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Olivier Keech

Étude des mécanismes cellulaires lors de la sénescence foliaire

From Green to Yellow
A Leaf Story

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ÉTUDE DES MÉCANISMES CELLULAIRES LORS DE LA SÉNESCENCE FOLIAIRE

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From Green to Yellow – A Leaf Story

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Resumé

Lors de son jaunissement, une feuille subit aussi bien des modifications morphologiques que métaboliques. Ce processus est appelé « sénescence ». Une meilleure compréhension des mécanismes de la sénescence représente un challenge très important non seulement pour la recherche fondamentale mais aussi pour de futures applications en biotechnologie. La thèse présentée ici porte sur d'importants aspects relatifs aux mécanismes cellulaires et métaboliques rencontrés lors de la sénescence foliaire et ce, en apportant une attention particulière à l'implication des mitochondries lors de ce processus.

Dans un premier temps, nous avons développé deux méthodes pour isoler, à partir de feuilles d'*Arabidopsis*, soit des mitochondries conservant leurs fonctionnalités, soit des mitochondries hautement purifiées. Ces méthodes furent utilisées afin d'étudier le rôle des mitochondries dans l'équilibre redox des cellules mais aussi dans le but de déterminer les capacités mitochondriales lors de la sénescence foliaire. Plus précisément, nous avons comparé l'induction de la sénescence foliaire grâce à différents traitements à l'obscurité. Cette comparaison entre des feuilles individuellement placées à l'obscurité et des feuilles provenant d'une plante entièrement disposée à l'obscurité révéla des stratégies métaboliques très différentes. En intégrant des mesures de l'activité photosynthétique, de la respiration et de microscopie laser confocale avec des analyses de transcriptomique et de métabolomique, nous suggérons que le métabolisme d'une feuille provenant d'une plante placée longuement à l'obscurité entre dans un état de « veille » dans le but de maintenir la machinerie photosynthétique fonctionnelle le plus longtemps possible; dans ce cas, les capacités mitochondriales diminuent. *A contrario*, les mitochondries issues de feuilles individuellement soumises à l'obscurité sont beaucoup plus actives et peuvent par conséquent fournir l'énergie et les squelettes carbonés nécessaires à la dégradation des constituants cellulaires facilitant ainsi la remobilisation des nutriments. Par ailleurs, nous avons aussi mené des investigations sur la dynamique du cytosquelette lors d'une sénescence induite par l'obscurité. La mobilité mitochondriale fut affectée dans les feuilles individuellement soumises à l'obscurité par la dégradation précoce des microtubules ce qui ne fut pas le cas dans les feuilles issues d'une plante entièrement placée à l'obscurité. De plus, un certain nombre de MAPS (microtubules-associated proteins) semblent être impliquées dans l'agrégation des microtubules autour des chloroplastes.

Dans son ensemble, cette thèse apporte d'importantes informations quant aux ajustements métaboliques ainsi qu'aux mécanismes cellulaires prenant place lorsque des feuilles d'*Arabidopsis* sont soumises à une obscurité prolongée. En particulier, nous pensons que les mitochondries ont un rôle prépondérant lors de la sénescence foliaire et que selon le statut métabolique de la plante, les régulations mitochondriales peuvent apparaître divergentes.

Mots clés: *Arabidopsis thaliana*, chloroplastes, cytosquelette, obscurité, métabolisme, microscopie, mitochondries, microtubules, sénescence, système redox.

Abstract

When switching from green to yellow, a leaf undergoes both morphological and metabolic changes. This process is known as senescence and improved understanding of its mechanisms is important both from a fundamental scientific perspective but also for biotechnological applications. The present thesis reports on several important aspects regarding the cellular and metabolic mechanisms occurring during leaf senescence with an emphasis on the mitochondrial contribution to this process.

As a first step, we developed methods to isolate either highly functional crude mitochondria or highly purified mitochondria from leaves of *Arabidopsis thaliana*. These methods were further used to study mitochondrial contributions to cellular redox homeostasis and to estimate the mitochondrial capacities in leaves undergoing senescence. In particular, we compared the induction of senescence by different dark treatments in *Arabidopsis*. The comparison between individually darkened leaves and leaves from whole darkened plants revealed different metabolic strategies in response to darkness. Integrating data from measurements of photosynthesis, respiration and confocal laser microscopy with transcriptomic and metabolomic profiling, we suggested that metabolism in leaves of the whole darkened plants enter a “stand-by mode” with low mitochondrial activity in order to maintain the photosynthetic machinery for as long as possible. In contrast, mitochondria from individually darkened leaves are more active and may provide energy and carbon skeletons for the degradation of cell constituents, facilitating the retrieval of nutrients. We also investigated the dynamics of the microtubular cytoskeleton during dark-induced senescence. Mitochondrial mobility was affected by an early disruption of the microtubules in individually darkened leaves but not in whole darkened plants. In addition, several microtubules associated proteins (MAPs) seemed to be involved in the bundling of the microtubules around the chloroplasts.

Altogether, the work presented in this thesis highlights several important steps regarding the metabolic adjustments and the cellular mechanisms in Arabidopsis leaves submitted to prolonged darkness. In particular, we suggest the mitochondria to fulfill specific and important functions during leaf senescence since the role of mitochondria in leaves experiencing prolonged darkness appears very dependant on the whole metabolic status of the plant.

Keywords: *Arabidopsis thaliana*, chloroplasts, cytoskeleton, darkness, metabolism, microscopy, mitochondria, microtubules, senescence, system redox.

FROM GREEN TO YELLOW
A LEAF STORY

Olivier Keech

Nancy-Université
Université
Henri Poincaré

Octobre 2007

A vous qui m'avez soutenu et cru en moi…

Il n'est point de bonheur sans liberté, ni de liberté sans courage ! [Périclès]

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List of papers

The following work is based on the here below papers, which will be referred to by their Roman numerals.

I. **Keech O**, Dizengremel P, Gardestrom P (2005). Preparation of leaf mitochondria from *Arabidopsis thaliana*. Physiol. Plant. 124: 403-409.

II. Rouhier N, Gelhaye E, Villarejo A, Srivastava M, **Keech O**, Droux M, Finkemeier I, Samuelsson G, Dietz KF, Jacquot JP, Wingsle G (2005). Identification of plant glutaredoxin targets. Antioxid. Redox Sign. 7: 919-929.

III. Gama F*, **Keech O***, Eymery F, Finkemeier I, Gelhaye E, Gardestrom P, Dietz KJ, Rey P, Jacquot J-P, Rouhier N (2007) The mitochondrial type II peroxiredoxin from poplar. Physiol. Plant. 129: 196-206.

IV. **Keech O**, Pesquet E, Ahad A, Askne A, Nordvall D, Vodnala SM, Tuominen T, Hurry V, Dizengremel P and Gardeström P (2007). The different fate of mitochondria and chloroplasts during dark-induced senescence in *Arabidopsis* leaves. Plant Cell Environ. (*doi: 10.1111/j.1365-3040.2007.01724.x*)

V. Ahad A, **Keech O**, Sjödin A, Stenlund H, Moritz T, Jansson S, and Gardeström P (2007). Leaf metabolism during dark induced senescence in arabidopsis integrating metabolomics and transcriptomics. (Manuscript)

VI. **Keech O***, Pesquet E*, Sjödin A, Jansson S, Tuominen H, Ahad A, and Gardeström P (2007). Early disruption of the microtubules during dark-induced senescence. (Manuscript)

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Preface

In this preface I would like to say a few words about being a PhD-student. The following thesis reports the results of my research during my PhD time but it does not show how my thoughts have matured during these years. Beyond the work and the experiments, successful or not, I learnt a lot about the others, about myself, about my strengths and my weaknesses. This cannot be written in any manuscript or article, but it appears to be essential in the process of becoming a doctor. Becoming a researcher does not only include being able to run experiments or to write articles. It is also to be able to interact with human beings from first grade students to professors and that also includes persons from different professional horizons. We need to be able to present our work to the public, to make it as clear and as simple to understand as we can. We need to work on our skill to insufflate our knowledge and passion for sciences to others. Furthermore, failures and disillusions forged us along the time with courage, patience, endurance, modesty and humility. Finally, and maybe the most important thing, I have learnt from "you", from every single person irrespective of their social belongings I have interacted with during these years. The evolution appears to be personal but cannot be achieved without the others. Always remember: we own our fate!

Olivier K.

A PhD journey…

12th of July 2007. Well well well, here I am, starting to write my thesis to get the grade of PhD in Plant Sciences. I have about a month to summarize by this text my work as PhD student. I guess there are several ways to start writing a thesis and I don't really know which one is best or even if there is one better than another one…However, in order to summarize my work during the last four and a half years and also to help the reader to understand this PhD time, I think I need to let my mind going back in the past and tell you what was going on for me at that time! I started my PhD with a project in collaboration between the Umeå Plant Science Centre (Umeå, Sweden) under the supervision of the professor Per Gardeström and the department of Ecology and Forest Ecophysiology (Nancy, France) under the supervision of the Professor Pierre Dizengremel. The subject was basically defined as 2 proteomic studies: one was about a comparison of the mitochondrial proteomes during cold stress in Arabidopsis leaves and the other one was about the modifications of the mitochondrial proteome during ozone stress in Arabidopsis leaves. As mentioned here above, the 2 projects dealt with mitochondrial proteomes from Arabidopsis leaves which first implicated to be able to get these mitochondrial proteomes. Back in the beginning of 2003, there were a couple of articles available purposing methods for isolation of mitochondria from Arabidopsis (Cf paper I and references therein). However, none of them truly dealt with Arabidopsis leaves. The authors clearly explained that their preparation was not pure enough for suitable proteomic analysis and consequently, they decided to work with dark grown cell-suspension cultures. However, our aim was clear and we wanted to work with leaves (mitochondrial metabolism is tightly coupled to the photosynthetic machinery which makes cell metabolism so interesting!). So, I started to work on establishing a protocol to isolate pure and functional mitochondria from Arabidopsis leaves. After 6 months of work (spending my days in the cold room…One told me Umeå was cold!) and going through the literature, I came to the point saying that we should change my subject and focus on leaf senescence and ozone instead of cold and ozone. Both Per and Pierre agreed and I must admit now that I am very thankful to them for the freedom they let me during all those years! After a year and a half, I finally established 2 suitable protocols yielding either highly functional crude mitochondria or highly purified mitochondria, see paper I (Keech *et al.*, 2005). Through collaborations with the lab of Jean-Pierre Jacquot and Nicolas Rouhier (Nancy, France), we further used these protocols to first

determine potential targets of plant glutaredoxins, see paper II (Rouhier *et al.*, 2005) and then to characterize the mitochondrial peroxiredoxin IIf in poplar, see paper III (Gama *et al.*, 2007).

However, in order to study the potential modifications of the proteome during leaf senescence, we first needed a system where we could get senescing leaves at different stages. We got 2 choices: 1/ use leaves from natural aging Arabidopsis plants 2/ use a system where we can induce leaf senescence. We opted for the second choice. In 2001, Weaver and Amasino (2001) described, mainly based on a molecular biology approach, a faster induction of senescence in individually covered Arabidopsis leaves in comparison with the leaves from a whole darkened plant. We thought this system to be extremely interesting because i) the system is very easy to handle ii) we can discriminate the effects of starvation and/or darkness from the effects of senescence iii) we can easily get an important amount of fresh material to isolate mitochondria. However, before running any proteomic studies, we needed to describe in details these two experimental setups. Based on a detailed quantification of organelles by confocal laser microscopy, photosynthetic and respiratory measurements, we showed that these two experimental setups exhibited different metabolic adjustments in response to darkness, see paper IV (Keech *et al.*, 2007). At the same time, Dr. Abdul Ahad, postdoc in Per Gardeström's group at that time, started a project focusing on metabolic and transcriptomic comparisons of individually darkened leaves with leaves from whole darkened plants. The results of this work are presented in paper V (Ahad *et al.* manuscript). Finally, since we suspected the cytoskeleton to be involved in the cellular rearrangement observed and described in paper IV, we present in paper VI (Keech *et al.* manuscript) the results of our up-to-date investigations.

Consequently, and before I let the reader going though the following thesis, I need to clarify 2 things about my work and my primary objectives: First, no data on comparison of mitochondrial proteomes are presented in the present thesis and second, even if I did some preliminary work on ozone stress, no data are included in this thesis in order to make a clearer story.

INTRODUCTION GÉNÉRALE

A contrario des animaux, les plantes ne peuvent se déplacer. Ne pouvant échapper à leur environnement, elles n'ont pour solution que de pouvoir s'adapter aux différentes contraintes dans le but d'assurer leur croissance et en conséquence leur pérennité. Les contraintes environnementales peuvent être d'origine très diverse et sont souvent regroupées sous le terme de stress abiotique. Froid, sécheresse, déficit en nutriments, pollution des sols, ozone, excès ou manque de lumière représentent quelques exemples de facteurs influençant le développement d'un végétal. En fonction de la nature du stress et de son intensité, une plante peut choisir différentes stratégies afin de s'adapter et de survivre aux conditions environnementales. Le sacrifice d'un ou de plusieurs organes facilement remplaçables, en particulier les feuilles, fait partie de ces stratégies adaptatives. Ce processus porte le nom de sénescence, du latin *senescere*, « devenir vieux ». Dans les régions tempérées, la chute des feuilles en automne est la manifestation la plus évidente du phénomène de sénescence foliaire. Toutefois, ce phénomène ne s'applique pas qu'aux arbres se préparant à l'hiver mais représente pour un végétal une stratégie d'adaptation à de multiples contraintes environnementales.

La sénescence foliaire est un processus organisé visant essentiellement à la mobilisation et à l'exportation de nutriments de la feuille vers d'autres organes « puits » tels que les méristèmes, les bourgeons, les feuilles en développement, les fruits et les organes de stockage (e.g. troncs, tubercules). Ce processus est marqué par la rapide dégradation des chloroplastes ce qui par voie de fait entraîne un remaniement métabolique considérable au sein des cellules photosynthétiques. Cependant, dans le but d'assurer le bon fonctionnement cellulaire jusqu'à la complète mobilisation des nutriments (azote, phosphore, ions métalliques et composés minéraux), les cellules doivent conserver un niveau d'énergie suffisant et par conséquent maintenir l'activité mitochondriale. Toutefois, le rôle des mitochondries lors de la sénescence foliaire reste mal connu et bien souvent hypothétique du fait de la difficulté de travailler *in vivo* sur ces organites.

