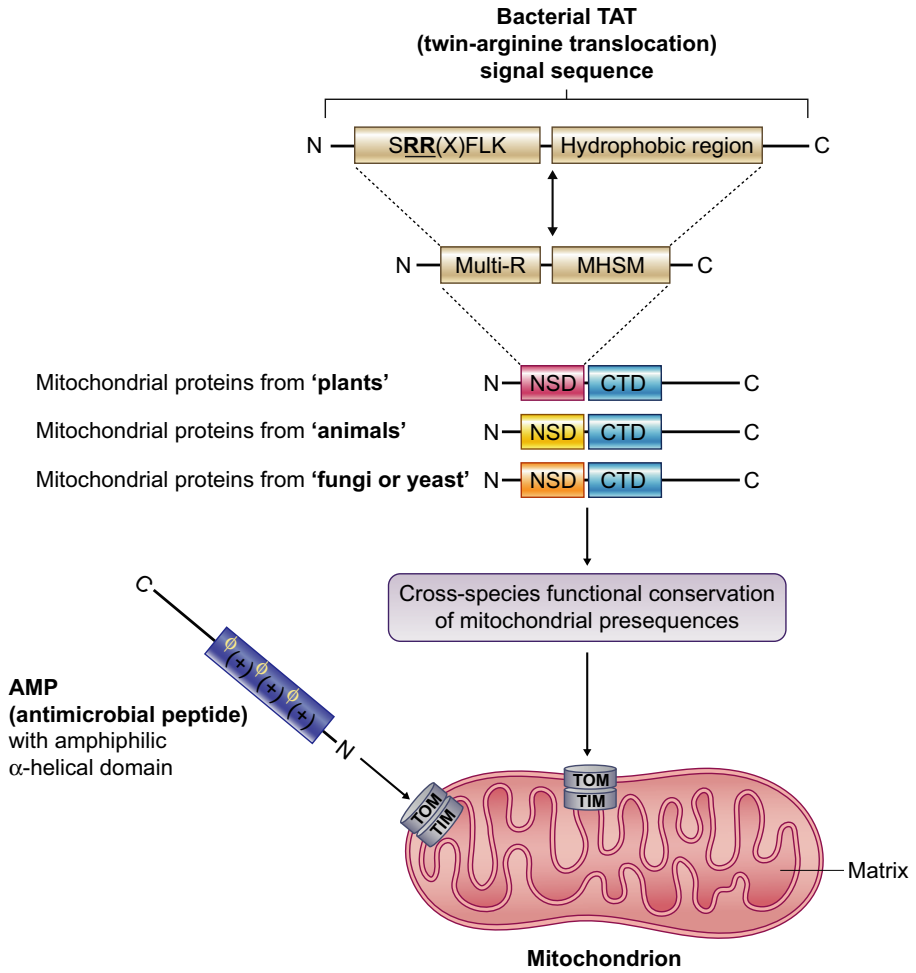

Fig. 3 Functional conservation of presequences throughout eukaryotic species and possible origins of presequences. Nonplant presequences are capable of delivering proteins to plant mitochondria. Moreover, bacterial twin-arginine translocation (TAT) signal sequences, which are also composed of two invariant arginine residues and a small hydrophobic region, can functionally substitute the N-terminal specificity domain (NSD) of mitochondrial presequences. Another possible origin of presequence, antimicrobial peptide (AMP), also can substitute the presequence for protein import into mitochondria. (+), positively charged amino acids; Φ, hydrophobic amino acids; TOM/TIM, translocase of outer/inner membrane.

