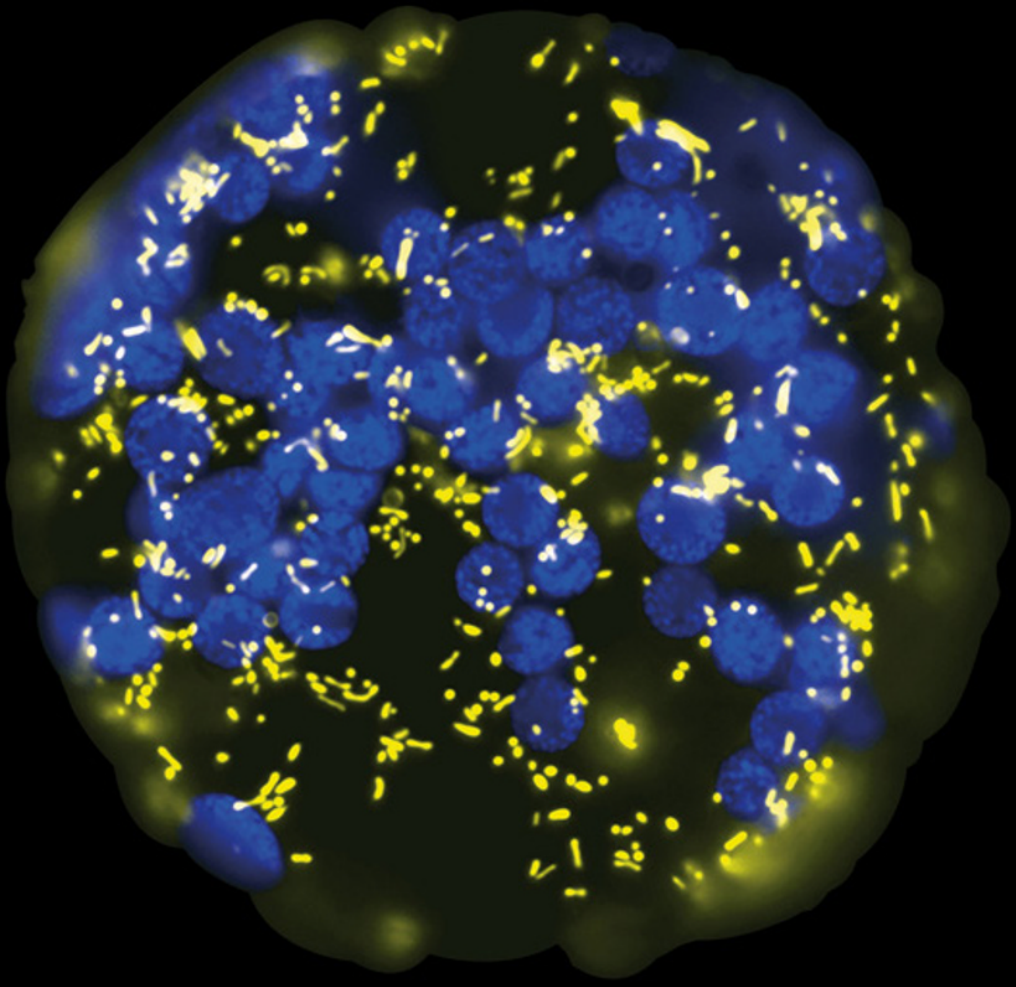
Annual Plant Reviews, Volume 50

Plant Mitochondria

Second Edition

Edited by **David C. Logan**



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This edition first published 2018

Registered Office(s)
John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA
John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

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Library of Congress Cataloging-in-Publication data applied for

9781118906576

Cover design: Wiley
Cover image: Courtesy of David C. Logan

Set in 10/12pt Palatino by SPi Global, Pondicherry, India

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PREFACE

Welcome to the second edition of *Plant Mitochondria*. The first edition was published in 2007, which, perhaps depending on your age, was either a long time ago or almost as if it were yesterday. While we can accept differences in human perception of the passage of time, it becomes more conceptually difficult to understand that time is not an absolute: two people moving through time at different speeds will experience events in that timeline at different relative times. The publication of Albert Einstein's 1905 paper 'On the electrodynamics of moving bodies', which became known as his special relativity paper, was a seminal moment for physics, and science in general (Einstein, 1905). However, at the same time, the organelles fuelling Einstein's extraordinary thinking did not have an agreed name (Cowdry, 1918), nor, indeed, did we know that the fuelling was even performed by organelles, of whatever name: identification of mitochondria as the site of oxidative metabolism took another 40+ years. Research in physics operates at a pace and scale different to that of biology!

As biologists, we use time, in our experiments, all the time. We are interested in the rate of change of an activity or behaviour. And central to all biology is evolution, which is change over time. As Theodosius Dobzhansky famously wrote in his essay of the same title, 'Nothing in biology makes sense except in the light of evolution' (Dobzhansky, 1973). A true statement cannot be more true, just as a falsehood is a lie, but in the case of mitochondria, we can say the statement is particularly apt; indeed, perhaps the corollary is valid, and nothing in the evolution of life on earth makes sense without considering mitochondria?

The world at the time of publication of the first edition of this book was very different from the world of 2017. The first iPhone was released in 2007, cloud computing took off in 2007 (for example, Dropbox was started in 2007), Google introduced Android, and Amazon introduced the Kindle. These advances changed the way many of us interact with the world around us, with parallel developments in social media: Facebook had only opened up to individuals with private email addresses in September 2006, and Twitter, launched in July 2006, was showing traffic of 400 000 tweets per quarter in 2007, rising to 50 million per day in February 2010, and now stands at 500 million tweets per day! Social media has revolutionized the way many people communicate science. However, 2007 also marked the end of a period of economic growth and optimism that culminated in a massive loss of optimism and a global financial crash from which the world still reels. This led to 'austerity', budget cuts and drastic reductions in the funding of basic scientific research, as the reduced funds available are earmarked to support research some believe is more likely to lead to economic recovery.

Despite years of austerity for fundamental plant biology research funding, we have seen major breakthroughs in our understanding of plant mitochondria, and thus a new edition of this book was timely. The evolving story of the mitochondrion, the story of the evolving mitochondrion, is the longest in the history of the eukaryotic cell. To paraphrase Roy Batty, the mitochondrion has seen things other organelles wouldn't believe. But, in what ways has our understanding of plant mitochondria advanced in 10 years?

We have seen dramatic advances in next-generation sequencing since 2007, and use of this technology has had a profound influence on our understanding of the evolution of mitochondrial genomes. The availability of sequence data and bioinformatic advances were also critical to the discovery of PPR proteins as editing factors, and subsequently, the amino acid code they use for RNA recognition (Barkan *et al*., 2012). And, more recently, advances in genome sequencing led to the discovery of the first mitochondriate eukaryote, amongst over 300 mitogenomes analysed, to lack complex I (Skippington *et al*., 2015).