Les mitochondries proviennent de l'endosymbiose d'une α-protéobactérie dans une cellule hôte il y a plus de deux milliards d'années. Leur principal rôle consiste dans la production d'énergie via leurs chaînes de transfert d'électrons. A cela, s'additionnent plusieurs fonctions essentielles assurant aussi bien le maintien de l'homéostasie que l'anabolisme et le catabolisme d'acides

aminés ou de lipides. Depuis une quinzaine d'années, de nombreuses études ont porté sur l'implication des mitochondries lors de l'apoptose. Bien que les mitochondries apparaissent comme les principaux régulateurs de la mort cellulaire chez les animaux, leur participation durant le processus de mort cellulaire programmée (PCD) reste incertaine du fait de rapports divergents. La question du rôle des mitochondries au cours de la sénescence foliaire demeure quant à elle presque inexplorée.

Par conséquent, c'est dans cette thématique que s'inscrit le travail de thèse ici présenté. Afin de faciliter notre travail en laboratoire, nous avons décidé de travailler sur un modèle de sénescence induite par l'obscurité et ce avec la plante modèle *Arabidopsis thaliana*. La dégradation précoce des protéines et des ARNs au cours de la sénescence entraine rapidement la perte de l'activité photosynthétique. Les cellules de la feuille devant toutefois survivre durant le recyclage de la plupart des nutriments, ont besoin d'ajuster leur métabolisme. Dans un premier temps, nous avons développé deux méthodes afin d'isoler des mitochondries de feuilles d'Arabidopsis. Ces protocoles furent ensuite utilisés dans le but de définir certaines régulations redox d'homéostasie spécifiques aux mitochondries mais aussi dans le but de déterminer la contribution mitochondriale lors des régulations métaboliques au cours de traitements à l'obscurité prolongée. Combinant d'une part une étude de microscopie confocale à des mesures sur les activités photosynthétique et de respiration et intégrant d'autre part des données de transcriptomique et de métabolomique, nous avons comparé les régulations métaboliques entre des feuilles individuellement placées à l'obscurité (le reste de la plante restant dans un cycle photopériodique normal) et des feuilles provenant d'une plante entièrement disposée à l'obscurité.

INTRODUCTION

1. Senescence

1.1 Introduction to senescence

Through the plant kingdom, there is a large range of lifespans. Certain tree species can live for several centuries whereas other plants, such as Arabidopsis, can complete their life cycle in a few weeks. Beyond this first remark, we also want to notice that individual organs of a plant such as leaves, flowers and vessels have lifespans that can substantially differ from that of the whole organism. For example, in a tree, a trunk consists of wood, which is the result of dead vascular tissue. So, it is important to keep in mind that there is a high rate of cell death occurring throughout the life of the plant (Guarente *et al.*, 1998). When contemplating the longevity and ultimate fate of plant tissues, laws of thermodynamics must be kept in mind. A plant cell requires energy input for its creation and maintenance and in theory an individual cell could be maintained alive forever if enough energy was provided. Yet, somatic tissues in plants have limited life spans (Bleecker and Patterson, 1997). Interestingly, the lifespan of leaves for example may vary from few days to as long as 20 to 30 years in species of *Araucaria* (Woolhouse, 1967).

When considering an individual leaf, its development primarily requires nutrients from the rest of the plant. But as soon as the photosynthetic apparatus is achieved and functional, the leaf converts from a nutrient sink to a nutrient source and the payoff can be established. It is advantageous to maintain leaves only for as long as they contribute to the survival of the plant. However, when the leaf gets mature, its life spans truly depends on environmental factors, both abiotic and biotic such as extreme temperatures, drought, shading, ozone and pathogen infection for instance (Woolhouse, 1967; Smart, 1994; Gan and Amasino, 1997; Thompson *et al.*, 2004). In temperate regions of the world for example, the shortening days and colder temperatures of the approaching winter limit the leaf productivity which leads the leaves from trees and other perennial plants to turn to magnificent yellow, orange and red colors. Finally, a massive

programmed cell death occurs and leads to death and loss of the leaves. This phenomenon is often referred to in the plant biology literature as leaf senescence. Specifically, the foliar senescence relates to the process by which nutrients are mobilized from the dying leaf to other parts of the plant to support their growth. Nutrient availability, particularly nitrogen, has been a major limit to growth and reproductive success throughout plant progression (Guarente *et al.*, 1998). Moreover, plants are fixed in a particular location in the soil and deplete their local environment. Thus, it becomes very easy to consider that plants have evolved mechanisms for dealing with obsolete organ systems, especially when we know that photosynthetic organs are very rich in valuable nutrients for the plants.

1.2 What is leaf senescence?

Foliar senescence is often regarded as a particular type of programmed cell death (PCD) (Nooden *et al.*, 1997; Pennell and Lamb, 1997; Delorme *et al.*, 2000; Lim *et al.*, 2003; Thomas *et al.*, 2003; Van Doorn, 2005) and its main purpose in plants is for nutrients mobilization and recycling. Throughout the scientific literature, common features are stated to describe the evolution of this regulated process within an organ such as a leaf. Chloroplasts are the first affected by the cellular degeneration process. Proteins and RNAs are degraded, causing the photosynthetic machinery to be rapidly impaired. Nutrients such as nitrogen, phosphorous, sulphur, minerals and metals ions are drawn back from the senescing leaves (source) to be recycled in other parts of the plant (sink) (For reviews see: (Smart, 1994; Buchanan-Wollaston, 1997; Nooden *et al.*, 1997; Himelblau and Amasino, 2001; Hortensteiner and Feller, 2002; Buchanan-Wollaston *et al.*, 2003). The loss of chlorophyll, one of the main biological markers for leaf senescence, begins from the outer parts of the leaf and proceeds inwards giving to the leaf this characteristic yellowish color. The tissues near the vascular system are the last ones to senesce, since they are needed for nutrient allocation (Quirino *et al.*, 2000). Nowadays, the understanding of senescence mechanisms is part of both fundamental scientific questions and an economical challenges to increase the yield of the crops by prolonging the photosynthetic activity and to minimize the post-harvest quality loss in vegetables (Gan and Amasino, 1997).
Leaf cells ongoing senescence undergo many biochemical and structural changes controlled by an important programming of gene expression. However, even though the catabolism leading

chloroplasts to become gerontoplasts is under direct nuclear control (Feller and Fischer, 1994; Gan and Amasino, 1997; Van Doorn, 2005), mitochondria are known to remain intact until rather late during the process most probably in order to supply energy mainly for reallocation of nutrients (Feller and Fischer, 1994; Smart, 1994; Collier and Thibodeau, 1995; Bhalerao *et al.*, 2003; Lim *et al.*, 2003; Keskitalo *et al.*, 2005). Nevertheless, no clear evidences are given about the respective roles of these two organelles during the leaf senescence. For instance, it has been recently suggested that chloroplasts could regulate leaf senescence by increasing the reduction level of electrons transporters and by generating reactive oxygen species (ROS), which are further thought to damage cell structures and functions (Zapata *et al.*, 2005).

1.3 What can trigger leaf senescence?

There are many factors that can initiate senescence in leaves and it is obvious that there are many different pathways involved in controlling the process (Smart, 1994; Gan and Amasino, 1997). As examples, we would like to mention that leaf senescence can be induced by a number of different environmental stresses such as pathogen infection (Quirino *et al.*, 1999; Beers and McDowell, 2001), nutrient deficiency (Feller and Fischer, 1994), drought (Pastori and Trippi, 1993; Pic *et al.*, 2002; Munné-Bosch and Alegre, 2004), ozone (Paakkonen *et al.*, 1997; Puffett *et al.*, 1997; Ojanpera *et al.*, 1998; Miller *et al.*, 1999; Pell *et al.*, 1999) or even UV-B (Pradhan *et al.*, 2006). However, a recent study from Costa *et al.* (2006) showed that UV-C delays postharvest senescence in broccoli florets. Overall, it seems that many signalling pathways controlling gene expression in response to different stresses are also involved in leaf senescence (Buchanan-Wollaston *et al.*, 2003). For instance, and taking the HR (HR: hypersensitive response, a disease- resistance response that results from incompatible pathogen interactions) as example, one could think that a senescing leaf is more susceptible to pathogen infections and consequently the defense-related genes induced during leaf senescence would be a normal preventive response to potential pathogenesis accompanying the senescence process. However, Quirino *et al.* (1999) demonstrated that defense-related genes are still induced during leaf senescence of Arabidopsis plants grown in sterile conditions which in turn indicates that the expression of defense-related genes (and most likely many other stress-related genes in other stress cases) might be an integral part of the senescence program. These considerations are in

line with an interesting study based on the Arabidopsis Genechip system (Affymetrix) where Chen *et al.* (2002) reported that 2/3 of the transcription factors genes induced by stress treatment were also induced during leaf senescence which suggests extensive overlapping in the responses to these stress treatments. However, depending on the nature and the intensity of the stress apply we are still unable to explain clearly whether stress causes the onset of senescence or whether the senescence itself induces stress responses.

1.4 Ozone as an example of stress leading to leaf senescence

1.4.1 What is ozone?

Ozone (O_3), also called "super-oxygen", is the tri-atomic form of oxygen and is naturally formed by the UV (ultra-violet) rays of the sun (photochemical reaction) and by lightning (bioelectrical reaction). Ozone may also be formed synthetically by passing air or oxygen over an UV lamp (photochemical reaction) and through a high-voltage field (bioelectrical reaction). Some of the oxygen molecules are split into two separate oxygen atoms. These singlet atoms then form semi-stable bonds with the oxygen molecules. These polyatomic oxygen molecules are highly reactive because the third oxygen atom, also known as a "Hungry Atom", is very willing to break away from this semi-stable bond and gives hydroxyl radical. The hydroxyl radical serves as the main scavenger in the atmosphere, reacting with a variety of compounds such as hydrocarbons, hydrogen sulfide, and carbon monoxide that would otherwise accumulate and would poison most of breathing organisms. Due to its reactivity, ozone has a very short life span (about 20 min.).

In the nature, two kinds of "Ozone" can be distinguished; the stratospheric ozone and the tropospheric ozone. Most of ozone resides in the stratosphere (layer of the atmosphere between 10 and 40 km above the earth's surface) and is essential to protect life on the earth by acting as a shield against solar UV-B irradiation (280nm-320nm) (Zinser *et al.*, 2000). Tropospheric ozone which appears in the atmospheric layer from the surface up to about 10 km is mainly the result of human activity (the burning of fossil fuels commonly gathered under the name of petroleum increases the production of carbon compounds that reacts in sunlight with oxygen to form ozone in the troposphere (Buchanan *et al.*, 2000)). Interestingly, troposheric ozone may be essential for

survival of many organisms by playing a key role in enhancing human health and well being since O_3 is involved in chemical reactions that clean the troposphere of some pollutants but is at the same time drastically harmful to life because it reacts with any oxidizable compound (organic or inorganic) (http://earthobservatory.nasa.gov/Library/Ozone/). In plants, the discovery of the phytotoxicity of O_3 during the mid 1950's (Haagen-Smit *et al.*, 1952) prompted widespread studies on the effects of O_3 on their growth and development (Ashmore, 2005).

1.4.2 What are the effects of ozone on plants?

The biochemical and molecular mechanisms underlying O_3 phytotoxicity have only begun to be unraveled in recent years. As shown by the figure 1, ozone will enter into the leaf through the open stomata, will diffuse in the apoplastic water phase and react very quickly with cellular components such as proteins and lipids giving in this way a pool of toxic reactive oxygen species (ROS) such as hydrogen peroxide (H_20_2), superoxide anion (0_2^-) and hydroxyl radical (OH^-)(Pell *et al.*, 1997; Schraudner *et al.*, 1997; Zinser *et al.*, 2000; Dizengremel, 2001; Rao and Davis, 2001; Langebartels *et al.*, 2002). Particularly hydroxyl radicals can react with membrane lipids to generate lipid peroxides that can initiate a series of reactions producing damaging reactive oxygen intermediates. These damaging free radicals and their products react with proteins, DNA and membrane lipids to cause an impairment of photosynthesis, electrolyte leakage and accelerated senescence usually associated with O_3 exposure.

Ozone exposure induces genes involved in ethylene biosynthesis (Miller *et al.*, 1999; Overmyer *et al.*, 2000). The O_3 toxicity is related to ethylene emission which might react with volatile compounds released in the apoplast. This interaction with ethylene but also with some other more complex hydrocarbons (isopropene, alpha-pinene) might be part of the mechanisms leading to injuries (Schraudner *et al.*, 1997). Recently, it has also been suggested that under field conditions ethylene could amplify ozone effects in european beech (*Fagus sylvatica* L.) (Nunn *et al.*, 2005). However, it must be kept in mind that phytotoxicity depends on the ozone flux within the leaf apoplast which in turn depends on stomatal opening linked to various climatic factors. On the other hand, the degree of sensitivity of plants to ozone can be related to the level of ascorbate (mainly in apoplasm) as well as to the intracellular capacity of regeneration of this antioxidant (D'Haese *et al.*, 2005).