IV. Principle underlying sequence motif organisation of chloroplast transit peptides and mitochondrial presequences

We now have a better understanding of the origin and sequence motif organisation principles of the diverse transit peptides and presequences. Previously, through systematic analysis of diverse transit peptides, we showed that the transit peptides can be grouped into at least seven subgroups based on sequence motifs critical for protein import into chloroplasts (Lee *et al.*, 2008). Furthermore, the sequence motifs can be exchangeable between these subgroups when placed in a proper distance and context (Lee *et al.*, 2009, 2015). These findings led to the proposal that diverse transit peptides might have been generated through the selective assembly of potential sequence motifs that can support protein import into chloroplasts (Li & Teng, 2013; Lee & Hwang, 2018). This explains the lack of consensus sequence among transit peptides. For presequences, considering the compatibility between CTDs of the transit peptide and presequence, it is plausible that diverse presequences also can be generated by a design principle similar to that of transit peptides. Then, what is the origin of the NSD of the presequence, consisting of MAR and the MHSM? Recently, we showed that the NSD of the presequence can be functionally replaced with twin-arginine translocation (TAT) signal sequences, which play a crucial role in protein export in bacteria and translocation of proteins into the thylakoid lumen in chloroplasts (Cline, 2015). The TAT signal sequences also have two invariant arginine residues and a short moderately hydrophobic domain (Fig. 3) (Cline, 2015; Lee *et al.*, 2020). Moreover, TAT signal sequences can substitute the NSD of the presequences in both plants and nonplant species (Lee *et al.*, 2020). Furthermore, the replacement of the N-terminus of transit peptides with TAT signal sequences led to mitochondrial targeting in yeast and animal cells. Thus, we propose that a TAT signal sequence might have been recruited and evolved as the NSD for specific and efficient mitochondrial targeting during the evolution of presequences. However, this proposal is different from the antimicrobial peptide-based hypothesis. Mitochondrial presequences have markedly

overlapping physicochemical properties with antimicrobial peptides (AMPs), which led to the idea that they might have originated from antimicrobial peptides (Fig. 3) (Wollman, 2016). Moreover, Venus with N-terminally fused AMPs were imported into chloroplasts or mitochondria in *Chlamydomonas reinhardtii* (Garrido *et al.*, 2020).

V. Possible evolutionary scenarios of signal sequences for chloroplast and mitochondrion proteins

Now, a fundamental question is what kind of evolutionary processes led to such a relationship between presequences and transit peptides. We can think of two possible scenarios for the relationship (Fig. 4). One hypothesis is that the transit peptide evolved first by incorporating many sequence motifs that support specific targeting to chloroplasts, and subsequently, multiple arginine residues (MAR) and MHSM were incorporated into the N-terminal region of the transit peptide to confer specific targeting to a newly evolving organelle, mitochondria (hypothesis 1) (Fig. 4). We showed that a transit peptide can be changed to a presequence in two successive steps; firstly insertion of MAR to prevent chloroplast targeting and secondly insertion of MHSM to relocate cytosolic proteins to mitochondria (Fig. 2). However, the change might have occurred via one step; the N-terminal fusion of a TAT sequence to a transit peptide could simultaneously bring both motifs, MAR and a MHSM. The other is that the presequence evolved first from a sequence with certain sequence motifs such as MAR but gradually acquired additional sequence motifs to enhance the targeting efficiency. The original sequence might have been AMPs that had an amphiphilic α-helix, therefore acquiring the MAR from the beginning (Fig. 4). Then, the elimination of the N-terminal MAR gave rise to a new signal sequence that confers specific targeting to a newly evolving organelle, chloroplast (hypothesis 2) (Fig. 4). In hypothesis 2, we propose the loss of the MAR in the transit peptide evolution because it is found in the presequences of the animal and fungal mitochondrial proteins (Lee *et al.*, 2020).

Of the two hypotheses, hypothesis 1 appears to be more consistent with the targeting signal evolution of other organellar proteins; in endomembrane compartments, addition of a sorting motif such as dileucine or YXXΦ motifs to plasma membrane proteins gives rise to a new targeting specificity to tonoplast (Dasilva *et al.*, 2006; Wang *et al.*, 2014). This hypothesis implies that the chloroplast evolved earlier than mitochondrion. Therefore, hypothesis 1 is less consistent with the current concept of chloroplast and mitochondrial evolution; mitochondria evolved earlier than chloroplasts (Dyall *et al.*, 2004; Archibald, 2015). The current concept is supported by phylogenetic analysis of chloroplast and mitochondrial genes and by the fact that chloroplasts are present only in plants and algae. Hypothesis 2 is more consistent with the current view that mitochondria evolved earlier than chloroplasts. However, it also brings many questions. Why did the presequence evolve to have many motifs including MAR that functions as a chloroplast-evading motif? A simpler signal sequence can specifically deliver proteins into an endosymbiont-derived organelle, the chloroplast. One possible answer would be that the