We have seen fresh views on the photorespiratory pathway, which enables continued operation of the Calvin–Benson cycle, rather than being a wasteful process. And interactions between the two processes apparently include regulatory feedback between glycine decarboxylation in the mitochondrion and CO_2 fixation in the chloroplast (Hagemann & Bauwe, 2016).

Our understanding of other signalling processes between mitochondria and other cell components, and how these signals regulate mitochondrial activity, has increased apace in the past 10 years. We have also seen advances in our understanding of retrograde signalling, for example via NAC transcription factors (de Clercq *et al*., 2013; Ng *et al*., 2013), and there is growing evidence for retrograde signalling as a means to regulate nutrition, with a potential role for mitochondria as nutrient sensors (Vigani and Briat, 2015).

Signals induce changes in activity and one means to alter protein activity is by protein modification, but until recently we knew little about modification to mitochondrial proteins. However, lysine acetylation has now been identified as a common modification of mitochondrial proteins, and *Arabidopsis* sirtuin 2 was identified as the first plant mitochondrial lysine deacytylase (Finkemeier *et al*., 2011; König *et al*., 2014).

Finally, I end this preface with microscopy, the scientific tool first used to investigate mitochondria in the late 19th century. Our knowledge of mitochondrial cell biology has advanced dramatically since 2007, aided by the development of better imaging systems and the relatively massive computing power at our disposal to drive image analysis. These have allowed precise quantitative analysis of changes in the dynamics and, even more excitingly, the physiology of each individual mitochondrion, in real time. These advances have underpinned work identifying energy transients in individual mitochondria within living plant cells, *in situ*, and components of

mitochondrial calcium regulation (Schwarzländer and Finkemeier, 2013; Schwarzländer *et al*., 2012a, b, 2014; Wagner *et al*., 2015).

Advances in our understanding of plant mitochondria are made through the actions of research scientists, and communicating those advances is a vital part of their job. The purpose of this book is to communicate to you some of the most important aspects of plant mitochondrial biology, and who better to serve as the conduit for that communication than the researchers responsible for those very advances? The chapter authors are experts in their field – many of the advances in plant mitochondrial biology over the past 10 years arise from the primary research output of these authors or members of their teams. I would like to thank them all for their excellent contributions to plant mitochondrial biology, for staying with this project through its long gestation and, in many cases, for being great friends to have within the community.

David C. Logan
June 2017
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Annual Plant Reviews (2018) **50**, 1–10
doi: 10.1002/9781119312994.ch1

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Chapter 1

BIOBLASTS, CYTOMIKROSOMEN AND CHONDRIOSOMES: A SHORT INCOMPLETE HISTORY OF PLANT MITOCHONDRIAL RESEARCH

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1.1 Discovery

Advances in microscopy in the nineteenth century, spurred on by the new cell theory, enabled pioneering cell biologists to make the first descriptions of granular bodies within the eukaryotic cell (see Scott and Logan 2004 for a brief history of cell biology). While some of these granules were probably mitochondria, the various fixation and staining methods employed at the time made their unambiguous identification impossible (Cowdry, 1918; Hughes, 1959). Unambiguous identification of mitochondria and an absence of agreed defining features inevitably led to very complex terminology for what we now simply know as mitochondria. This lack of defining characteristics meant that many structures considered by some investigators to be mitochondria either were not or were composed of mitochondria as well as other uncharacterized organelles (e.g. endoplasmic reticulum, Golgi). As documented by Cowdry (1918), there were nearly 100 names in the literature for mitochondria, or structures confused with mitochondria, ranging from A (apparato reticulare interno, the early name given to Golgi and confused

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with mitochondria) to Z (zentralkapsel, which may also have been applied to structures that were in fact Golgi).

Mitochondria were first named 'cytomikrosomen' by La Valette St George in 1867 following observations of highly refractive bodies, forming the nebenkern, that could be stained in living insect sperm cells with Dahlia, which was routinely used as a stain for protein at the time (Douglas, 1935). Other investigators, such as Albert von Kölliker, who has been credited with the first isolation of mitochondria in 1888 (Lehninger, 1964), Hermann Henking, discoverer of the *x* chromosome, and Toyama, reported similar structures in insect cells before two sets of detailed studies were published independently around the turn of the century by Friedrich Meves and Carl Benda (Cavers, 1914). In 1898, Benda coined the term 'mitochondria', derived from the Greek *mitos*, a thread, and *chondros*, a grain, although this new name was not immediately universally accepted (Tribe and Whittaker, 1972).

1.2 Complexity of nomenclature

In his extensive 1918 review, Cowdry is clearly exasperated with the complexity of nomenclature, writing that the complications and confusion are due to 'hasty individual action in elaborating new names, often only to discard them in a new paper in favour of some other'. Some researchers sought to convey information about organelle morphology, others about physiology or chemistry. Indeed, Benda's term 'mitochondria' was not immediately accepted because observations had shown that mitochondria sometimes existed in forms other than grains or threads. New terms were therefore introduced, some by Benda, to subdivide mitochondria into different morphological forms, for example 'chondriokonts' for rod-like structures, 'mitochondries' for granules, 'chondriosphären' for spheres, 'chondriomites' for filaments of granules, 'chondriocontes' for straight or curved threads. Thankfully, the term 'mitochondria' won through and thus we are saved from having to learn and understand myriad names for what is the same structure, albeit in a different morphological state.

1.2.1 Discoveries of mitochondria in plants

The first recorded observation of mitochondria in plant cells (of *Equisetum* sp.) has been attributed (Wayne, 2010) to Wilhelm Hofmeister in 1851 (discoverer of the alternation of generations, amongst his many other pioneering contributions to plant biology) but a more detailed report of mitochondria in plant tissues was made by Meves in 1904 (Cavers, 1914; Millerd and Bonner, 1953), who found them in tapetum cells in the anthers of the white water lily, *Nymphaea alba.* Many further studies, often also using tapetum cells, followed up on Meves' work and inevitably led to new controversies, this time regarding the origin and function of mitochondria, just as a consensus was being

reached on their name. With regard to origin, some researchers believed that plant mitochondria were of nuclear origin, originating as protuberances of the nuclear membrane or from chromatin. However, in 1910, papers were published by Lewitsky and by Pensa who both concluded that mitochondria occurred neither in, nor did they arise from the nucleus, but that they instead underwent division (Cavers, 1914). So far so good, as it turned out, but both these researchers, along with Forenbacher in 1911 and Guillermond in 1911 and 1912, believed their results demonstrated that mitochondria gave rise to plastids, going against the prevailing Schimper–Meyer theory of the *sui generis* origin of chloroplasts (Cavers, 1914). This view was a red flag to Meves who, according to (Cavers, 1914), 'demanded more definite proofs that chondriomes can be distinguished from small chromatophores and that the actual transformation of the former into the latter can be actually seen directly in the living cell, as for instance in filamentous algae'. It was not long before other researchers re-examined the mitochondria-plastid link and concluded that there was no question of a morphological relationship between mitochondria and chloroplasts (Cavers, 1914).