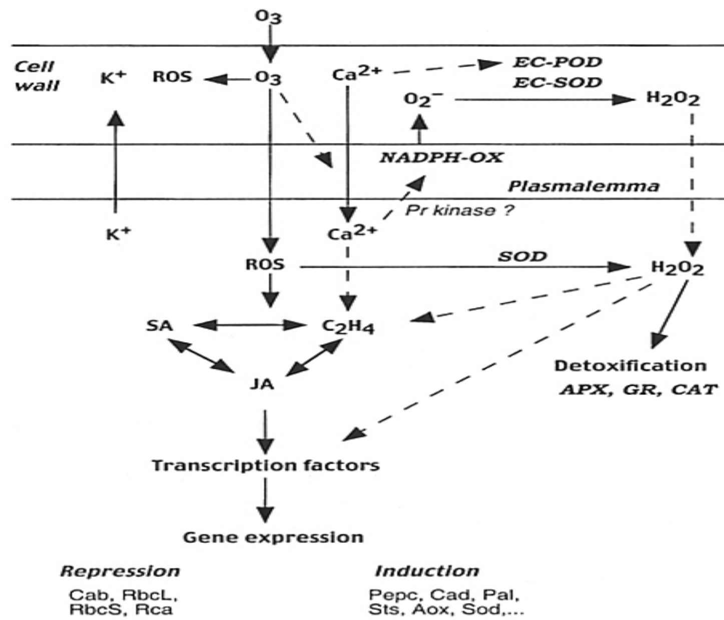


Figure 1. Possible cellular mechanisms of ozone-induced defence reactions in trees. Aox, alternative oxidase; APX, ascorbate peroxidase; Cab, chlorophyll a/b- binding protein; Cad, cinnamyl alcohol deshydrogenase; CAT, catalase; EC-POD, extracellular peroxidase; EC-SOD, extracellular superoxide dismutase; GR, glutethione reductase; JA, jasmonic acid; Pal, phenylalanine ammonia lyase, Pepc; phosphoenolpyruvate carboxylase, RbcL, RbcS, large and small subunits of Rubisco; Rca Rubisco activase; ROS, reactive oxygen species; SA, salicylic acid; Sts, stilbene synthase. (Dizengremel 2001). Reproduced with permission.

Because ozone formation requires sunlight, periods of high ozone concentration coincide with the growing season and ozone damage to plants can occur without any visible signs.

It has been shown that ozone may reduce biomass production and consequently crops' yields (Sharma and Davis, 1994; Gregg *et al.*, 2003). But according to the NCLAN (National Crop Loss Assessment Network), crops are not equal when they are exposed to ozone as shown in Figure 2.

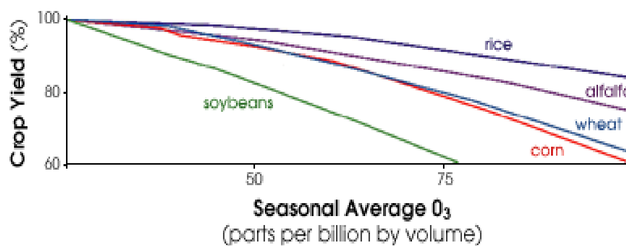


Figure 2. This graph from a study by the Environmental Protection Agency shows the reduction in yield of crops exposed to ozone. At an ozone concentration of 60 parts per billion, soybeans yields decrease to about 75 percent of normal, while wheat, corn, and alfalfa yields decrease to about 90 percent of normal. (http://earthobservatory.nasa.gov/Library/OzoneWeBreathe/ozone_we_breathe3.html)

1.4.3 Ozone and senescence

Many efforts are made today to determine precisely the mode of action of ozone and for many years, scientists have been trying to correlate physiological and biochemical events occuring in the plants with the intensity of ozone stress (Schraudner *et al.*, 1997). Phytotoxicity of ozone can be divided into acute and chronic exposures (Sandermann, 1996; Pell *et al.*, 1997). The former one leads most of the time to a quick cell death whereas the latter one may cause the acceleration of the normal rate of foliar senescence especially by a chlorophyll and protein loss within the foliar cells (Brendley and Pell, 1998; Rao and Davis, 2001; Rao *et al.*, 2002).

In many plant species, an accelerated foliar senescence has been reported as one of the harmful effects of O_3 (Pell *et al.*, 1997; Brendley and Pell, 1998; Bielenberg *et al.*, 2002; Langebartels *et al.*, 2002). This was also recently observed for several aspen stands at the free-air (CO2+ O_3) exposure site Aspen FACE (in northern Wisconsin) (Karnosky *et al.*, 2005). A study from 1999 revealed that Arabidopsis O_3-induced senescence involves many (although not all) of the genes associated with natural leaf senescence (Miller *et al.*, 1999). The findings of Miller *et al.* (1999) have been strengthened by two recent studies. D'Haese *et al.* (2006) observed in Arabidopsis the simultaneous induction of salicylic acid synthesis and genes involved in programmed cell death and senescence. Recently, Gupta *et al.* (2005) showed higher expression of senescence-associated genes (SAGs) and genes involved in the flavonoid pathway after long-term exposure of *Populus tremuloides* to elevated tropospheric O_3 in the Aspen FACE facility. Moreover they showed that many signaling and defense-related genes were also up-regulated and a lower expression of several photosynthesis and energy-related genes was observed under O_3, in normal or elevated CO_2 treatment.

Consequently, and as we have previously mentioned, ozone exposure leads to a reduction of the photosynthetic capacity by chlorophyll degradation and by an accelerated loss of both the activity and the quantity of Rubisco and Rubisco activase (Glick *et al.*, 1995; Brendley and Pell, 1998; Fontaine *et al.*, 1999; Dizengremel, 2001; Pelloux *et al.*, 2001). At the same time, the catabolic pathways (glycolysis, pentose phosphate pathway) and mitochondrial respiration are increased (Dizengremel, 2001). However, the role of increased mitochondrial respiration from

photosynthetic tissues during the plant response to O_3 remains insufficiently defined. It seems then essential to investigate further their metabolic role and involvement into the biochemical events and regulatory pathways facing ozone stress.

1.5 Signalling during senescence

1.5.1 Sugars signalling

Leaves are specialized organs for photosynthetic activity and their lifespan truly depends on their photoassimilates production. Interestingly, in green leaves and under light conditions, low sugar levels enhance photosynthesis, and the accumulation of glucose and sucrose represses the transcription of photosynthetic genes (Rolland *et al.*, 2002). During leaf senescence, one of the most early and common event occurring is the rapid impairment of photosynthesis. It has even been proposed several times that the rapid decline of photosynthetic activity could be a signal for the induction of leaf senescence (Smart, 1994; Bleecker and Patterson, 1997). In addition, the sugar starvation resulting from a reduction in the photosynthetic capacity is also thought to be linked with the induction of leaf senescence (Hensel *et al.*, 1993). This is further supported by the observation that the dark induced expression of many SAGs (senescence associated gene) is repressed in the presence of sucrose (Chung *et al.*, 1997; Fujiki *et al.*, 2001). In line with the theory that decreased sugar concentration would trigger senescence, it has been shown that transgenic tomato plants that overexpress the hexokinase (which catalyzes hexose phosphorylation) exhibit an accelerated senescence although their actual sugar content were lower than those from wild type plants (Dai *et al.*, 1999). Despite these findings, the role of sugars in the induction of leaf senescence under natural conditions is still unclear and remains rather controversial.

There is an extensive data set on a wide range of species showing that the soluble sugar concentration often goes up at the onset of leaf senescence (Nooden *et al.*, 1997; Keskitalo *et al.*, 2005). Similarly, Wingler *et al.* (2006) found that glucose and fructose accumulated in Arabidopsis leaves until late senescence. Moreover, a recent study from Pourtau *et al.* (2006) confirmed that Arabidopsis leaf senescence was induced rather than repressed by sugars. It must be kept in mind that the sink–source balances may affect the partitioning of sugar within a plant

and can consequently induce leaf senescence. Young leaves are sink organs until their complete maturation, whereas old leaves remain source organs by providing sugars to the rest of the plant and especially to the expanding leaves. When young leaves of sunflower and bean plants have fully developed their own photosynthetic machinery their demands for sugars begin to decrease. Such limited demands may lead to the accumulation of carbohydrates in the old leaves and their induction of senescence. However, when young leaves are shaded and therefore cannot operate photosynthesis and produce sugars, the older leaves of the same plant do not accumulate sugars and their senescence becomes retarded (Ono *et al.*, 2001). Taken altogether, these results indicate that senescence may be induced by carbohydrate accumulation and not by starvation. However, this seems highly dependant on a combination of environmental factors. Finally, sugar control of senescence is influenced by many other, environmental factors that affect leaf senescence, such as light conditions, CO_2 concentrations, nitrogen supply, stress and pathogen and it is highly possible that environmental signals are integrated by sugar signaling. For a very good review on the subject, we redirect the reader to Wingler *et al.* (2006).

1.5.2 Hormones signalling

Cytokinin and ethylene represent maybe the two best examples for hormonal involvement in the control of senescence. The signal that initiates the onset of developmental senescence appears to involve cytokinin. It has been known for many years that cytokinin levels decline in senescing leaves and that treatment with cytokinin can delay leaf senescence. To deliver cytokinin specifically to leaves at the onset of senescence, Gan and Amasino (1995) used the promoter of one of the senescence-associated genes (SAG12, coding for a cystein protease uniquely active during senescence) to drive expression of the gene coding for an enzyme involved in the cytokinin biosynthesis (the *Agrobacterium ipt* gene). The transgenic tobacco plants were shown to remain green and non-senescent for an extended period of time and a clear improvement of several traits important in agronomy, including a 50% increase in both seed yield and total biomass was observed. However, it is important to note that cytokinin alone may not be sufficient to delay all of the symptoms associated with leaf senescence (Oh *et al.*, 1996).

As we mentioned above, the other phytohormone commonly involved in senescence signalling is the ethylene. It seems that ethylene was discovered to induce senescence firstly by looking at the trees growing near by the streetlamps. Before incandescent streetlamps, gas lighting was employed. The earliest lamps required that a lamplighter toured the town at dusk, lighting each of the lamps by striking the flame when the gas supply was activated. However, it was noticed that leaves from trees growing nearby those streetlamps showed an accelerated-senescence. It was later understood that during gas combustion in the lamps ethylene was produced. Ethylene is essential for the ripening of many fruits, and plants exposed to ethylene show premature senescence. Grbic and Bleeker (1995) showed that the leaves of an ethylene insensitive mutant of *Arabidopsis* (*Etr1*) were delayed in their onset of senescence. In addition, certain *Arabidopsis* mutant lines that have been identified as showing delayed senescence turn out to have defects in genes in the ethylene signalling pathway (Oh *et al.*, 1997). However, in all these cases senescence occurs normally once the process has begun. Hence, it has been concluded that ethylene is a modulator of leaf senescence; its presence will speed up the senescence process but it is not essential for senescence to occur. Leaves have to be a certain age to be ready for the ethylene signal, young leaves treated with ethylene do not senesce (Buchanan-Wollaston *et al.*, 2003).

Furthermore, other hormones such as abscisic (ABA), salicylic acid (SA), jasmonic acid (JA) and giberellic acids (GA) are also reported to be more or less involved in the signalling pathways linked with senescence. The plant hormones ABA, SA, JA are known to promote senescence, whereas GA is known to inhibit senescence and promote flowering. It is becoming increasingly clear that various hormone-mediated signalling pathways form an interactive network. However, although the reports related to ethylene and cytokinins were fairly consistent and were pointing at the same direction, the complex interconnecting pathways of these phytohormones during senescence makes the whole conception rather unclear. This is partially due to a sum of divergent reports and observations about the implication of these phytohormones in signalling pathways during senescence. Finally, some evidences for a link between these plant hormones, senescence- associated processes, and ascorbic acid are also rising. Due to its essential function as a co-factor for the biosynthesis of giberellic acids and abscisic acid, ascorbate appears to influence not only the endogenous level but also signalling of these phytohormones during

senescence. Barth *et al.*(2006) hypothesized that low levels of ascorbate cause accelerated flowering and senescence under long-day conditions and delayed flowering and senescence under short-day conditions through alterations in phytohormone levels that are at least partially dependent on photoperiod. However, the role of ascorbate in regulating the final stages of plant development and its involvement during senescence remains to be elucidated.

1.6 Genes regulation during leaf senescence

More than 20 years ago, Watanabe and Imaseki (1982) highlighted important changes in mRNA translation during leaf senescence. Since the past decade, numerous studies about gene expression analysis during leaf senescence have become available and have revealed that leaf senescence is also characterized by substantial changes in gene expression. For example, the abundance of transcripts encoding proteins involved in photosynthesis decreases sharply during senescence. In contrast, a group of genes, often called senescence associated genes (SAGs) and mainly encoding degradative enzymes such RNases, proteinases or lipases are specifically up-regulated during leaf senescence (Gan and Amasino, 1997; Buchanan-Wollaston *et al.*, 2003). In addition, several genes involved in nutrient mobilization and reallocation processes are known to be up-regulated during leaf senescence (Brugiere *et al.*, 2000; Masclaux-Daubresse *et al.*, 2005; Pageau *et al.*, 2006).

In 2001, He *et al.* screened 1300 Arabidopsis enhancer trap lines and identified 147 lines expressing GUS specifically in leaves ongoing senescence. Using these lines, they analyzed the effects of 6 senescence promoting factors: ethylene, JA, ABA, brassinosteroids, dehydration and darkness. Interestingly, they noticed that none of these factors could induce up-regulation of all the lines (He *et al.*, 2001). This thus reinforces the idea of some overlap between age-dependant senescence and senescence induced by other factors, although distinct sets of SAGs are induced under each induction condition. In a more recent study, Guo *et al.* (2004) have identified almost 2500 ESTs representing a collection of genes specifically expressed during Arabidopsis leaf senescence. Among them, the authors found more than 130 transcriptional regulators and 182 genes whose products are components of signal transduction pathways in senescent leaves. One hundred and sixteen of these genes are predicted to be involved in protein turnover.

Although Arabidopsis is likely the most used species for plant biology nowadays; it remains a model of senescence for annual species. Further studies then dealt with perennial species. For example, Bhalerao *et al.* (2003) and Andersson *et al.* (2004) reported transcriptomes of Aspen leaves (Populus tremula) during natural autumn senescence. Nevertheless, they observed that transcripts during autumn senescence had very much in common with the leaf transcriptomes from annual plants ongoing senescence.

As a conclusion for this part about senescence, we reprinted this scheme proposed by Buchanan-Wollaston *et al.* (2005).