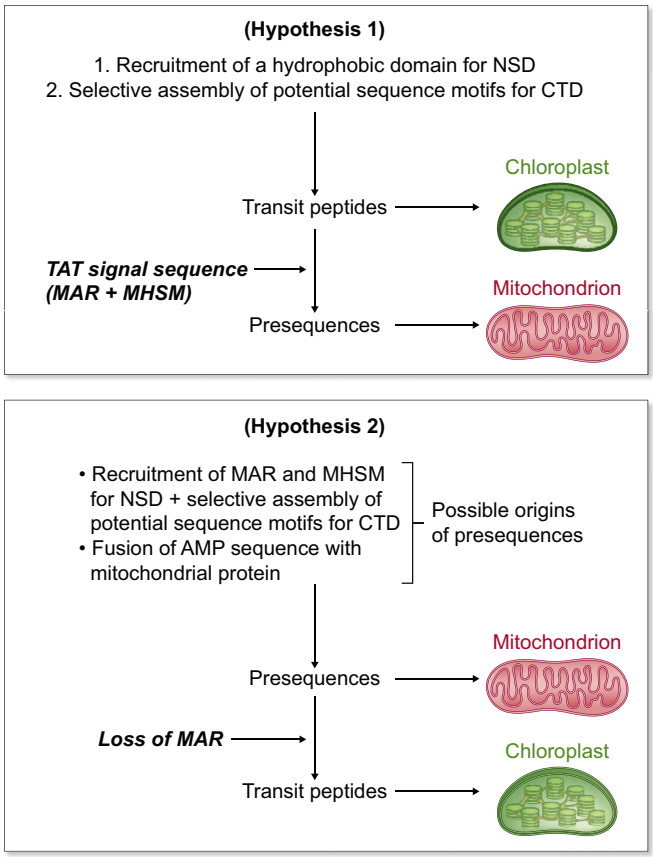

Fig. 4 Two hypotheses for the evolution of chloroplast and mitochondrial import signals. Hypothesis 1 proposes that a transit peptide was generated by recruitment of a hydrophobic domain for N-terminal specificity domain (NSD) and selective assembly of potential sequence motifs for C-terminal translocation domains (CTD). Subsequently, mitochondrial specificity motifs (MAR and MHSM) were inserted into the N-terminal region of a transit peptide to give rise to a presequence. Hypothesis 2 proposes that the presequence was generated: (i) by recruitment of MAR and MHSM for NSD and selective assembly of potential sequence motifs for CTD, or (ii) through utilisation of host sequence coding for antimicrobial peptide (AMP). Subsequently, loss of multiple arginine residues from a presequence gave rise to a transit peptide.

original sequence recruited as a presequence already contained sequence motifs including MAR. The origin of presequences could be AMPs (Wollman, 2016). However, one puzzling question is how loss of a sequence motif from a signal sequence can give a specific targeting to a newly evolving organelle. This can occur when two targeting pathways have a hierarchical relationship as shown in endomembrane compartments (Kunze & Berger, 2015). It is also possible that the targeting signal of mitochondria might have worked well for the second endosymbiotic organelle, chloroplasts, after certain modification such as removal of MAR. It has been hypothesised that pre-existing mitochondrial targeting mechanisms gave rise to chloroplast targeting and, at the same time, further evolved via the addition of a chloroplast-evading motif to reduce the frequency of mistargeting (Cavalier-Smith, 2006). However, our results are not consistent with this hypothesis; animal presequences efficiently delivered proteins into plant mitochondria and vice versa, and fungal presequences also delivered proteins into

plant mitochondria, albeit with a lower efficiency (Fig. 3) (Lee *et al.*, 2020). Moreover, the removal of MAR from animal and fungal presequences resulted in chloroplast targeting in plant cells (Lee *et al.*, 2019, 2020). Furthermore, once protein targeting mechanisms were established in the cell, we believe that they are resistant to change because the whole protein targeting system is highly complex consisting of two parts, the signal sequence and the molecular machinery, both of which have a high degree of complexity.

VI. Conclusion

The cellular system such as protein targeting mechanisms for chloroplasts and mitochondria would have been crucial for organelle evolution and also coevolved with these organelles. Therefore, the protein targeting mechanisms are molecular fossils that contain information for their evolutionary processes. Here, based on the relationship between transit peptides and presequences, we formulated two possible scenarios to explain the intriguing hierarchical relationship. Both scenarios have strength and weakness. In addition, many hypotheses were proposed in the field. One of them proposed AMPs as the origin of presequences. Therefore, in the future, further in-depth studies will be necessary to unravel the evolutionary relationship between chloroplasts and mitochondria. One topic of future studies would be the relationship of signal sequences of dually targeted proteins that are imported into both chloroplasts and mitochondria. Another topic would be the relationship of signal sequences of chloroplast and mitochondrion outer envelope membrane proteins whose targeting signals are completely different from those imported into chloroplasts or mitochondria (Lee *et al.*, 2014). It will be interesting to see which hypothesis is supported by the relationship of signal sequences of dually targeted proteins or chloroplast and mitochondrial outer membrane proteins.

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