1.3 Mitochondria are dynamic

In the early 1900s, a time when some researchers refused to accept that mitochondria were specific, non-artefactual, independent, heritable constituents of the cytoplasm, others were convinced that mitochondria were a structure of considerable importance given their ubiquity across the animal and plant kingdoms. Among them was a husband and wife team of embryologists, Warren and Margaret Lewis (Margaret was also probably the first person to culture mammalian cells), who are credited with being the first to focus on the remarkable dynamics of the intriguing new organelle (Lewis and Lewis, 1914).

In their 1914 paper, Lewis and Lewis wrote of mitochondria in living tissue:

> [they] are almost never at rest, but are continually changing their position and also their shape. The changes in shape are truly remarkable not only in the great variety of forms, but also in the rapidity with which they change from one form to another.

Furthermore, the Lewises were able to witness mitochondria fusion and division:

> granules can be seen to fuse together into rods or chains, and these to elongate into threads, which in turn anastomose with each other and may unite into a complicated network, which in turn may again break down into threads, rods, loops and rings.

The Lewises are clearly enthralled by the dynamism they witness and end their paper with questions about mitochondrial biogenesis and function that, just as authors claim now, must wait for a more extensive study.

That extensive study was published the next year and runs to 62 pages (Lewis and Lewis, 1915). At the end of this remarkable piece of work, which describes the morphology and dynamics of mitochondria, their staining properties and their relation to other cell structures, the Lewises return to the question of the origin and function of mitochondria. A logical process then follows: they note that mitochondria have been found in almost every kind of cell, in plants, animals and protozoa. They remind readers that mitochondria have been claimed to form fibrillae in a variety of tissues, and to form secretory granules in the salivary, gastric and mammary glands, and to aid formation of the retina cells, and that they form the external shell of Foraminifera protists. We are further reminded of claims of direct or indirect roles in fat generation, and in the biogenesis of leucoplasts, chloroplasts and chromoplasts. The Lewises find all these claims difficult to reconcile. They believed instead that the mitochondria 'are too universal in all kinds of cells' to function in such specific ways, and, given what is known of biochemistry, considered it 'practically impossible' for mitochondria to form all these different structures. They conclude succinctly: 'They [mitochondria] are, in all probability, bodies connected with the metabolic activity of the cell' (Lewis and Lewis, 1915).

Despite the Lewises' detailed description of fusion and division of mitochondria in 1915, 82 years passed before identification of the first genetic mediator of mitochondrial fusion (the *Drosophila melanogaster fzo* gene) (Hales and Fuller, 1997) and a further 2 years before publication of the first mitochondrial division gene, *DNM1* (Sesaki and Jensen, 1999) (see Chapter 4). In the intervening years, researchers, having finally generally agreed on the name 'mitochondria', and that they were true organelles, instead focused their efforts on discovering mitochondrial function.

1.4 Mitochondrial function and outputs

The view held by the Lewises, that mitochondria were the sites of cellular oxidation, had been first proposed by Kingsbury (1912). Earlier, Altman had proposed his 'bioblasts' as the elementary particle of life, a view at least partially shared with Meves and Benda who, based on their observations of transfer of mitochondria from sperm to egg at fertilisation, were both of the view that mitochondria transported heritable characteristics. Indeed, Meves was careful to declare that his belief in a genetic role for mitochondria was in addition to the nuclear chromosomes – a view well ahead of its time. Despite Meves' standing, and this extensive hypothesis about a role in inheritance, most researchers believed plant mitochondria, as with animal mitochondria, were involved in nutrition. Kingsbury commented that although much morphological work had been performed using fixation and staining, there was

'too little cognizance of what kind of substances such a technique would be likely to preserve and bring out'. Kingsbury suggested that reducing power and protoplasmic activity were linked and that the mitochondria were the structures responsible for the consumption of oxygen in respiration. However, as noted by Cowdry (1924), determination of function required a greater knowledge of mitochondrial chemistry.

1.4.1 Vital staining of mitochondria with Janus green B and identification of mitochondria as sites of redox

A key event in the determination of mitochondrial function can be traced back to the demonstration by Leonor Michaelis in 1900 that mitochondria were capable of producing an oxidation-reduction change in the vital stain Janus green B (Tribe and Whittaker, 1972). Indeed, Lehninger (1964) stated that one of the most significant steps in our understanding of the function of mitochondria came from the development first of crystal violet as a mitochondrial stain by Benda in 1898 and then the vital staining of mitochondria with Janus green B. In 1913, Warburg demonstrated that the oxidation of metabolites was associated with insoluble, granular elements of the cell (Kennedy and Lehninger, 1949; Tribe and Whittaker, 1972), although he did not link these observations to mitochondria. This link was provided by Albert Claude who purified the 'respiratory particles' from rat liver by differential centrifugation and showed that they stained with Janus green, thereby identifying them as mitochondria as seen by light microscopy.

Further confirmation was provided by pioneering work in Albert Claude's laboratory by George E. Palade, that combined subcellular fractionation and subsequent biochemistry with electron microscopy, not only to confirm the isolated particles as mitochondria but also to subsequently define the structures of the mitochondria. By combining structure and functional studies in this way, Palade did much to invent the field of cell biology. In 1953, Palade and Fritiof S. Sjöstrand published their results on mitochondrial ultrastructure (Palade, 1953; Sjostrand, 1953). The two models were slightly different, with Palade proposing the existence of the cristae mitochondriales which form invaginations from an inner membrane, while Sjöstrand believed the inner membrane was not continuous with the outer and that the matrix, proposed by Palade, was a fixation artefact. Sjöstrand was, however, correct about the organelle having a double membrane, which was more clearly presented in his thinner ultramicrotome sections, although even on this point Palade had not been adamant since he had stated that 'in favourable electron micrographs the mitochondrial membrane appears to be double' (Palade, 1953).