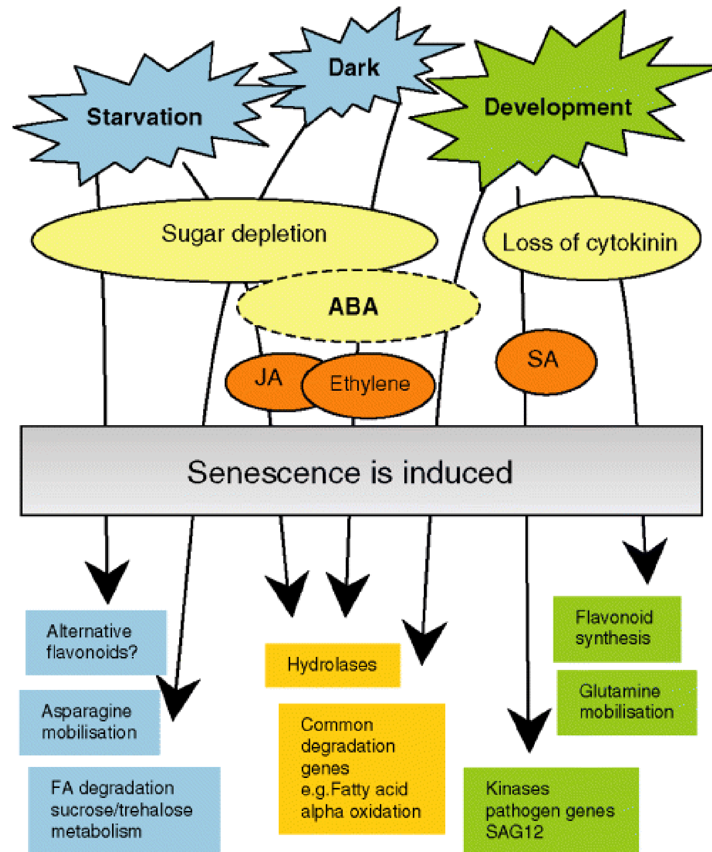


Figure 3. Model illustrating the similar pathways and alternative pathways that operate in three types of senescence. From Buchanan-Wollaston *et al.* (2005). Reproduced with permission.

2. Mitochondria

2.1 Introduction

Since when do we talk about mitochondria?

Mitochondria were described during the 19th century where early studies from cytologists reported the presence of subcellular granules similar in size and shape to bacteria in a variety of different cell types. Kolliker described conspicuous "granules" aligned between the striated myofibrils of muscle and was the first to isolate mitochondria from insect muscles. Fleming also observed "filaments" in the cytoplasm of other cell types. In 1890, Robert Altman discovered a method of staining these structures with fuchsin that made it possible to demonstrate their occurrence in nearly all types of cells. Consequently, he postulated that these granules were the basic units of cellular activity. Accordingly to their size and shape similar to bacteria, he named them "bioblasts". In 1898, the term mitochondrion was coined by the german microbiologist Carl Benda; in Greek "*mitos*" means "thread" and "*khondros*" "granule". Benda made valuable observations on their form and distribution in preparations stained with alizarin and crystal violet (Scott and Logan, 2007). In 1900, L. Michaelis selectively stained mitochondria with the dye Janus Green B. Since this staining method is based on redox properties (the dye must be oxidized to become colored), Michaelis proposed that mitochondria were cellular oxidizing agents. During the period 1900–1930, most cytologists recognized the mitochondrion as a well-defined and ubiquitous organelle, although at that time there was no real agreement about its function. Separation of mitochondria by differential centrifugation of cell homogenates was first attempted with some success by Bensley and Hoerr in 1934. The method was further perfected by Claude in the early 1940's and by Hogeboom, Schneider, and Palade in 1948. It was only during the late 1940's that mitochondria were identified as the site of oxidative energy metabolism (Logan, 2003). The development of improved methods of fixation and thin sectioning for electron microscopy enabled Palade and Sjostrand to describe independently in 1953 the basic structural plan of the internal membranes of the mitochondria. Since then, mitochondria have become subject of intensive research. They have been shown to be involved in many human diseases and the understanding of their integration and regulation within the cell metabolism appears crucial for further treatments and cures. Concerning plant tissues, Millerd *et*

al. (1951) were the first to isolate mitochondria, allowing a long series of work leading to better understand the specific functional characteristics of these organelles when comparing to animal counterparts (Douce, 1985). However and with no regard to their phyla, roles of mitochondria in cell metabolism remain under intensive investigations.

2.2 The origins of mitochondria

Two theories for the origins of mitochondria have been proposed through the years. The first theory is based on a non-symbiotic hypothesis where mitochondria would have evolved from compartmentalization of existing genetic material within the ancestral proto-eukaryote. However, complete genome sequences for many mitochondria, as well as for some bacteria, together with the nuclear genome sequence of yeast have provided a coherent view of the origin of mitochondria. In particular, conventional phylogenetic reconstructions with genes coding for proteins active in energy metabolism and translation have confirmed another theory: the endosymbiosis hypothesis (Andersson and Kurland, 1999). Interestingly, more than a century ago, Altman had already speculated that bioblasts were capable of an independent existence, yet formed a colonial association with the cytoplasm of a host cell, and that it was through this association that the host cell acquired the properties of life (Tzagoloff, 1982). The endosymbiosis theory postulates the capture, about 2 billion years ago, of an α-proteobacterial endosymbiont by a nucleus-containing eukaryotic host cell (Gray, 1999; Gray *et al.*, 1999; Lang *et al.*, 1999; Gray *et al.*, 2001). Possibly, the mitochondria of animals, fungi and plants have originated from a single symbiosis preceding the divergences of these kingdoms (Fig. 4).

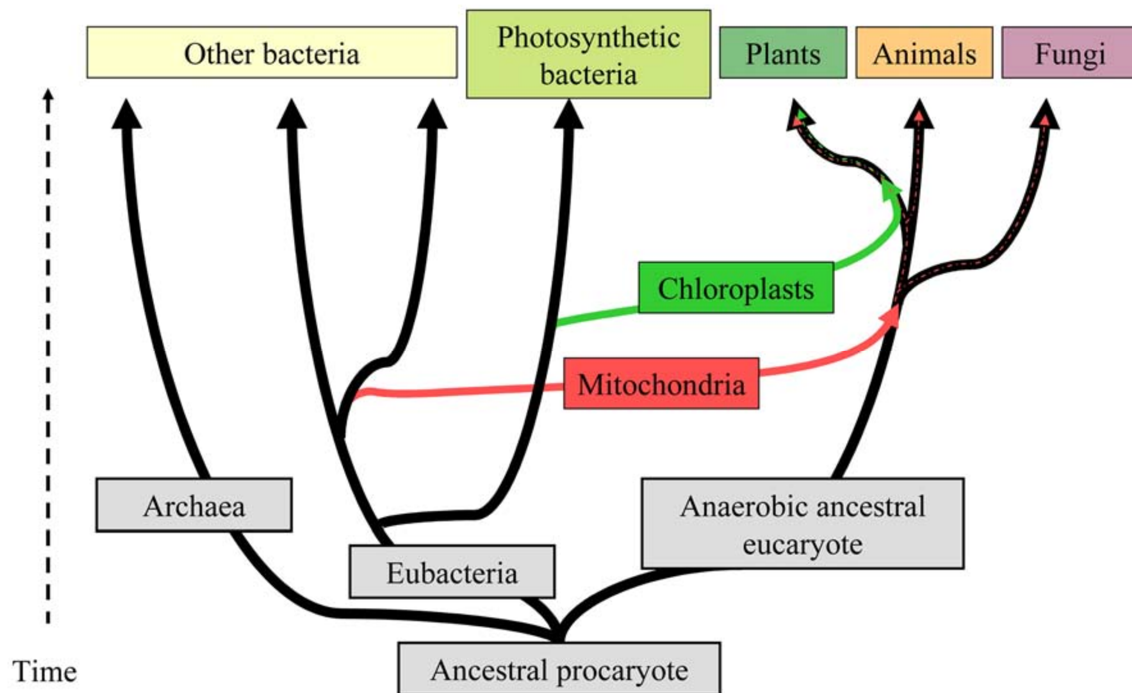


Figure 4. Model illustrating the endosymbiosis theory.

2.3 The basic functions of the plant mitochondria

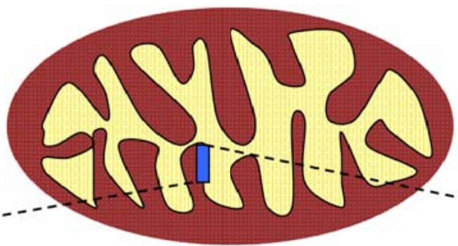
Energy production is most likely the main function of plant mitochondria. By the oxidation of substrates leading to synthesis of ATP, plant mitochondria can supply energy to the rest of the cell. Yet, mitochondria play a central role in the life of a cell by fulfilling many other essential functions by being involved in anabolic pathways, redox balance, cell death and differentiation, mitosis, along with more specialized cell functions including calcium homeostasis and thermogenesis, reactive oxygen species (ROS) and reactive nitric oxide species signaling, ion channels, and metabolite transporters. However, in this chapter, we do not aim at giving an exhaustive list of the functions fulfilled by mitochondria for two main reasons: first, it is almost impossible since we do not know all those functions and second even if we were trying, we would most probably lose the reader among the different pathways. However, in order to give a better understanding of the following work and its drawn conclusions, we will briefly present the main functions of plant mitochondria that we regrouped under 3 main themes: Electron transport chain, tricarboxylic acid cycle and photorespiration.

2.3.1 The mitochondrial electron transport chain

As we just mentioned, one of the primary functions of plant mitochondria is to produce and supply energy to the rest of the cell. The production of ATP (Adenosine triphosphate) is the result of the mitochondrial electron transport chain activity which takes place in the inner membrane of plant mitochondria. During this process also called oxidative phosphorylation, electrons resulting from oxidation of donors (NADH or $FADH_2$) are carried through the electron transport chain via redox reactions and achieve their "journey" by finally reducing O_2 into H_2O. The transport of electron is coupled with proton pumping from matrix to the inter-membrane space generating a transmembrane electrochemical proton gradient. The backflow of protons is further used for ATP synthesis i.e. phosphorylation of ADP into ATP.

In the mitochondrial electron transport chain (mETC), electrons are carried by proteins such as cytochromes (proteins with iron containing heme group), iron-sulfur proteins (with either 2Fe/2S or 4Fe/4S iron-sulfur center), and the small molecule ubiquinone (also called coenzyme Q). Major components of the electron transport chain are grouped in four multisubunit complexes: the NADH dehydrogenase complex (complex I), the succinate dehydrogenase (complex II), the cytochrome *c* reductase (complex III) and the cytochrome *c* oxidase (complex IV). In addition, the carrier lipid ubiquinone mediates transfer of electrons from the dehydrogenases to complex III and the monomeric protein cytochrome *c* shuffles electron between complex III and complex IV.

Complex I: The NADH dehydrogenase complex has a mass of about 1 MDa and is composed of over 40-45 distinct proteins. At least 10 cofactors are attached to this complex (one flavin mononucleotide and nine Fe-S clusters) (Heinemeyer *et al.*, 2007). Proton pumping occurs at the dehydrogenase complex when electrons from the oxidation of NADH,H^+ are transferred to ubiquinone which becomes reduced into ubiquinol. Four protons are pumped from the matrix side to the inter-membrane space of each pair of electrons passing through the complex I. Complex I can be inhibited by rotenone.



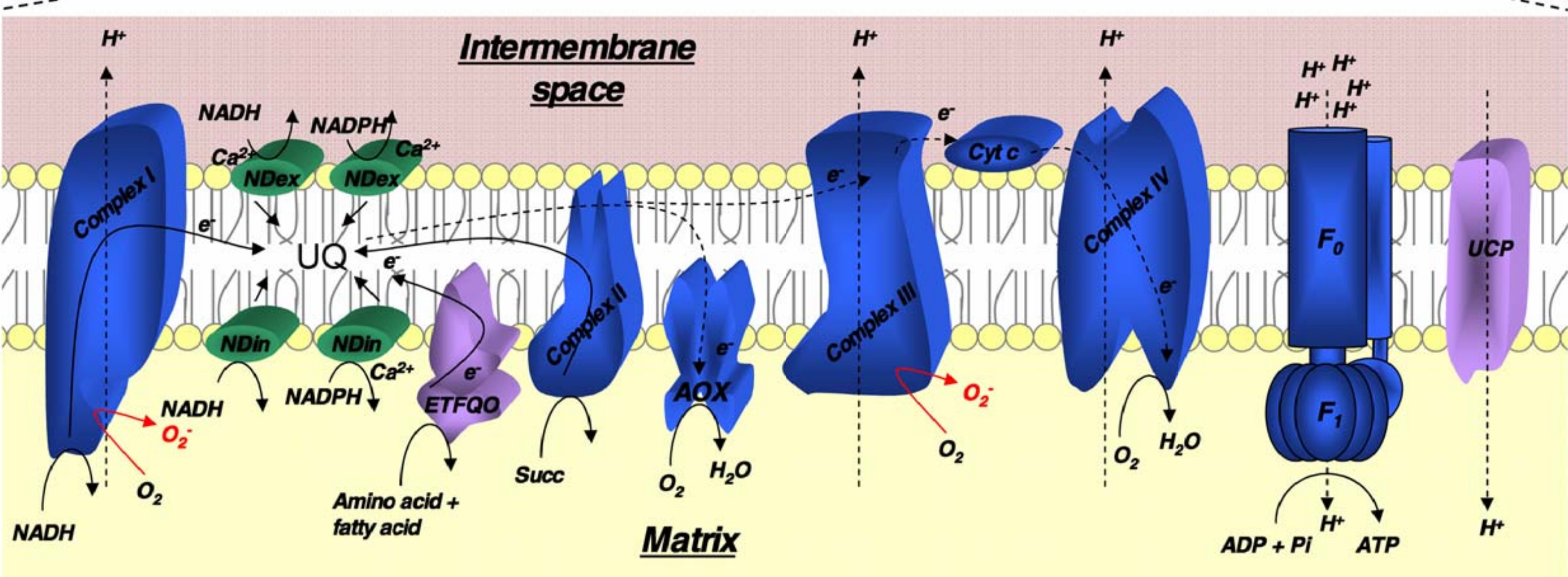


Figure 5. Plant mitochondrial electron transport chain. Complexes I, II, III, IV, cytochrome c and ATP synthase are common component to animals and plants. Plant specific components are added (NADH and NAHPH dehydrogenases, ETFQO, AOX and UCP). Reactive Oxygen Species formation is shown at complex I and complex III.

Complex II: The succinate dehydrogenase is the smallest of all four complexes and it does not pump any protons. It includes four types of subunits and five cofactors (one flavin adenine dinucleotide and three Fe-S clusters and one heme b) (Heinemeyer *et al.*, 2007). The complex II, to date the only membrane bound enzyme of the tricarboxylic acid cycle, oxidizes succinate into fumarate at an active site containing FAD (flavin adenine dinucleotide). Further, electrons reduce ubiquinone into ubiquinol.

Complex III: The ubiquinol-cytochrome *c* reductase is a functional dimer of about 500 kDa. Each monomer is composed of 10-11 proteins and 4 cofactors (three hemes and one Fe-S cluster). The complex III oxidizes ubiquinol and reduces cytochrome c (which can only accept one electron at a time). Four protons are pumped for each pair of electrons passing through the complex. Interestingly, the two largest subunits (also named core proteins) of the complex III are suspected to have a peptidase activity in plants. Isolated complex III from plants was shown to efficiently remove presequences from mitochondrial precursor proteins (Heinemeyer *et al.*, 2007). Complex III can be inhibited by antimycin A.

Complex IV: The cytochrome *c* oxidase has a mass of about 210 kDa and is made up of 13 subunits and four cofactors are attached to the complex (two heme a and two Cu^{2+}). Complex IV oxidizes cytochrome *c* and finally reduces oxygen into water which is the end product of the electron transport chain. Four electrons are necessary to reduce O2 into H2O. The complex IV can be inhibited by cyanide, azide and carbon monoxide.

ATP synthase: Sometimes also called the complex V, ATP synthase has a molecular mass of about 600 kDa. It is composed of two domains F0 and F1. F1, the part situated in the mitochondrial matrix, is composed of 5 different subunits (α, β, γ, δ and ε) and the F0 part, located in the inner mitochondrial membrane, is composed of ten subunits (a, b, c and several additional small subunits which are designated differentially in different organisms).

In addition to the four complexes described here above, plant mitochondria posses a few additional enzymes that play an important role in the mETC either as electron donors or acceptors.

-The uncoupling proteins (UCPs): Described in brown adipose tissues for the first time by Nicholls and Locke (1984) more than 2 decades ago, UCPs have later been evidenced in plants by Laloi *et al.* (1997). UCPs have a mass of about 32 kDa and they catalyze dissipation of the electrochemical gradient of protons in a fatty-acid dependant manner. In addition, they are activated by superoxide and aldehyde products of lipid peroxidation (Considine *et al.*, 2003; Smith *et al.*, 2004).

-The NAD(P)H dehydrogenases: Both the inner and the outer surface of the inner mitochondrial membrane has 2 additional dehydrogenases: one NADH and one NADPH types. They are not inhibited by rotenone and they transfer electrons to the pool of ubiquinone bypassing the complex I.

-The alternative oxidase: This enzyme has a molecular mass of about 35 kDa (32 to 37 kDa according to species). It is plant specific and works as a dimer. Alternative oxidase can accept electrons before the complex III and will reduce O_2 into H_2O like the complex IV. However, this pathway is non-phosphorylating. The alternative oxidase can be inhibited by salicylhydroxamic (SHAM) acid and propylgallate (PG).

-The electron-transfer flavoprotein:ubiquinone oxidoreductase (ETFQO): Recently, Ishizaki *et al.* (2005; 2006) evidenced in the inner mitochondrial membrane of Arabidopsis a new protein involved in amino acid degradation and maybe also having a role in chlorophyll degradation. The electron-transfer flavoprotein:ubiquinone oxidoreductase (ETFQO) receives electrons from the matrix enzyme: the electron-tranfer flavoprotein.

-The glycerol-3-phosphate dehydrogenase (FAD-G3PDH): This enzyme was identified by Shen *et al.* (2003) in Arabidopsis. It is a flavoprotein located on the outer surface of the inner mitochondrial membrane and it oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate

2.3.2 The tricarboxylic acid cycle (Krebs cycle)

We just saw that one of the primary functions of mitochondria was the synthesis of ATP via oxidative phosphorylation. In addition to that, plant mitochondria are involved in many metabolic processes such as nitrogen fixation, biosynthesis of amino acids, fatty acids, tetrapyrroles and vitamin co-factors (such as folate, biotin or ascorbate) but also in catabolism of branched chain amino acids (Leu, Ile and Val) and fatty acids. The tricarboxylic acid cycle (TCA cycle and also called Krebs cycle or citric acid cycle) was first evidenced by Krebs and Johnson (1937) in pigeon muscles. Through glycolysis taking place in the cytosol, glucose is converted into pyruvate. Pyruvate is further imported to the mitochondrial matrix where it is converted into acetyl coenzyme A (acetyl CoA) by the pyruvate dehydrogenase complex. The acetyl group of acetyl CoA becomes oxidized to CO_2 together with the generation of the following redox equivalents (four NADH and one $FADH_2$ per molecule of pyruvate oxidized). Acetyl CoA can also results from the β-oxidation of fatty acids. This process occurs in peroxisome, glyoxysome and apparently also in plant mitochondria (Masterson and Wood, 2000; Masterson and Wood, 2001) although it remains controversial.

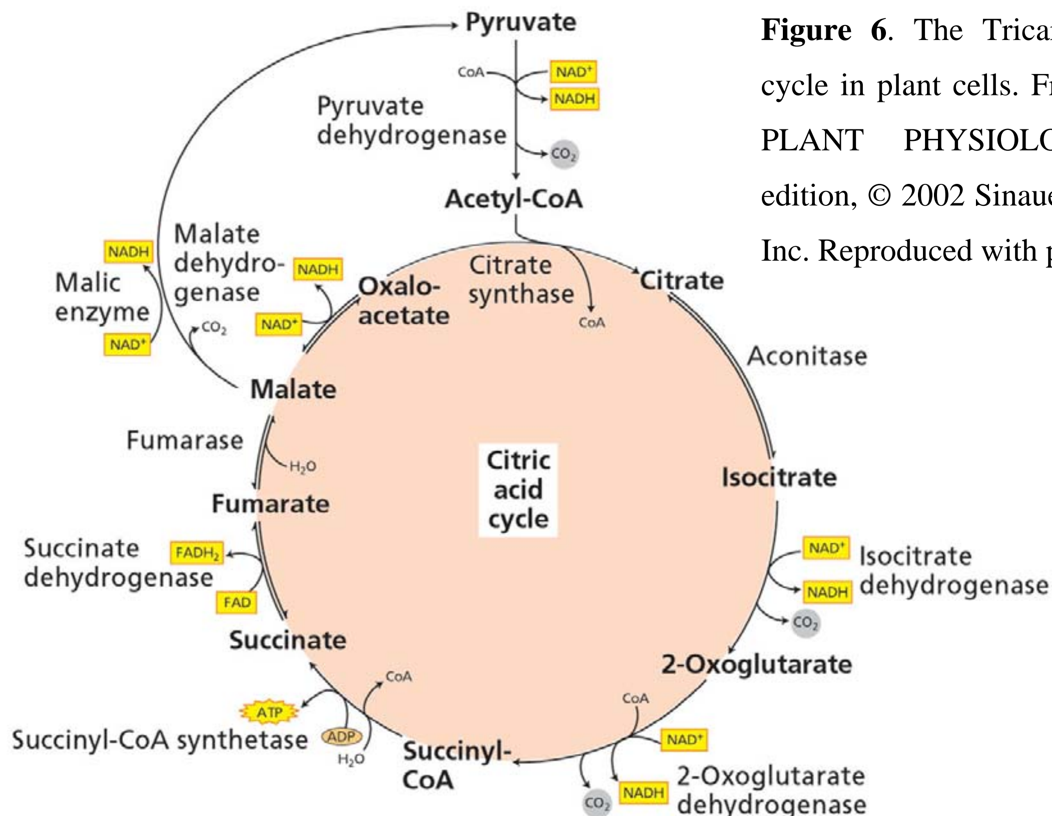


Figure 6. The Tricarbocylic acid cycle in plant cells. From the book PLANT PHYSIOLOGY, Third edition, © 2002 Sinauer Associates, Inc. Reproduced with permission.

By the above paragraph and the figure 6, we presented the TCA cycle in its entirety. However, in order to fully understand the essential role of the TCA cycle, it needs to be placed in the cell context. The TCA cycle plays an essential role by being at the inter-crossing of many cell metabolic pathways and consequently, by controlling the flux of carbon entering the TCA cycle, mitochondria are able to regulate the cell metabolic activity. For an excellent review, see (Nunes-Nesi and Fernie, 2007).

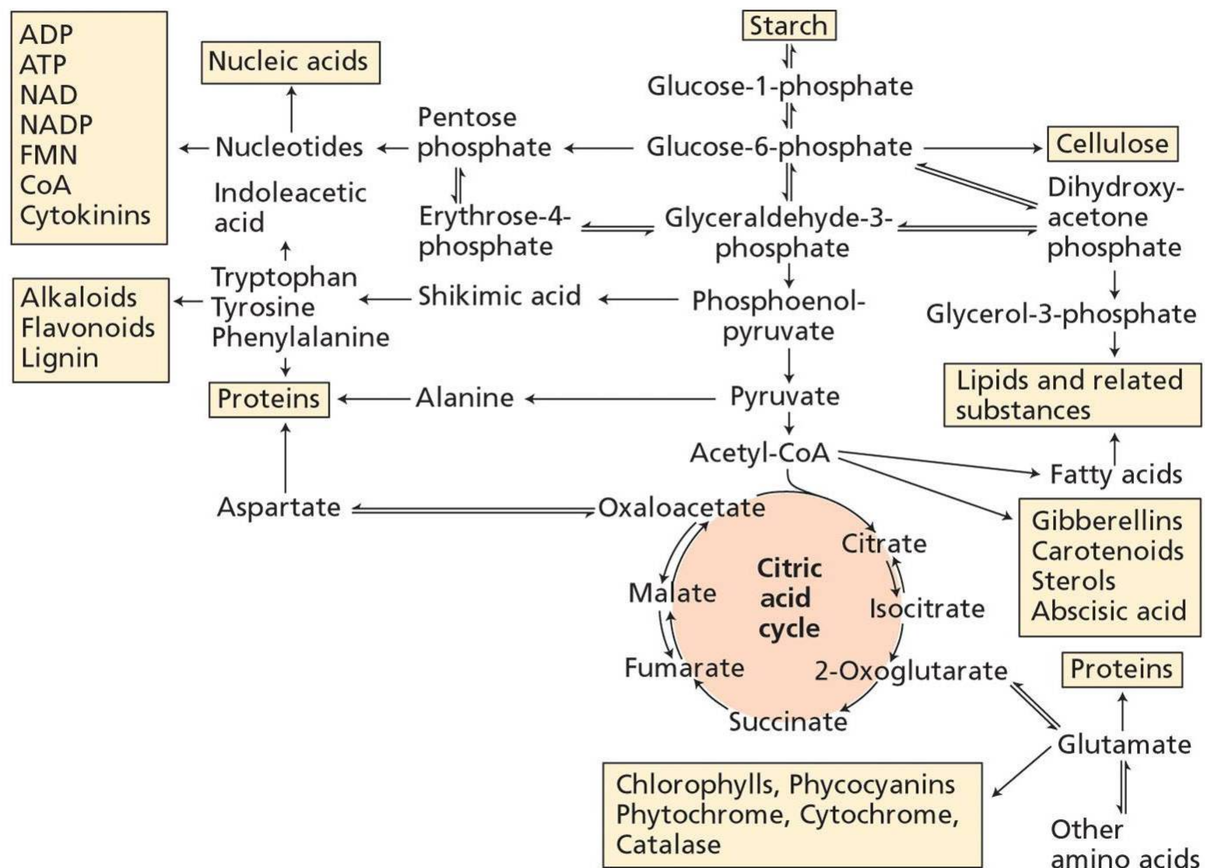


Figure 7. Example of interactions between TCA cycle and cell metabolism. From the book PLANT PHYSIOLOGY, Third edition, © 2002 Sinauer Associates, Inc. Reproduced with permission.

On the figure 7, we can notice the general involvements of the TCA cycle in several biosynthesis pathways. For example, the biosynthesis of amino acids is mainly localized in plastids. However, by the TCA cycle, mitochondria can supply α-keto-glutarate and oxaloacetate which once respectively converted to glutamate and aspartate will represent the backbone or carbon skeleton for amino acids synthesis.

2.3.3 The photorespiration

As early as 1920, Otto Warburg made the observation that O_2 inhibits photosynthesis. This phenomenon, originally known as the ''Warburg effect'' was later recognized as the light-dependent release of CO_2 by photosynthetic organisms, or photorespiration (Eckardt, 2005). The mechanistic basis of photorespiration was found in the dual nature of the RUBISCO (ribulose 1,5 bisphosphate carboxylase/oxygenase). Rubisco is a bifunctional enzyme that catalyzes both the carboxylation and oxygenation of Ribulose 1,5 biphosphate (RuBP). The photosynthetic carboxylation refers to CO_2 fixation and yields two molecules of 3-phosphoglycerate (3PGA) (i.e. the Calvin cycle) whereas the oxygenation process leads to the photorespiration (Fig. 8). Oxygenation of RuBP leads to the production of one molecule of 3-PGA and one molecule of the 2-carbon compound: the 2-phosphoglycolate. Since the two substrates (O_2 and CO_2) are competitive with regard to RUBISCO, an increase in CO_2 concentration leads to inhibition of oxygenase activity and vice versa. Temperature is an external factor enhancing the photorespiration. For example, on hot and dry days, stomata are closed and the O_2 concentration in the leaf exceeds that of CO_2, thereby increasing the competition for the common active site of the RUBISCO. Furthermore, the solubility of CO_2 diminishes more rapidly than that of O_2 as temperature increases.