Even before the contributions of Palade, Claude and their co-workers that were vital to linking biochemistry and cytology, Albert Lehninger was convinced that mitochondria were the sites of oxidative energy transduction (Kennedy, 1992). One of Lehninger's key discoveries was the inhibition of

fatty acid oxidation and oxidative phosphorylation in particulate cell extracts by exposure to hypotonic buffers. This observation was the subject of subsequent graduate studies by Eugene L. Kennedy which allowed Kennedy and Lehninger (1948) to conclude that fatty acid oxidation, oxidative phosphorylation and the reactions of the Krebs cycle took place in a single organelle bounded by a semi-permeable membrane. Next, using the newly described Palade method of differential sucrose density gradient centrifugation to purify mitochondria, Kennedy and Lehninger (1948, 1949) were able to present convincing evidence that the active organelle was the mitochondrion. In 1953, after over 50 years of use, the Janus green B reaction was formally linked to the reoxidation of the reduced dye by mitochondrial localized cytochrome oxidase (Lazarow and Cooperstein, 1953). The identification of cytochromes themselves as respiratory pigments was made by Keilin in 1925, who stated that they were a common biochemical feature of higher plants, animals and yeasts (Keilin, 1925). Despite Otto Warburg's refusal to accept their role (Slater, 2003), Keilin correctly identified cytochromes *a*, *b* and *c* as being major constituents of the respiratory chain, and they were later confirmed as being localized to mitochondria by Chance and Williams (1955).

While the studies just described paved the way for elucidation of individual reactions, their substrates, enzymes and products, and the association of these reactions into pathways, they did not complete the line-up of respiratory pathways open to plants. Not long after the discovery of cytochromes, Genevois in 1929 described a respiratory pathway in sweet pea (*Lathyrus odoratus*) that was resistant to cyanide, and hence independent of cytochromes (reviewed in Rogov *et al*., 2014). This alternative oxidation pathway was later associated with mitochondria in cellular preparations from *Arum maculatum* spadix by James and Elliot (1955), and found also to exist in other kingdoms, including yeast. The multiple roles of the alternative oxidase (AOX) have been debated for some time (including thermogenesis, energy overflow, resistance to cytotoxic compounds and antioxidant properties), but at its core this terminal oxidase provides plant mitochondria with a non-ATP-generating pathway in the electron transport chain that aids in cellular homeostasis (Vanlerberghe, 2013).

1.5 Mitochondrial DNA

By the 1960s, evidence was starting to grow that mitochondria contained their own nucleic acids (Nass and Nass, 1963a,b) (see Chapters 2 and 3), and were capable of producing proteins independently of cytoplasmic ribosomes (Haldar *et al*., 1967). The extension of mtDNA studies to plants (Suyama and Bonner, 1966) led to increasing interest in the transcriptional and translational machinery contained within these organelles (see Chapter 6 for a review of RNA metabolism). The ribosomal component of plant mitochondria was characterized in a series of biochemical experiments by Leaver and Harmey

(1972, 1973, 1976), who demonstrated that these ribosomes contained a 5S rRNA subunit, which is absent in animals and yeast. The mitochondrion is thus viewed as semi-autonomous. The mitochondrial genome encodes a few proteins, but they are vital, and these proteins are synthesized on mitochondrial ribosomes, from mRNA transcripts encoded in the mtDNA, transcribed and edited within the mitochondrion (see Chapter 6 for a review of RNA editing). But semi-autonomy is best reserved to thinking about the provision of the mitochondrion with the protein complement necessary for function. But that function is not autonomous – the mitochondrion is part of the cell.

While we can purify mitochondria, obtain snapshot information on their component materials and measure their activities, we must remain fully aware that we have ripped the mitochondria from their natural habitat and are no more likely to see natural behaviour from them than from a polar bear in Edinburgh Zoo. The signalling between mitochondria and nucleus, and indeed between mitochondria and other organelles, that is known to be important for function (see Chapter 7) has been lost. Isolated mitochondria will be stressed (see Chapter 8 for a review of mitochondria biochemistry and stress), and any 'recovery' probably more hopeful than actual. Luckily, technology allows more and more investigations to be performed *in vivo*; advances in imaging technology and sensors provide physiological readouts at incredible resolution. And development of synthetic biology, fuelled by knowledge gleaned from studies on isolated organelles, will allow experimenters to determine the extent to which the complex 3D ultrastructure of the plant cell, and its dynamism, is necessary for function.

1.6 Mitochondria, photosynthesis and carbon cycling

Plant mitochondria were shown to be a central part of maintaining efficient photosynthesis in the late 1970s, when they were identified as being the site for glycine oxidation (see Chapter 10). In C_3 plants, around 25% of photosynthetic output can be lost through the oxygenation reaction of Rubisco, which leads to the production of phosphoglycolate. After processing by chloroplasts and peroxisomes to glycine, this metabolite is shuttled to the mitochondria where it is oxidized, allowing further processing by peroxisomes to glycerate where it can re-enter the photosynthetic pathway. Studies by Kisaki *et al*. (1971), Woo and Osmond (1977) and Moore *et al*. (1977) showed that the enzyme activity responsible for glycine decarboxylation was localized to the mitochondria.

1.7 A trigger for death

The living-giving role of mitochondria in eukaryotes was well established by the middle of the twentieth century, but the role of mitochondria in programmed cell death took longer to become established in plants

than in metazoans. However, there is now a good deal of evidence to suggest that this organelle is a central part of the response (see Chapter 11). In animals, the induction of apoptosis (cf. programmed cell death) leads to several mitochondrial processes, including the translocation of Bax from the cytosol to the outer mitochondrial membrane, and the release of cytochrome *c* from the inter membrane space to the cytoplasm. Cytochrome *c* interacts with cytosolic factors that lead to the induction of caspase activity, a group of cysteine proteases that degrade cellular components in an orderly fashion (for review, see Desagher and Martinou, 2000, and Martinou and Youle, 2011). While there are no caspase homologues in higher plants, there is clear evidence for the early release of cytochrome *c* in plant programmed cell death (Balk *et al*., 1999). In addition, a family of proteins dubbed 'metacaspases' act in a similar manner to mammalian caspases (Lam and Zhang, 2012), indicating that the cell death pathway is relatively conserved (see Chapter 11).

1.8 Known knowns, known unknowns and unknown unknowns of mitochondrial biology

This introductory chapter has provided a brief historical overview of the key early discoveries in plant mitochondrial research. Inevitably, there are huge gaps; for example, there was no mention of Fe-S metabolism, arguably more important than aerobic respiration to some organisms. But the beauty of this book is that you can simply flick to Chapter 5 and fill that gap.

As the mass of research published on plant mitochondria grows, it becomes increasingly difficult to keep abreast of the subject. The amount of published research 'lost' to history increases. There is an increasing amount of information that is known but that we, as individuals, do not know. At least we know we do not know some details. Indeed, if we were being honest with ourselves, we may admit to not knowing more than just the details about some aspects of the subject of our research. So, we are comfortable in our ignorance of the known unknown. In that regard, review articles and books like this one, with chapters written by experts, are extremely important in reminding us all about those personal known unknowns.

Famously, in 2002, the serving US Secretary of State, Donald Rumsfeld, said during a press briefing:

> There are known knowns. There are things we know that we know. There are known unknowns. That is to say, there are things that we now know we don't know. But there are also unknown unknowns. There are things we do not know we don't know.

Many thought this statement nonsensical, but the concept of the unknown unknown, that is, the existence of things we do not know, as a species, we do not know, meaning even their existence is beyond our current conceptual

framework, probably arose with the dawn of consciousness. This book provides you with a selection of chapters reviewing the known knowns of the wonderful world of mitochondria, and the authors comment often on the known unknowns. However, as experts and not soothsayers, we cannot comment on the unknown unknowns, but it is exciting, and realistic, to think that some novel and unexpected mitochondrial function may yet be discovered.

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Annual Plant Reviews (2018) **50**, 11–32
doi: 10.1002/9781119312994.ch2
http://onlinelibrary.wiley.com

Chapter 2

MITOCHONDRIAL DNA REPAIR AND GENOME EVOLUTION

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2.1 Plant mitochondrial genomes are large and variable

As soon as plant mitochondrial genome sizes were determined, it was clear that the evolutionary trajectories of mitochondrial genomes in plants were very different from those in animals. Rather than the small compact circular molecules found in animals, plant mitochondrial genomes are large, complex and contain abundant and variable amounts of non-coding DNA (Mackenzie and McIntosh, 1999; Schuster and Brennicke, 1994). The smallest complete mitochondrial genomes from free-living angiosperms are found in the *Brassica* genus, and are approximately 220 kb in length (Chang *et al*., 2011; Grewe *et al*., 2014), roughly 10 times the size of animal mitochondrial genomes. The smallest currently known angiosperm mitochondrial genome, only 66 kb and missing many genes found in free-living angiosperms, is found in the parasitic plant *Viscum scurruloideum* (Skippington *et al*., 2015). The largest sequenced genome so far is the 11.5 Mbp genome of *Silene conica* (Sloan *et al*., 2012), and most genomes, no matter how large, contain a mere 30–60 functional genes.

2.1.1 Low mutation rates in genes

DNA sequencing also revealed additional differences: genes in plant mitochondria have very low mutation rates, measured as substitution rates in rRNA or synonymous substitution rates in protein-coding genes, while the non-gene content is large and variable (Drouin *et al*., 2008; Palmer and Herbon, 1988; Richardson *et al*., 2013; Wolfe *et al*., 1987), exhibiting frequent rearrangements and stoichiometric shifts between one part of the genome and another. These stoichiometric shifts are thought to be occasionally

Annual Plant Reviews, Volume 50: Plant Mitochondria, Second Edition. Edited by David C. Logan.

responsible for phenotypic traits such as male sterility (Woloszynska, 2010) and have therefore been the subject of study from an agronomic point of view. Further DNA sequence analysis of multiple taxa has shown that these features are characteristic of the angiosperms, gymnosperms and lycophytes, while the streptophyte and bryophyte lineages are more consistent with the small compact genome features of animals and fungi (Hecht *et al*., 2011; Knoop, 2004; Oldenburg and Bendich, 2001; Smith and Keeling, 2015). The remainder of this chapter will be limited to discussing angiosperm mitochondrial genomes.

2.1.2 Genome Organization

Many of the sequenced plant mitochondrial genomes can be assembled into circular sequences, but searches for genome-size circular molecules have been largely unsuccessful (Backert and Borner, 2000; Backert *et al*., 1997; Bendich, 2004; Oldenburg and Bendich, 2015; Sloan, 2013). Many plant mitochondrial genomes, but not all, contain large repeats of several kilobases of DNA. Homologous recombination occurs frequently between different copies of these repeats, leading to multiple isomeric rearranged forms (Klein *et al*., 1994; Unseld *et al*., 1997). In a number of species, the sequence assembles into two or more independent circles (Shearman *et al*., 2016; Sloan *et al*., 2012) and linear molecules are also known (Handa, 2008). The alternative forms are not always equimolar (Mower *et al*., 2012) so the exact structure and abundance of different subgenomic molecules remain unclear. Repeats of 50–600 bp are often present, although these do not typically recombine in wild-type plants (Arrieta-Montiel *et al*., 2009; Forner *et al*., 2005). Interestingly, there are usually no repeats between 600 bp and a few kb, leading us to label the 50–600 bp repeats whose recombination is diagnostic of an aberrant repair process 'repeats of unusual size' (ROUS). The overall structure of the genome and gene content are also variable.

Recent analysis of a basal angiosperm allowed examination of the phylogenetic distribution of gene content (Richardson *et al*., 2013). The patterns are complex, and include apparent horizontal transfer of tRNAs from the plastid. This study also examined mitochondrial genomes for conservation of gene clusters, and found it to be limited. The largest clusters contained three genes, and the only one conserved across all taxa was the *rrnS-rrn5* cluster. Other frequently found clusters are *rpl5-rps14-cob*, *nad3-rps12* and *nad4L-atp4*. There is evidence in *Arabidopsis thaliana* that these clusters are also co-transcribed (Forner *et al*., 2007; Hoffmann *et al*., 1999), although the *rps14* gene is often transferred to the nucleus, leaving the mitochondrial copy between *rpl5* and *cob* as a transcribed pseudogene (Aubert *et al*., 1992; Figueroa *et al*., 1999; Ong and Palmer, 2006; Quinones *et al*., 1996). No significant synteny beyond these clusters has been found, due to the rearrangements that frequently occur in plant mitochondrial DNA (Palmer and Herbon, 1988).

2.1.3 Genome replication

Recent work has provided another piece in the puzzle of how mitochondrial genomes replicate and rearrange. Mutations in the *recG1* gene (a nuclear gene encoding a mitochondrially targeted protein) show the interesting phenotype of permitting the persistence of autonomously replicating subgenomic circles (Wallet *et al*., 2015). Following restoration of RECG1 function, the circles become unstable and are either reintegrated into the genome by homologous recombination at one of two sites, or lost. The proposed biological function of the wild-type RECG1 protein is to restart stalled replication forks. It must also inhibit replication of circular molecules that involve an R-loop or D-loop in the replicative cycle (Wallet *et al*., 2015). This implies that normal mitochondrial replication uses a mechanism that does not include such loops. Information about the genes involved in DNA replication, recombination and repair has been recently reviewed (Gualberto and Newton, 2017).

2.2 The mutational burden hypothesis

Enough sequence data have been obtained from the angiosperms to use comparative and theoretical approaches to ask why the genes have such low mutation rates and yet the genomes are so large, dynamic and full of seemingly non-functional DNA. Lynch and co-workers (Lynch *et al*., 2006) proposed the mutational burden hypothesis (MBH) to explain an inverse correlation between mutation rate and genome size. The MBH posits that any nucleotide in any context is a potential target for a deleterious mutation, and thus selective pressure and drift will drive genome sizes smaller in a high mutation rate environment. This hypothesis nicely explains many genome sizes, including the small size of animal mitochondria, but may not completely explain the large sizes of plant mitochondria. In recent years, exceptions have been found – several plant species have been shown to have both increased mitochondrial mutation rates and greatly expanded genome sizes (Sloan *et al*., 2012), in contrast to the predictions of the mutational burden hypothesis.