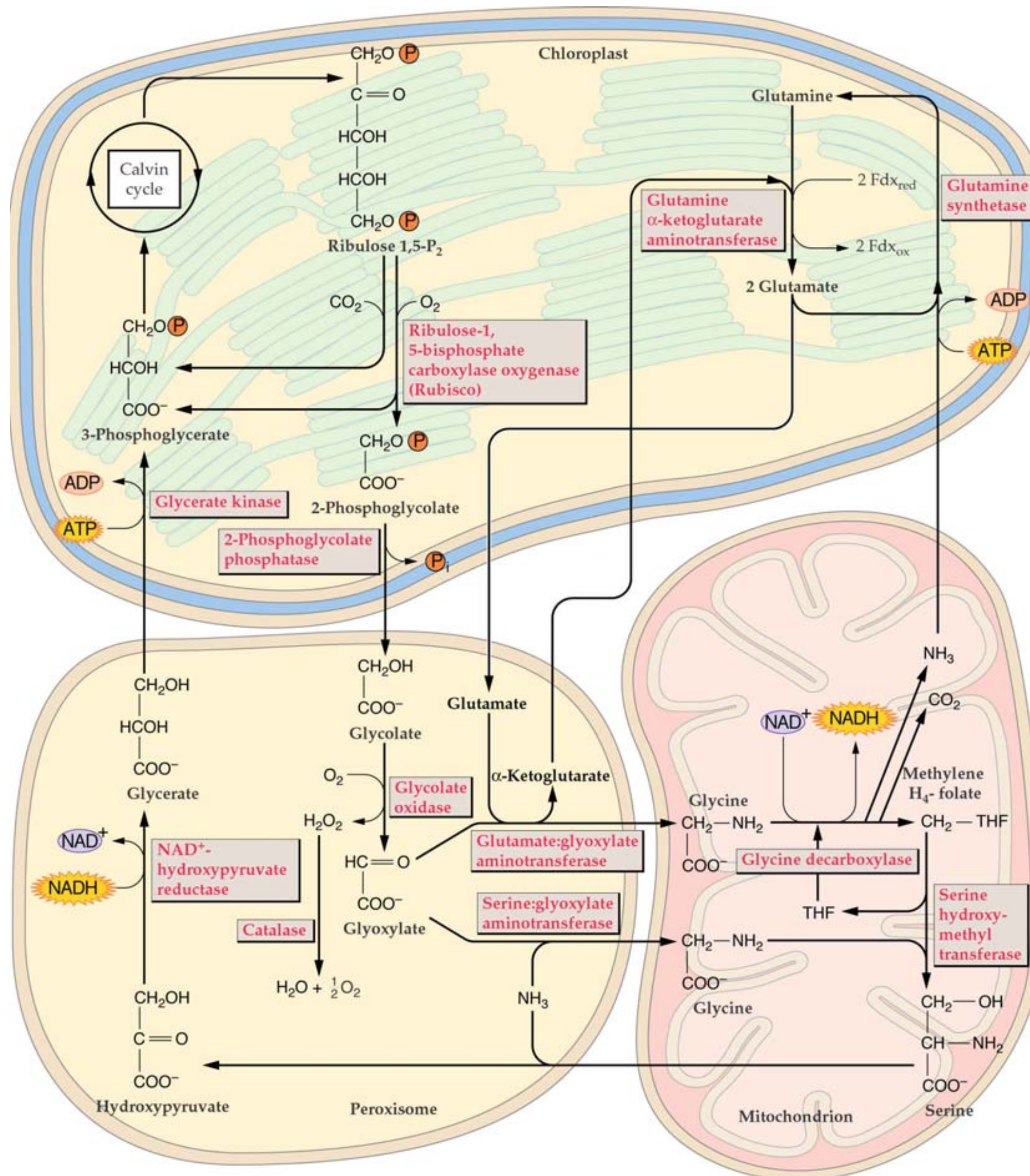


Figure 8. Scheme on C2 metabolism: photorespiration. From Biochemistry and molecular biology of plants edited by Buchanan, Gruissem and Jones. 2000. Copyright John Wiley & Sons Limited. Reproduced with permission.

Photorespiration involves three types of organelles: chloroplasts, peroxisomes and mitochondria (Fig. 8). The first step of the pathway involves the dephosphorylation of 2-phosphoglycolate which releases Pi within the chloroplast but also prevents accumulation of 2-phosphoglycolate, a potent inhibitor of triose-P isomerase. In its dephosphorylated form glycolate is exported to the

peroxisome where it is converted to glyoxylate. The hydrogen peroxide generated is detoxified by catalase. Glyoxylate is then converted into glycine by two different enzymes: serine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase After its transfer to the mitochondrion, glycine gets converted into serine by the glycine decarboxylase complex. Glycine decarboxylase complex has four different subunit (P, H, T and L), which catalyze the transfer of a methylene group from glycine to tetrahydrofolate with the concomitant release of NH_3 and CO_2, and reduction of NAD^+ to NADH (we can remark that NH_3 is used by glutamine synthetase in the chloroplast). The serine hydroxylmetyltransferase catalyses then the transfer of the methylene group to another glycine molecule to form serine. Exported back to peroxisome, serine is converted to hydroxypyruvate via serine:glyoxylate aminotransferase. The last of the peroxisome steps consists in the reduction of hydroxypyruvate into glycerate by an NADH-dependent hydroxypyruvate reductase. Finally, glycerate is exported to the chloroplast where it is phosphorylated to 3-phosphoglycerate and reenters the Calvin cycle.

Despite the net loss of carbon (up to 25% of the carbon that is fixed during photosynthetic carbon assimilation), the photorespiration allows a partial recycling of carbon. The photorespiratory cycle is essential for plant growth, as demonstrated by photorespiration mutants that are non-viable in normal air (around 0.04% CO_2) and grow only in elevated CO_2 (1% to 2% CO_2), conditions under which RuBP oxygenation is suppressed (Somerville, 2001). However, the outcome of photorespiration remains a loss of CO_2 and energy in photosynthetic cells. The biological function of photorespiration is not clear. One possibility is that photorespiratory cycle is necessary under conditions of high light intensity and low CO_2 concentration (i.e. when stomata is closed under water stress) to dissipate excess ATP and reducing power from the photosynthesis light reactions, thus to prevent damage to the photosynthetic apparatus (Somerville, 2001). Finally, photorespiration is also seen as an evolutionary adaptation. Due to the increasing amount of O_2 in the biosphere, plants had to find a way to salvage the loss of carbon which seems to be an inevitable consequence of the RUBISCO reaction mechanisms.

3. Aim: Why senescence and mitochondria?

After reading the two previous chapters, one obvious question that directly comes to the reader is: Why senescence and mitochondria? Through this new chapter, I will try to lead the reader towards our aim and hypothesis about the here presented thesis. In order to facilitate their understanding, I will first address 3 points:

3.1 The mitochondrial contribution to programmed cell death

In animal programmed cell death (PCD), also called apoptosis, mitochondria are shown to play a crucial role by generating ROS via the mitochondrial electron transport chain and by integrating several signals such as oxidants and Ca^{2+} overload which further leads to PCD by the release of cell-death mediators such as cytochrome c, AIF (Apoptosis Inducing Factors) and the endonuclease G (Green and Reed, 1998; Lam *et al.*, 2001; Li *et al.*, 2001; Arnoult *et al.*, 2002). Yet, in plants, the situation concerning the role of mitochondria during PCD becomes more obscure and still remains under important investigations.

3.2 Senescence / programmed cell death: where are the boundaries?

Over the last decade, scientists have tried to define the boundaries between senescence and PCD but this issue is still a matter of debate (Thomas *et al.*, 2003; van Doorn and Woltering, 2004; Van Doorn, 2005). The death of an organ is generally called “senescence” whereas the death of a cell often obviously refers to the programmed cell death process (Of course, the death of an organ results from the combined and rather synchronized death of cells). Nevertheless, as Thomas *et al.* (2003) argued, reversibility might serve to distinguish senescence fundamentally from programmed cell death (PCD), as does the fact that viability is essential for the initiation and progress of cell senescence. This seems to be in line with the idea raised by Delorme *et al.* (2000) after their study on a metalloproteinase from cucumber. They suggested that either PCD could occur only at the culmination of the senescence program or that the processes are distinct with PCD being triggered at the end of senescence. This consequently would mean that PCD and

senescence should be clearly dissociated by their fundamental mechanisms but associated in a common fate of a cell or an organ ongoing death process.

3.3 Economical perspectives

As we previously mentioned, the understanding of leaf respiration during aging and senescence is part of very important economical challenges. We can take the recent use of Modified Atmosphere Packaging (MAP) as an illustration.

In order to preserve fruits and vegetables and to increase their shelf-life time, plant respiration is studied to design adequate packaging for shipping and conservation. By correctly manipulating the amount of O_2 and CO_2 within the packaging, MAP enables the produce to live longer by delaying respiration, ripening and ethylene production. This in turn reduces browning, retards textural softening, preserves vitamins and extends the overall freshness of the packaged produce (http://www.convex.co.nz/prod_refresh_technical.html). This demonstrates the importance of a correct understanding of the cellular and metabolic mechanisms occurring during maturation, aging, senescence and death.

3.4 Aim and Hypothesis

We just mentioned that mitochondria are known to be involved in animal apoptosis but their role during plant PCD is still enigmatic and rather controversial. Furthermore, even though PCD and senescence are both assimilated to the death process, the mitochondrial contribution to the process of leaf senescence is unknown (despite a few sparse reports) and surprisingly remains rather aside from current researches. However, during leaf senescence, chloroplasts are primary targets of catabolism, which leads to the rapid impairment of photosynthesis. Consequently, we assumed that such loss of photosynthetic capacity might lead to important perturbations and reprogramming in the plant cell metabolism. Since chloroplasts loose their functionalities during leaf senescence, we further hypothesized that mitochondria could have a crucial role by supplying energy and the adequate carbon skeletons to the rest of the cell in response to the potential demand due to catabolism and nutrient recycling. Therefore, the following study will

present the result of our investigations in our attempt to clarify the rearrangements of the cell metabolism and the potential role played by mitochondria during the leaf senescence.

4. Isolation of mitochondria: a useful tool

Leaves are the main photosynthetic organs of the plant, but due to their photosynthetic activity, their metabolism has to adjust constantly. As a result, studies of isolated cell organelles are essential for the understanding of the processes at the cellular and subcellular levels in photosynthetic cells. Leaf cell metabolism represents a very complex network of inter-crossing metabolic pathways between organelles. However, in order to obtain clear results, we often have to work with isolated organelles. These isolated organelles have to be sufficiently pure and intact to adequately represent their function in the intact cell. Since our interest was on mitochondria, we decided to first develop a protocol for isolation of mitochondria from Arabidopsis leaves suitable for further studies.

4.1 Why Arabidopsis?

During the last decade, *Arabidopsis thaliana* Heynh., a small Brassicaceae, became the model plant for intensive research worldwide. Its small, diploid genome, fully sequenced (The Arabidopsis Genome Initiative, 2000), has provided invaluable tools for genomic and proteomic studies. The information compiled in databases makes Arabidopsis a model plant for many scientists, which offers opportunities for detailed studies of cellular functions that is not possible with other species. Several reports had already described the isolation of mitochondrial Arabidopsis leaf fractions for specific studies (Somerville and Ogren, 1982; Turano *et al.*, 1997; Berkemeyer *et al.*, 1998; Yasuno and Wada, 1998; Fan *et al.*, 1999; Johansson *et al.*, 2004; Taira *et al.*, 2004). However, most of these reports lack detailed information on the purity and intactness of the preparations obtained, and they do not consider respiratory properties, an important aspect of the functionality of isolated mitochondria.

4.2 Where are the difficulties?

At first, a practical problem associated with Arabidopsis is that the plant is small, and thus, it is difficult to obtain large quantities of its leaves. It was suggested that a hydroponic cultivation system would be useful for increasing the leaf mass (Norén *et al.*, 2004). This might improve the situation somewhat, but the yield of leaves from Arabidopsis will always be limited.

In leaves, photosynthetically active cells (i.e. mesophyll cells and guard cells of stomata) contain chloroplasts. During extraction process, the grinding of the leaves often leads to considerable contamination by fragments of broken chloroplasts, which causes problems for the isolation and study of other cell fractions from leaves. In addition to thylakoids, peroxisomes that have a rather similar density as mitochondria generally contaminate the mitochondrial fraction. Therefore, an alternative method, adopted by several scientific groups (Davy de Virville *et al.*, 1994; Kruft *et al.*, 2001; Millar *et al.*, 2001; Werhahn *et al.*, 2001), has been to work with cell-suspension cultures of non-green tissues in order to obtain preparations with good respiratory properties (Douce and Packer, 1987) and, thus, greatly reduce peroxisome and chloroplast (especially thylakoids) contamination; the two main contaminants of the mitochondrial fraction from green leaves, irrespective of the plant species.

Finally, isolation of functionally intact leaf mitochondria can be a very difficult task. First, one needs to establish the right composition of extraction solutions as well as their correct combination for the extraction, to track the mitochondria during the isolation procedure, to run purity and integrity tests. Moreover, mitochondria have important dynamics which can influence their isolation.

Even though isolation procedures yielding highly purified and functional mitochondria have been described for many species (for review see (Douce, 1985)), we have not been able to obtain good preparation of leaf mitochondria from Arabidopsis by most of the techniques and protocols cited in this book and other articles. Consequently, we present in paper I (Keech *et al.*, 2005) two different procedures for isolating mitochondria from Arabidopsis leaves. Those two procedures are also briefly described in Figure 9. Method A produces highly functional mitochondria with respect to respiratory properties using a small quantity (about 5 g) of leaf material. However, the resulting preparations are contaminated by chloroplast material. The second protocol, method B,

requires more leaves (at least 20 but better with 50 g) and leads to highly pure mitochondria, but they have weaker respiratory coupling than those isolated with the first method.