2.2.1 Problems with the MBH and mutation rate measurements

There are a number of assumptions behind the MBH, and in the measurement of mutation rates. The first assumption is that mutations in non-coding DNA are potentially deleterious. This may be true in many situations, particularly in compact genomes. However, as the size of the non-coding part of a genome increases, it is more likely that many mutations are completely or nearly neutral. There have been relatively few studies on the cost of large genome size. One recent study found that in bacteria, increased plasmid size has a negative effect on fitness (Adler *et al*., 2014), but the rapid division of bacteria may not represent plant fitness costs very well.

In plants, replicating the mitochondrial genome is probably also a very minor expenditure of energy compared to the other costly activities of the cell, including replicating the much larger nuclear genome, transcription, protein synthesis and active transport. In any case, there are extant species with extremely large mitochondrial genomes, so selection for reduced size appears to be minimal or non-existent. In *A. thaliana*, nearly two-thirds of the genome shares no known similarity with any other sequences in any organism outside the sister taxa of the Brassicales (Christensen, 2013) and similar results can be found with the intergenic regions of many angiosperm mitochondria. These regions include fragments that appear to be derived from mitochondrial genes, plastid genes, nuclear genes and horizontal transfer from other species, but the only consistently found sequences in mitochondrial genomes are the known rRNA, tRNA and protein-coding genes (Richardson *et al*., 2013). The relatively poor conservation of the intergenic regions of plant mitochondria argues against there being any selection on these sequences at all (Graur *et al*., 2013).

Additional evidence is found in the comparison of the mitochondrial genomes of two species of mistletoe, *Viscum scurruloideum* and *Viscum album*. In these two species of parasitic plant, there has been massive gene loss, particularly of the genes encoding components of respiratory complex I (Skippington *et al*., 2017). However, in spite of this apparent selection for gene loss, it is not accompanied by selection for DNA loss, as the *V. scurruloideum* genome is 66 kb and the *V. album* genome is 565 kb. The dramatic loss of non-coding DNA seen in *V. scurruloideum* also suggests that the non-coding DNA in *V. album* is non-functional.

Another important assumption is that synonymous substitutions accurately measure the neutral mutation rate. RNA editing in plant mitochondria artificially reduces these rates in the following way. Most of the editing is deamination of cytosine to uracil in the mRNA, although some uracil to cytosine editing also occurs (Takenaka *et al*., 2013). Editing is accomplished by pentatricopeptide repeat (PPR) proteins, which recognize short upstream sequences in the RNA to give them specificity for the edited base (Barkan and Small, 2014).

To understand the impact of RNA editing on mutation rate calculations, consider as an example the sequence GGT-ACC-GTT-GCG-GCC-CCT, encoding the amino acid sequence Gly-Thr-Val-Ala-Ala-Pro. All of these amino acids have four-fold degenerate codons, so changes in the first two bases of each codon would be counted as non-synonymous substitutions, while changes in the third bases would be synonymous. If the first C residue in the proline codon of the mRNA (underlined) is edited to a U, the edited mRNA would encode a leucine at that site, instead of the proline encoded in the DNA. A transition mutation of C to T at that position would encode leucine and would be effectively a synonymous substitution, even though conventional mutation analysis would count it as a non-synonymous substitution and would not include that position in the total of potential synonymous sites.

Worse still, because the editing PPR proteins recognize upstream sequence, changes in upstream codons could eliminate editing, producing an amino acid change in the protein, although not at the site of the mutation. Cis-acting regulatory sequences for editing will thus be counted as neutral synonymous sites, even when changes at those sites would result in an amino acid change that may not be neutral at all.

RNA editing therefore causes the number of potential synonymous sites to be overcounted, lowering the synonymous substitution rate. If a mutation from C to T occurs at an edited site, it will then release the upstream cis-regulatory sequences from selection for editing accuracy, allowing them to be synonymous substitutions again. Current tools for sequence alignment and measurement of mutation rates do not allow this information to be taken into account, so the simplest way to avoid these errors is to delete edited codons and a likely upstream cis-regulatory region from the alignments and analysis, which has the effect of slightly increasing the measured neutral mutation rate in genes (Christensen, 2014).

Codon usage bias could also affect the rate of synonymous substitutions. An analysis of codon usage showed that four-fold degenerate positions in codons were not random, showing both an A+T and pyrimidine bias (Sloan and Taylor, 2010). Additional insight into the question of whether synonymous substitutions are really neutral has emerged from comparison of genes and pseudogenes. As mentioned above, *rpl5*, *rps14* and *cob* are clustered and co-transcribed in many angiosperm mitochondria. In some species, *rps14* has been relocated to the nucleus and the mitochondrial copy is a pseudogene, yet *rpl5* and *cob* are apparently still co-transcribed. Selection for transcription of *cob* from the *rpl5* promoter maintains the gene arrangement, but selection on the *rps14* pseudogene sequence is eliminated. A comparison of synonymous substitution rates in *rps14* genes and substitution rates in *rps14* pseudogenes shows that the synonymous substitution rate is a few-fold higher than the neutral substitution rate in pseudogenes (Wynn and Christensen, 2015). Both of these studies suggest that there is a small effect of selection on synonymous substitution rates, but this effect is not sufficient to explain why plant mitochondrial genes mutate at 10% the rate of plant nuclear genes and 1% the rate of animal mitochondrial genes, while the non-genes mutate so quickly that alignment is not possible except among very close relatives.

Another important assumption is that mutation and DNA repair are uniform across the genome, affecting expansion of non-coding regions and substitution in coding regions alike. Investigation of this assumption, and the clear involvement of double-strand break repair processes in mitochondrial genome maintenance and evolution (Davila *et al.*, 2011), has led to greater understanding of the molecular processes of DNA repair that may lead to the paradoxical patterns of genome evolution in plant mitochondria. Analysis of the evolutionary patterns seen in plant mitochondrial genomes led to hypotheses of what mechanisms of DNA repair are available in plant mitochondria, and the consequences of those repair pathways on genome evolution

(Christensen, 2013, 2014). This analysis suggests that plant mitochondria do not have the complete suite of potential repair mechanisms available, and that the evolutionary patterns reveal the consequences of both selection and DNA repair mechanisms on genomes. Additional evidence continues to accumulate, suggesting that the mutational burden hypothesis does not apply well to plant mitochondria (Smith, 2016).

2.3 DNA repair-based hypothesis

The types of DNA damage that occur in mitochondria, the available mechanisms of repair, and selection on the repaired DNA products must be taken into account in understanding the patterns of plant mitochondrial genome evolution. The MBH can explain the selective pressure to reduce genome size in animal mitochondria with their high mutation rates, but in plant mitochondria, the genome sizes correlate poorly with mutation rates, suggesting that other factors are involved. The mutation rates in the junk DNA of plant mitochondria are extremely difficult to measure because non-coding DNA is poorly conserved and difficult to align, suggesting that mutation rates in non-coding DNA are significantly higher than in genes. The poor conservation also suggests that these sequences have no selected function and are junk (Graur *et al*., 2013, 2015).

The first attempt to measure mutation rates in junk DNA of plant mitochondria took advantage of the close similarity of the genomes of two ecotypes of *A. thaliana* (Christensen, 2013). The non-coding DNA could be aligned because the ecotypes diverged about 200 000 years ago. Alignment with another member of the Brassicales, *Raphanus sativa*, was much less complete (illustrating how quickly non-coding DNA diverges), but allowed polarization of 39 of the 103 differences between the two ecotypes of *A. thaliana*. This analysis revealed that most of the mutations in non-coding DNA were of the following three types: G:C to A:T transitions, presumably due to unrepaired deaminated cytosine; G:C to T:A transversions, presumably due to unrepaired oxidized guanine; or deletion of one base from homopolymeric runs, presumably due to replication slippage.

There was also one 1.8 kb insertion consisting of five fragments from elsewhere in the genome joined together (Forner *et al*., 2005). This insertion creates duplications in the size range of 50–600 bp that can recombine with homeologous sequences elsewhere in the genome under some conditions (Arrieta-Montiel *et al*., 2009; Janicka *et al*., 2012; Miller-Messmer *et al*., 2012; Shedge *et al*., 2007; Zaegel *et al*., 2006). Thus the prevalent mutational mechanisms produced the 1.8 kb chimeric duplication, several 1 bp deletions and a number of substitutions. The substitutions are consistent with guanine oxidation or cytosine deamination that escaped repair, while the single-base deletions are probably due to DNA polymerase slippage (Bebenek *et al*., 2008; Garcia-Diaz *et al*., 2006). The duplication is likely to have been produced by

non-homologous end-joining. It is interesting to note that the duplication event added 1.8 kb to the genome, while all the deletions combined removed only 35 nucleotides, consistent with genome expansions over time.

Transcribed intergenic regions mutate more frequently than genes, and with a similar spectrum to non-transcribed intergenic regions, ruling out transcription as a mechanism for the mitochondrion to distinguish between genes and junk in order to repair them differently (Christensen, 2014). The A+T and pyrimidine biases seen in degenerate codons (Sloan and Taylor, 2010) might also be explained by guanine oxidation and cytosine deamination being the most common causes of damage.

A model was proposed (Christensen, 2013, 2014) that simplifies and unifies many of these observations under the umbrella of double-strand break (DSB) repair. Double-strand breaks are the most serious damage that can occur to a genome, and there are numerous pathways that can be used to repair them (Figure 2.1). Among these are gene conversion which is very accurate, and microhomology-mediated end-joining and break-induced

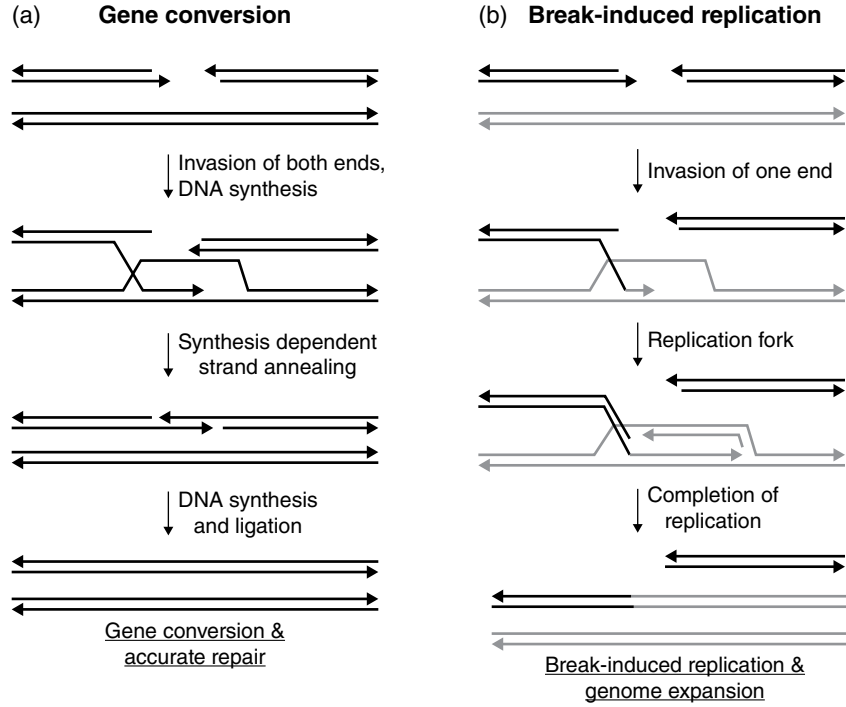

Figure 2.1 Model of two types of DNA repair. The black and grey lines indicate different sequences. (a) The consequences of co-ordination of both ends following a break. (b) The consequences of invasion of a single DNA end, which ultimately leads to genome expansions. Invasion occurs at an ectopic site due to a small region of homology. From Christensen AC (2013) Genome Biol Evol ***5***: 1079–1086 by permission of Oxford University Press and the Society for Molecular Biology and Evolution.

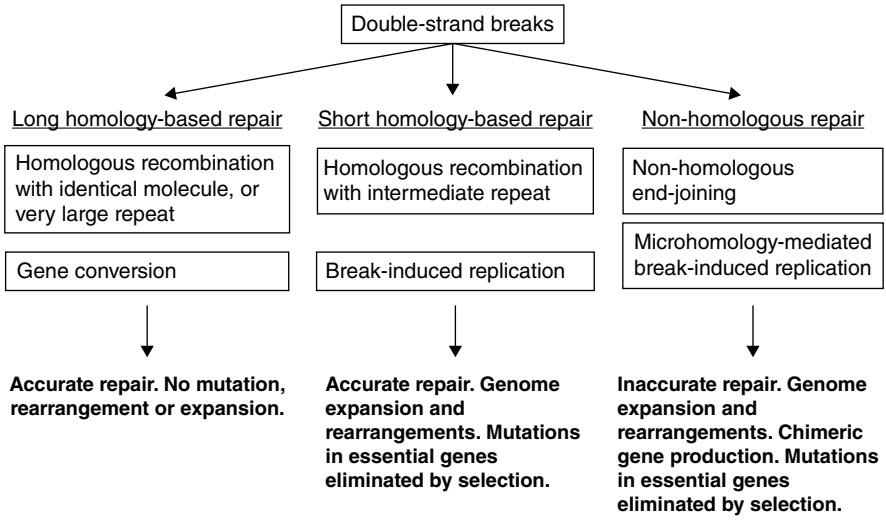

Figure 2.2 Model for the pathways of double-strand break repair in plant mitochondria. From Christensen AC (2014) Genome Biol Evol **6**: 1448–1453 by permission of Oxford University Press and the Society for Molecular Biology and Evolution.