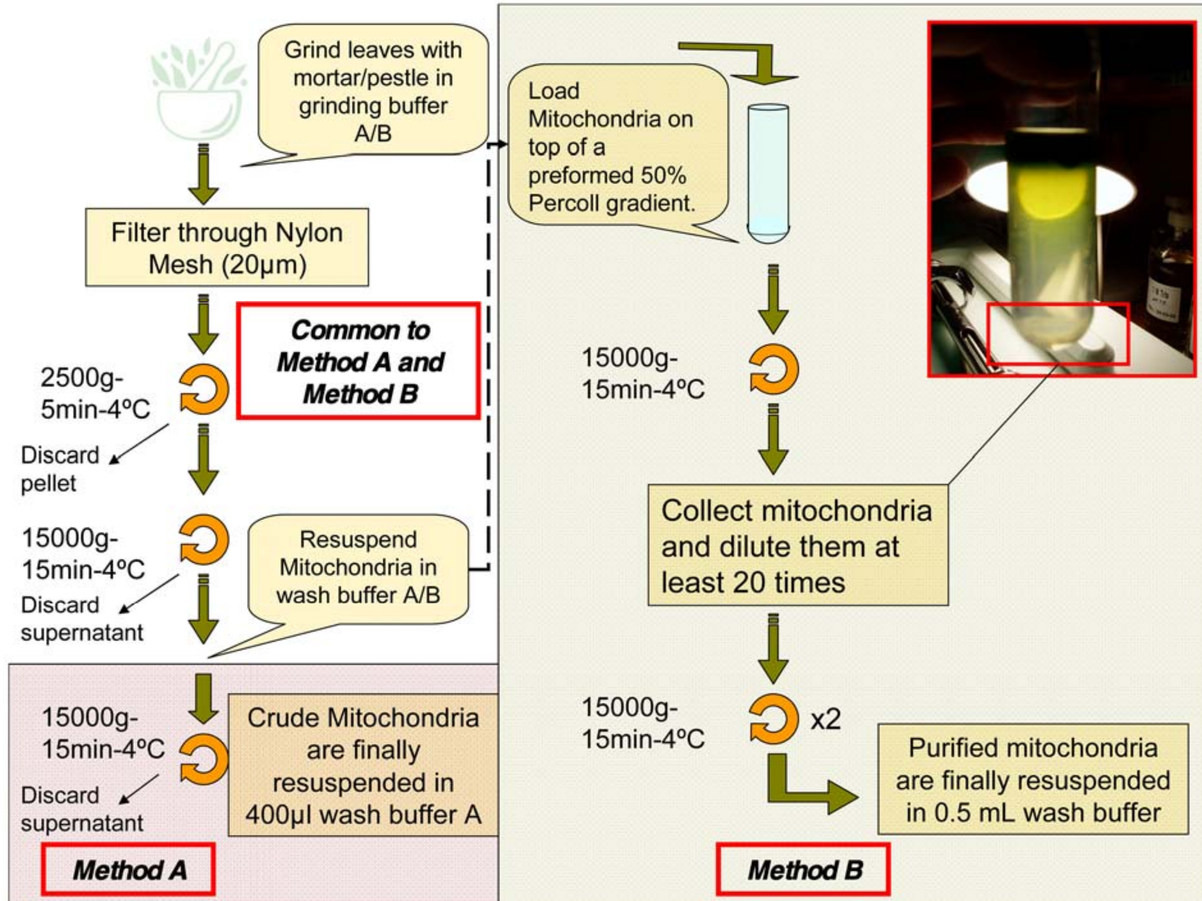


Figure 9. Flow chart of the two procedures for isolation of leaf mitochondria.

4.3 Comments about the two methods

One of the main differences between method A and method B proposed in paper I is the concentration of EDTA (ethylenediamine tetraacetic acid) added in the grinding buffer and in the wash buffer. Mitochondria kept a better coupling (i.e. respiratory properties) when a high concentration (10mM) of EDTA was used in the grinding medium. However, when we tried to purify the crude fraction of mitochondria extracted accordingly to method A on a Percoll gradient, we could not get a good purification. The mitochondrial fraction was always contaminated by thylakoids.

Even though we do not have a clear explanation for this, we suggest a high concentration of EDTA to limit damages caused by the lipases released during the extraction process. This would explain the better coupling of mitochondria extracted by method A. Arabidopsis is known to have several lipases, notably the 2 families of phospholipases C and D (Dhonukshe *et al.*, 2003) which need Ca^{2+} as cofactor (Wang, 2005). The EDTA can chelate both Ca^{2+} and Mg^{2+}. The chelation of Ca^{2+} would consequently reduce the activity of the phospholipases limiting the release of free fatty acids and free-head groups (Wang, 2005) destabilizing the biological membranes. However, since EDTA also chelates Mg^{2+}, it might be possible to get a separation of grana stacks into series of single thylakoids by the lack of magnesium (Popovic *et al.*, 1979). This would result in a huge set of thylakoid clusters from many different sizes and densities and would then explain the presence of thylakoids all over the Percoll gradient and our troubles to get good purification of mitochondria with method A.

Another point we would like to mention about the composition of the buffers used in the two methods described in paper I is about the Percoll gradient. To our knowledge, there is no other protocol to isolate mitochondria where a 50 % Percoll gradient has been used. Here, we do not want to highlight the “modest novelty” of using a 50 % Percoll buffer but more the fact that the combination of a preformed gradient and a 50 % Percoll buffer can make the trick! However, we come to question about the mitochondria that are able to migrate to such densities. For information, during centrifugation for purification of mitochondria, the auto-generated Percoll gradient is often between 25 and 32 percent Percoll. Consequently, we came to think that several subpopulation of mitochondria coexisted in leaves and we have basically been able to isolate one of these subpopulations with mitochondria having higher density. This is purely speculative but however in line with remarks from Dr D.C. Logan (personal communication) about the dynamics of plant mitochondria. Mitochondrial population can vary in number and in mass according to the phase of the cell cycle and the physiological status of the cell (Scott and Logan, 2007).

4.4 A tool for further studies

It took more than a year to establish a successful protocol. However, as we previously mentioned here above, we also tried several procedure previously described to isolate mitochondria from Arabidopsis leaves. One of those consisted on a combination of differential centrifugations and phase partitioning (Bergman *et al.*, 1980). By using this technique, adapted for Arabidopsis though, we also obtained rather good results to get purified mitochondria from Arabidopsis leaves. We actually used this technique to identify plant glutaredoxin (Grx) targets (paper II) through a collaborative work with N Rouhier and JP Jacquot. However, as one can see in paper II, few targets of Grx found in the Arabidopsis mitochondrial extract were actually localized in peroxisomes or in chloroplasts. It is known that up to date, none of the protocol for extraction of mitochondria from leaves, irrespective to the species, leads to perfectly pure fraction. Even a very recent protocol developed by Dr. H. Eubel (personal communication) and based on free flow electrophoresis cannot yield to a totally pure fraction of leaf mitochondria.
As one can see through papers III, IV and VI, the protocol developed in paper I has been widely used for different purposes. In Paper III (Gama *et al.*, 2007), in order to characterize the PrxIIf (a miotochondrial peroxiredoxin), we used the method A to obtain fraction enriched with mitochondria from poplar leaves. Method A was also used in paper IV (Keech *et al.*, 2007) to evaluate the mitochondrial respiration during dark-induced senescence in Arabidopsis leaves.

Finally, isolation of highly purified mitochondria (method B) has been used in several other experiments related to different projects, including a few preliminary experiments in the proteomic field. However, since none of them has been finalized yet, the results are not presented in this thesis. So, to conclude, the establishment of such protocol was very laborious and time consuming for a PhD project but after all it appears to be of importance for many of the studies we led and are still leading.

5. Dark induced senescence as a model

The experimental setup

Amongst external factors triggering leaf senescence, the shading phenomenon has caught the attention of scientists. Indeed, in order to study leaf senescence, scientists have tried to mimic the natural shading of leaves by using darkening treatments which lead to the senescence of the organ due to drastic modifications of the photoperiod. This dark-induction of senescence might be done either by placing excised leaves in the dark or dim light (Simon, 1967; Satler and Thimann, 1983; Feller and Fischer, 1994; Jimenez *et al.*, 1998; Pruzinska *et al.*, 2005; van der Graaff *et al.*, 2006) or by setting the whole plant in the dark or by covering just one or a few leaves still attached to the plant (Feller and Fischer, 1994; Weaver and Amasino, 2001; Buchanan-Wollaston *et al.*, 2005; Zelisko *et al.*, 2005). The two latter ones may present an undisputable advantage for physiological studies because they do not dissociate the leaf from the rest of the plant thus allowing potential signals throughout the plant. Consequently, we decided to use the experimental design proposed by Weaver and Amasino (2001) where they reported, by a study based mainly on both loss of chlorophyll and expression of senescence associated genes (SAG's), a much faster induction of senescence in Arabidopsis leaves individually covered than in the leaves from a whole darkened plant (Fig. 10). However, we brought a few modifications to their experimental setup such as the photoperiod during growth, soil content or age of the plants. For further details, please refer to paper IV (Keech *et al.*, 2007).

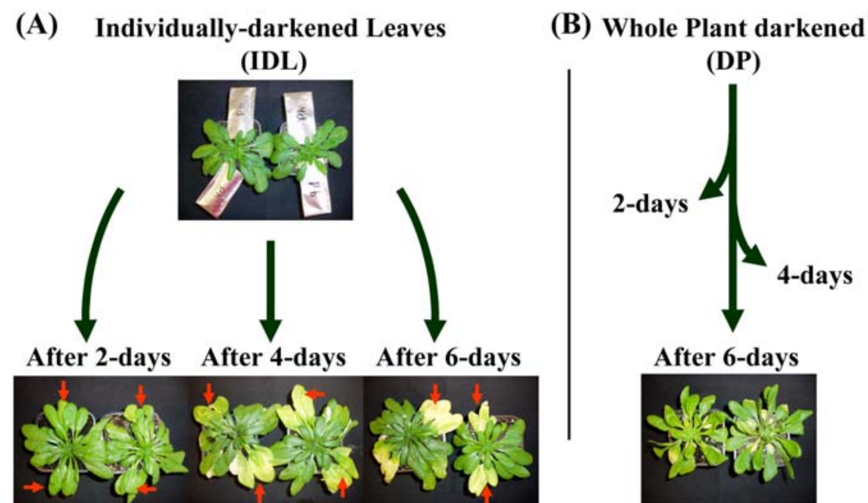


Figure 10. Scheme of the experimental setup. Individually-darkened leaves (IDL) in (A) and whole darkened plant (DP in (B). Leaves from rosette 6th to 12th were used for the experiments. From (Keech *et al.*, 2007). Reproduced with permission.

As shown in Figure 10, individually-covered leaves exhibited a noticeable yellowing and also tended to become a bit thinner after already 4 days of darkness (A). In contrast, leaves from plants darkened during 6 days did not exhibit an observable difference in their color in comparison to the plant which stayed in light (B). However, leaves tended to rise to the top and young leaves had longer petioles than their homologues from plants in light, two typical characteristics of the "shade-avoidance response".

As a model for leaf senescence, this experimental setup presents both advantages and disadvantages that we will try to summarize now.

Experimental setups with individually-darkened leaves have already been used before (references mentioned here above). As a matter of fact, it also facilitates the comparison with data from other groups and consequently enhances the scientific relevance of the presented results. Moreover, this system seems to present a biological relevance (see here below). Practically, this system is very simple to handle, synchronization of leaf senescence is fairly robust and both young and mature leaves do react to this drastic modification of the photoperiod. Note though that young leaves will have a delayed senescence in comparison with mature leaves. Nevertheless, the two types of leaves will undergo leaf senescence. However, a few parameters remain difficult to control. We noticed a slight increase of temperature and of humidity inside the plastic "mittens". In addition, the gas exchanges remain uncontrolled inside the mittens. However, in a recent study about transcriptomics between leaves senescing from natural aging and leaves ongoing dark-induced senescence, van der Graaff *et al.* (2006) showed that more than 75 % of the genes significantly up- or down-regulated in individually darkened leaves showed the same response during natural, age dependent, senescence. They concluded that developmental senescence and dark-induced senescence programs share many common pathways. In addition, in order to address whether the senescence was in fact induced by the darkness itself, and not by some artifact of the experimental design, Weaver and Amasino (2001) described a few important controls that we can summarize as follow:

-When leaves were covered with petroleum jelly on both adaxial and abaxial sides of the leaves (reducing the stomatal conductance), no yellowing was evidenced suggesting that reduced transpiration does not induce senescence in this experimental system.

-When leaves were covered with small boxes made of either black or clear x-ray film, the leaves covered with black boxes were induced to yellow, whereas the leaves covered with clear boxes

were not. Therefore, since the only difference between the two treatments was most probably the light received, it strongly supports the fact that a drastic change in the light intensity induces senescence.
As for individually-covered leaves, experimental setups where the whole plant is darkened have been widely used to induce senescence ((Weaver and Amasino, 2001; Buchanan-Wollaston *et al.*, 2005; Ishizaki *et al.*, 2005; Ishizaki *et al.*, 2006)). It is extremely simple to handle but the biological relevance can easily be questioned. Moreover, since absolutely no light is perceived, the plant undergoes severe starvation which might not be the case during natural leaf senescence.

To conclude, the biological relevance seems higher for individually-covered leaves than for the whole darkened plant. Indeed, during the development of Arabidopsis (notably under short days photoperiod), older leaves are progressively covered or shaded by new leaves due to the phyllotaxy of Arabidopsis. Whereas, the biological relevance of a plant experiencing prolonged darkness (several days) is far from being obvious (except maybe during a sunny day in Kiruna in beginning of January!). Consequently, even if a few parameters need to be improved and better controlled, we think that individually-covered leaf represents a good way to mimic leaf senescence and can be an interesting alternative to a whole plant switch to darkness. However, the comparison between the two treatments remains beneficial for our understanding about the specific regulations and adjustments of the metabolism during senescence.

6. Regulation of the metabolism

In this study, we decided to compare the regulation of metabolism (with an emphasis on photosynthesis and respiration) in both individually-darkened leaves (IDL) and in leaves from whole darkened plants (DP). We brought several modifications to the experimental system proposed by Weaver and Amasino (2001). Nevertheless, we validated our modifications by coming to the same and obvious point: senescence is faster in individually-darkened leaves than in whole darkened plant. However, in the next paragraph, we present several evidences showing that regulation of the metabolism in the two sorts of leaves (from IDL and from DP) will actually be the result of different adaptive strategies to dark treatment.