repair, which are less accurate (Chiruvella *et al*., 2013; Jasin and Rothstein, 2013). Importantly, these pathways can explain much of the paradoxical behaviour of plant mitochondrial genomes, whereby genes are repaired very accurately, the junk mutates and recombines, and the genome expands (Figure 2.2). Template-based and accurate mechanisms of DSB repair such as homologous recombination and gene conversion can account for accurate repair in genes, including the very high conservation of synonymous sites. Less accurate mechanisms of DSB repair such as non-homologous end-joining, break-induced repair or non-allelic homologous recombination at ROUS (50–600 bp) can account for the rearrangements, expansions and mutagenesis of the junk. Indeed, a recent review of DSB repair processes noted they can be '"Dr. Jekyll" in maintaining genome stability/variability and "Mr. Hyde" in jeopardizing genome integrity' (Guirouilh-Barbat *et al*., 2014). Clearly, there are other mechanisms of repair present in plant mitochondria (Gualberto and Newton, 2017; Gualberto *et al*., 2014) but the patterns seen over evolutionary time scales show that DSB repair processes are very important for genome evolution.

A key question is how the accurate mechanisms of repair are 'directed' to genes and the inaccurate mechanisms to the junk. The answer is probably that they are not directed in any sense, but that the outcomes of the different types of repair are filtered through selection on the resulting mitochondrial genomes. Bottlenecks or other mechanisms that would drive homoplasmy would presumably select against inaccurately repaired genes, while the

genome expansions, rearrangements and duplications will be subject only to drift (see Figure 2.2). The mechanism of repair ensures accurate inheritance of genes and has the side-effect of allowing duplications, rearrangements and the accumulation of junk DNA. Accurate repair of the genes is the selected feature, and the junk DNA is a spandrel – an unselected byproduct of the selected feature (Brenner, 1998).

2.4 Additional mechanisms of DNA repair

Many of the peculiar observations on plant mitochondrial genome evolution can be explained in this framework, although not all. Of course, additional repair mechanisms do occur in plant mitochondria (Table 2.1) (Gualberto and Newton, 2017; Gualberto *et al*., 2014). One repair pathway that has been shown

Table 2.1 Types of DNA repair present in plant mitochondria.

Type of DNA damage	Repair mechanism	Present in mitochondria?
Bulky adducts, cross-links, cyclobutane pyrimidine dimers	Nucleotide excision repair	Unknown
Cyclobutane pyrimidine dimers	Photoreactivation	Unknown
Deaminated, oxidized or alkylated bases	Base excision repair	Uracil-N-glycosylase* AP endonucleases?† Others not known
Double-strand breaks	Break-induced replication	Rare in wild type, much more common in some mutants§
Double-strand breaks	Homologous recombination	Crossing-over frequent at large repeats, rare at repeats of unusual size‡**
Double-strand breaks	Non-homologous end-joining	May be due to microhomology-mediated recombination†
Double-strand breaks	Single-strand annealing	May account for crossing-over at repeats of unusual size†
Double-strand breaks	Synthesis-dependent strand annealing	Likely present and leading to accurate gene conversion
Mismatched base pairs	Mismatch repair	A *mutS* homolog is present but may not perform mismatch repair‡§

*Boesch *et al*. (2009), †Gualberto *et al*. (2014), ‡Shedge *et al*. (2007), §Christensen (2014),
**Davila *et al*. (2011).

to occur in plant mitochondria is base-excision repair (BER), a pathway that removes damaged bases from the deoxyribose in the DNA, followed by further processing of the apurinic site to remove the deoxyribose-phosphate, and finally resynthesis using the complementary strand as a template. As noted above, one of the most common types of point mutational differences between two closely related strains of *A. thaliana* (Christensen, 2013) can be explained by cytosine deamination that escapes removal by the uracil-N-glycosylase enzyme (UNG). This type of damage may be very frequent, given that there is a repair system in place (Boesch *et al*., 2009) and yet it is one of the most common mutations seen.

The other frequent mutation, G:C to T:A transversions, can be accounted for by failure to repair an oxidized guanine residue. Two mechanisms may target this type of damage for BER. The OGG1 proteins remove the oxidized product 8-oxo-guanine (8-oxo-G) from DNA, leaving an apurinic site (Macovei *et al*., 2011). If an 8-oxo-G is not removed, it can be paired incorrectly with adenine during DNA replication, and mutY family glycosylases (MYH) remove adenines paired to 8-oxo-G (Markkanen *et al*., 2012a,b; van Loon *et al*., 2010). The apurinic sites would presumably be processed by the same short-patch BER as described for uracil removal. Although there is evidence from mutations that oxidized guanines occasionally escape repair, this type of damage is probably quite frequent, and it is likely to be repaired efficiently, suggesting that one or both of these specific systems will be found in plant mitochondria.

2.4.1 Mismatch repair and *MSH1*

Mismatch repair (MMR) has been suspected in mitochondria due to the presence of *MSH1*, a homologue of the *E. coli mutS* mismatch repair gene (Abdelnoor *et al*., 2003). However, the mutS family of proteins is involved in multiple DNA repair processes (Jiricny, 2013), and it has not been shown that the mitochondrially targeted MSH1 proteins participate in mismatch repair in either yeast (Sia and Kirkpatrick, 2005) or plants (Abdelnoor *et al*., 2003; Shedge *et al*., 2007). In plant mitochondria, *msh1* mutants show evidence of increased homeologous recombination at ROUS of 100–500 bases (Arrieta-Montiel *et al*., 2009; Davila *et al*., 2011; Shedge *et al*., 2007). Previous suggestions that there were microhomology-mediated recombination events in *msh1* mutants (Sakamoto *et al*., 1996) have been shown to be events involving ROUS that were unknown at the time of the first report (Shedge *et al*., 2007). It was suggested that the MSH1 protein functioned in homology surveillance in double-strand break repair to ensure that the invading template strand was identical to the damaged strand before allowing homologous recombination or gene conversion to occur (Shedge *et al*., 2007). The structure of the MSH1 protein of plants (Abdelnoor *et al*., 2003, 2006), the low synonymous substitution rate in genes and the high mutation rate in junk