6.1 A cytological approach

In French, we often say “voir c’est croire!” which literally means “to see is to believe!”. We carried out a rather detailed study by confocal laser microscopy in order to visualize the organelles content in leaves during the two darkening treatments. One of our primary ideas was to normalize mitochondrial respiration values and western blottings by an estimation of the number of mitochondria in the leaf. Interestingly, we observed a drastic reduction (around 65 %) of the number of mitochondria in IDL whereas the mitochondrial population was rather conserved in all cell types (epidermis cells, guard cells of stomata and mesophyll cells) of leaves from darkened plants (paper IV). We also noticed that in IDL, mitochondria were rounder and slightly bigger than mitochondria from both DP and light treatments. Chloroplasts were also influenced by the darkening treatments. After 6 days, we estimated a decrease in chloroplasts number which became small “gerontoplasts” to be around 60 % in IDL whereas only a loss of 30 % with no change in size was noticed in DP. In addition to this quantification but only in IDL, we noticed an important cytological rearrangement in mesophyll cells where chloroplasts and mitochondria were clustered together. Similar aggregations of organelles had also been reported in protoplast from senescing leaves of *Pisum sativum* (Simeonova *et al.*, 2004) and in PCD of mesophyll cells from cucumber (*Cucumis sativus* L.) (Selga *et al.*, 2005). Curious to understand how such cytological rearrangement might be possible, we investigated the dynamics of the microtubular cytoskeleton during dark-induced senescence (Paper VI). We noticed an early degradation of the microtubular network in individually-darkened leaves. Using the microarray data set from paper IV, we focused on a few specific proteins, the microtubules associated proteins (MAPs) that could have a role in the rearrangement of microtubules under certain conditions and especially could be involved in a kind of bundling of the microtubules.

6.2 Photosynthesis

In order to follow the photosynthetic capacity of chloroplasts, we measured several parameters such as F_v/F_m, ETR and ΦPSII from leaves undergoing the different treatments. All results showed the same trend (paper IV). After 6 days of treatment, the photosynthetic capacity was conserved in leaves from whole-darkened plant whereas it was progressively lost in individually-

darkened leaves. In addition, photosynthetic pigments were quantified by HPLC analysis. In individually darkened leaves, chlorophylls, xanthophylls and carotenoids remained constant during the first two - three days in darkness but when kept in the dark for longer periods, all pigment classes declined to 30-40 % of control levels after 6 days (Paper IV). In contrast, leaves from whole darkened plants maintained pigment contents similar to light controls during the same period with only a small decline at the end of the period studied. None of the treatments exhibited modification of the chlorophyll a/b (Paper IV). Furthermore, we could also notice that for both whole darkened plant and individually darkened leaves, the HPLC analysis did not reveal new peaks as by-products from pigments degradation as seen in autumn senescence of poplar leaves (Keskitalo *et al.*, 2005).

6.3 Mitochondrial Respiration

In vivo – We followed dark respiration in leaves individually-covered and from whole darkened plant. When expressed on a leaf area basis, respiration dropped of 50 % after 2 days for the two treatments and remained at this rate during the six days of the experiment. When expressed on a protein basis, dark respiration in leaves from whole darkened plant dropped the same way as when expressed on the leaf area basis whereas the dark respiration in individually darkened leaves increased significantly during the senescing process (Paper IV). Since a significant difference was noticeable between IDL and DP, we decided to investigate the *in vitro* mitochondrial capacity for respiration.

In vitro - For mitochondria from normally illuminated leaves, respiratory rates (expressed on a mitochondrial protein basis) were stable with all substrates during the 6 days experimental period. With mitochondria isolated from leaves of DP, respiratory rates were significantly lower with all the substrates tested after both 4 and 6 days of dark treatment. Interestingly, mitochondria isolated from IDL showed high respiratory rates throughout the dark treatment with all the substrates tested. In addition, no significant differences in respiratory rates were observed between mitochondria from normally illuminated leaves and mitochondria from IDL along the 6 days period of treatment (Paper IV).

The RCR provides an estimation of the coupling between electron transport and oxidative phosphorylation and it depends on the intactness of the inner mitochondrial membrane. For all treatments the general trend was that malate + glutamate and glycine gave higher RCR than NADH and succinate. The RCR values were very similar for mitochondria extracted from normally illuminated leaves throughout the experiment (paper IV). However, in mitochondria from both DP and IDL the RCR tended to decrease relative to normally illuminated leaves. For mitochondria from IDL, the RCR decreased throughout the 6 days whereas for mitochondria from DP, RCR dropped significantly for all substrates at day 2 but stayed fairly stable during the subsequent 4 days of the experiment.

6.4 Metabolomics

To get a better understanding of the metabolic changes during the respective treatments, we looked at the changes in content of specific metabolites in leaves and petioles (Paper V).
In leaves - Carbohydrates were, as expected much higher in light than in IDL and DP. Furthermore, in some cases the content in IDL was somewhat higher than in DP. A few specific amino acids, Gln and Gly in particular, were much higher in L as compared to dark treatments. However, during prolonged darkness in DP, many other amino acids and other nitrogen containing compounds increased. The N-metabolite progressively increased during dark treatment in DP whereas they remained low in IDL and in light (Phe, Asn, 3-cyanoalanine, ornitine, urea, 2,5-diaminovalerolactam). In addition, several common organic acids were high in light, intermediate in IDL and low in DP (succinate, malate, fumarate) whereas the citrate level was rather stable between the treatments.

In petioles - EDTA exudates from leaf petioles were also analysed by GC/ToF-MS. After one day in darkness the relative content of amino acids was low in petioles of both DP and IDL. After three days, several amino acids increased in both DP and IDL and to a large extent this increase mirrored to changes in the leaves. After six days in darkness the content of amino acids continued to increase in IDL but decreased in DP as compared to 3 days in darkness.

This supports the suggestion that amino acids are exported away from IDL thereby alleviating the problem of N-accumulation from degraded amino acids and contributing to the retrieval of nitrogen during senescence. In DP on the other hand the export does not seem to occur. During this condition the metabolism also enters a stand-by mode with decreased rate of respiration (Keech *et al.*, 2007) which would be appropriate in order to maintain viability as long as possible. During this condition of severe starvation amino acids accumulate as metabolic end products giving a way to store nitrogen in non-toxic compounds.

6.5 Conclusion

Altogether, data obtained from whole darkened plant (transcriptomics, metabolomics, physiological measurements and microscopy) suggest that under prolonged darkness Arabidopsis leaf metabolism could enter a “stand-by” mode to preserve photosynthetic capacities by slowing down the respiration to limit the consumption of substrates which most likely are in short supply during such long period of starvation. Let’s remark, as we previously mentioned, that prolonged darkness is obviously not a “natural physiological stress” for a plant. Ishizaki *et al.* (2006) recently estimated the loss of soluble carbohydrate content from whole darkened plants to be around 80 % after only 3 days of darkening. Consequently, it seems that the plant reacts by slowly degrading the cell wall as source of carbon since no sugars are available. This is consistent with early results from Journet *et al.* (1986) where they demonstrated that in carbohydrate starved sycamore cells, i.e. after the soluble carbohydrates and starch were depleted, the cells started to degrade cell components to obtain substrates for respiration. This was associated with a decreased rate of mitochondrial respiration that may be a direct consequence of substrate limitation (Aubert *et al.*, 1996). Moreover, Ishizaki *et al.* (2006) proposed that the mitochondrial complex ETF/ETFQO may play an essential role during carbohydrate deprivation by supplying alternative substrates, notably branch chain amino acids to the respiratory chain. However, the consequences of activity of this pathway for mitochondrial respiration remain unknown. Therefore, it seems likely that the differences in mitochondrial respiratory capacity we observed were linked to the depletion of “normal” reserves and a switch to other substrates affecting mitochondrial respiratory capacity. As a consequence, whole

darkened plant slowly reduces the amount of organelles but keep them functional most likely to be able to restart their photosynthetic activity as soon as the light comes back.

In contrast to DP, an accelerated senescence was observed in IDL. The photosynthetic apparatus was quickly degraded but the mitochondrial respiratory capacity remained high. The release of cytochrome *c* is a common hallmark of apoptosis in animals and has also been observed in few cases of plants PCD (Balk *et al.*, 1999; Sun *et al.*, 1999; Balk and Leaver, 2001) but not in all (Yu *et al.*, 2002). Interestingly, since the mitochondrial capacity to oxidize substrates is maintained during the accelerated senescence observed in IDL, it seems unlikely for cytochrome c to be released during dark-induced senescence. The reduction of the number of mitochondria in IDL after 6 days suggests that two populations of mitochondria could coexist: one population active and one population undergoing degradation. In consequence, two mitochondrial populations with different membrane permeability, one with retained coupling and one with low (or absent) coupling, may explain the decrease in RCR observed in IDL. In support of such a suggestion, Simeonova *et al.* (2004) observed that mitochondria from protoplasts isolated from senescing leaves of *Pisum satisum* were divided into two populations, one with mitochondria keeping a high membrane potential along the senescing process and another one with mitochondria losing their membrane potential during the process. A possible mechanism to explain the existence of these two populations may be related to production of reactive oxygen species (ROS). In mammalian cells undergoing PCD, production of ROS is supposed to trigger a mitochondrial permeability transition (MPT) which in turn dissipates the proton motive force causing an uncoupling of oxidative phosphorylation reviewed by Skulachev (1996). In plants, it has been commonly postulated that plant mitochondria could generate ROS and lose their membrane potential during PCD (Jones, 2000; Tiwari *et al.*, 2002; Yao *et al.*, 2002; Yao *et al.*, 2004). We thus speculate that ROS production could alter mitochondrial permeability during dark-induced senescence which could progressively lead to the impairment between oxidation and phosphorylation as observed in our study. The implication of ROS would also be reinforced by two facts: 1/ in paper III, we showed by western blotting that the amount of the prx-IIF does not change in IDL whereas more than 60 % of leaf mitochondria disappeared and 2/ the recent work of Logan and co-workers (Logan, 2006) reported that a large number of mitochondria swelled to at least double their volume after ROS treatment of Arabidopsis mesophyll

protoplasts. The latter point is in line with the bigger and rounder mitochondria we reported in paper IV. However, in paper VI, we also demonstrated that even very high concentration of hydrogen peroxide (one of the main ROS produced in a cell) did not induce the similar alteration of the microtubular cytoskeleton as seen during dark-induced senescence. So, we need to lead further investigations to understand properly the role played by reactive oxygen species during dark-induced senescence. In conclusion, we think that the remaining mitochondria in individually-darkened leaves could play an active role both to maintain sufficient ATP and also to provide proper carbon skeletons needed for degradations and reallocations occurring during the leaf senescence.

REMARKS

This thesis appears as some pioneer work for our group. We developed several "tools" to lead further studies and our preliminary investigations on adjustments of cell metabolism have highlighted the metabolic changes between an individually darkened leaf and a leaf from a whole darkened plant. These changes also included a divergent role of the mitochondrial metabolism between the two treatments. Research about mitochondria during leaf senescence has been rather superficial during the last decade whereas the involvements of mitochondria during programmed cell death were under deeper investigations. Nevertheless, we highlighted the fact that mitochondria could have a different role and behavior during leaf senescence than the ones commonly accepted during PCD which in turn questions once again about the boundaries between senescence and programmed cell death. In addition to this study, we, especially the new members of the group, are now dealing with questions related to the C/N fluxes, to the importance of Glycine decarboxylase complex and to the light dependant regulatory pathways during leaf senescence. So to conclude, I am glad to see that finally this thesis work is a basis for future investigations. Of course, things could have been done differently and/or maybe in a better way but nevertheless, this work have found its place in international conferences and especially have led us to deepen our understandings about the role of leaf mitochondria.

Finally, a few complementary remarks:

-Several times in this report, we mentioned the possibility to have different subpopulation of mitochondria in a leaf. Although no data are clearly supporting this idea, the point remains interesting to consider and I hope I will be able in the near future to investigate this idea.

- We also often mentioned the probabilities for ROS to be involved during dark induced senescence. However, we have not led any further studies yet.

-One of our strongest beliefs when we started this project was to see the glycine decarboxylase complex disappear during dark induced senescence. However, it was not the case which might suggest some other roles for the GDC than only its involvement in photorespiration.

- When working with senescence, one has to keep in mind that the complexity of leaf senescence is also suggested by the fact that it can be induced by numerous factors, biotic and abiotic.

CONCLUSION GÉNÉRALE

Au cours de ce travail de thèse qui s'est employé à approfondir les connaissances sur le rôle des mitochondries dans le mécanisme de sénescence foliaire, une comparaison a été effectuée entre la soumission à l'obscurité de feuilles individuelles et celle de plantes entières. Cette comparaison révéla des stratégies métaboliques très différentes. Lorsque qu'une plante entière est soumise à une obscurité prolongée (i.e. plusieurs jours), une rapide diminution permettant somme toute une conservation des capacités photosynthétiques fut observée. De plus, celle-ci fut concomitante à une diminution des capacités oxydatives des mitochondries et à une sur-régulation de gènes impliqués dans la dégradation de la paroi cellulaire. Par ailleurs, toutes les feuilles étant dans la même situation critique, l'accumulation d'acides aminés dans les feuilles nous laisse suggérer que la plante stocke son excès d'azote (pouvant être toxique) sous cette forme. Par conséquent, il semblerait que le métabolisme d'une plante soumise à une obscurité prolongée entre dans une sorte de mode « en veille » minimisant l'activité métabolique au maximum et probablement utilisant les lipides et les produits de la dégradation de la paroi cellulaire comme source métabolique alimentant la chaîne respiratoire. À l'inverse, les feuilles individuellement couvertes subissent une sénescence accélérée se traduisant pas une rapide dégradation de la machinerie photosynthétique ainsi qu'un démantèlement du cytosquelette microtubulaire affectant la mobilité des mitochondries. De plus, même si le nombre de mitochondries diminue de façon drastique, notamment dans l'épiderme, leurs capacités d'oxydation restent préservées tout au long de cette sénescence induite par l'obscurité. Il semble donc que les mitochondries aient un rôle prépondérant durant la sénescence en fournissant l'énergie et les squelettes carbonés nécessaires à l'efficace remobilisation des nutriments.

Pour conclure, il est évident qu'une meilleure compréhension des mécanismes de la sénescence représente un challenge très important non seulement pour la recherche fondamentale mais aussi pour de futures applications en biotechnologie. Par le présent travail, nous mettons donc l'accent sur les différentes régulations du métabolisme d'une feuille lorsque celle-ci est soumise à une obscurité prolongée. De fait, le devenir de cette feuille dépend grandement du statut métabolique général de la plante comme le suggèrent nos résultats.

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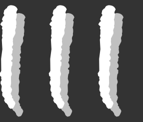

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