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Chapter 3

CONTROL OF LEAF CELL DEATH BY REACTIVE OXYGEN SPECIES

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ABSTRACT

The leaf is a complex organ composed of different tissues and cells that are subjected to a variety of environmental factors affecting the course of its aging, senescence and death. Most investigations on leaf death have been focused on the senescence of photosynthetic mesophyll cells, whose characteristic loss of chlorophyll becomes the reference symptom preceding leaf death. Autumnal and monocarpic leaf deaths are developmental processes genetically programmed but, at the cell level, many changes that precede them are similar to those of the senescence of shaded basal leaves and even to those of the stress-dependent leaf aging. Most knowledge of the molecular processes in programmed leaf death has been gained through the investigation with the last two deaths, provoked by developmental and environmental factors that in strict sense are not programmed leaf death. However, analogies with processes in programmed cell death in animals provide unifying views where reactive oxygen species (ROS, mainly singlet oxygen $^1O_2$, anion radical superoxide $O_2\bullet^-$ and hydrogen peroxide H_2O_2) emerge as key intracellular signals.

The steady state level of each ROS depends on generation and scavenging activities closely linked, respectively, to the photosynthetic electron transport and to systems that protect cells from the destructive action of ROS and, frequently, the destructive actions of ROS are hardly distinguishable from their signaling role in cell death. By controlling the levels of different ROS, chloroplasts regulate leaf death and assume roles similar to several roles attributed to mitochondria in programmed animal cells. High ratios ($^1O_2$ plus $O_2\bullet^-$)/H_2O_2 seem key to shoot the autocatalytic cell path to death where hormones and transcriptional factors join to change the feed-back regulation of cell processes to a feed-forward spiral of deleterious reactions. In this chapter, we will discuss the control of the levels of different ROS and their interaction with hormones and transcriptional factors in relation with programmed cell death in leaves.

INTRODUCTION

Except for a few plants where basal meristem persists, the leaf is an organ with limited growth where, when it reaches the characteristic form and size of adult stage, all its meristematic cells have differentiated into a variety of tissues among which mesophyll photosynthetic are the majority. Lacking meristematic cells, the life of an adult leaf is time-limited and spans from a few days in many herbaceous to several years in some conifers. Chlorophyll loss is the most characteristic symptom of leaf senescence preceding its death and it is one feature of the dismantling of chloroplast machinery.

Leaf death may be one aspect of the whole plant death, as in monocarpic, or it may occur while the whole plant remains alive, as in the autumnal leaf death of many perennials. Both, monocarpic and autumnal leaf deaths are developmental processes genetically programmed: only slightly (and frequently indirectly) affected by environmental factors. In addition, there are processes of leaf senescence and death, strongly affected by environmental factors, such as light intensity, temperature or nutrient availability. This is the case of the basal leaves of rapidly growing gramineans, whose senescence and death are linked to the simultaneous development of other organs of the plants. In both monocarpic and basal leaf, death is frequently delayed by scission of, respectively, reproductive buds and growing young leaves that, otherwise, act as sinks of the degradation products of senescent leaves. Finally, a leaf may die by a prolonged accumulation of hazardous damages, a process named aging, or by a burst of damages under biotic or abiotic stresses.

Except for the rapid death under biotic or abiotic stresses, leaf senescence preceding death is an organized process through which most valuable components of the leaves, as nitrogen present in proteins, are mobilized to young, storage or reproduction organs. This mobilization requires the ordered expression of genes encoding enzymatic activities that degrade, among others, chloroplast proteins (the main fraction of leaf protein, Dalling 1985) and reconvert amino acid nitrogen to exportable forms as glutamine and asparagine (Morita 1980; Feller and Fischer 1994). The commitment of plants to recover valuable nutrients (as nitrogen, phosphorus, potassium or magnesium) in seeds and growing young organs justifies the evolutionary selection of leaf senescence traits: ordered and efficient in dismantling cell structures and sending out components in coordination with the development of other organs in the plant.

The amount of Reactive Oxygen Species (ROS) increase in all types of leaf senescence and in aging, with respect to young and adult healthy leaves. ROS are mainly produced through side reactions in the photosynthetic machinery of chloroplasts (Apel and Hirt 2004; Sabater and Martín 2013a,b). ROS are highly reactive damaging agents that could demolish cell structures. In addition, ROS are signals that control the expression of genes that help cells to survive under different stress conditions or, alternatively, to lead them to death. Therefore, through ROS production and scavenging, chloroplasts have an active role controlling leaf senescence (Zapata et al. 2005; Chen et al. 2010; Doyle et al. 2010; Van Doorn and Yoshimoto 2010; Sabater and Martín 2013a,b). Probably, the damaging effect of ROS is a side effect that does not interfere with the organized processes of cell death and evidences suggest that the relative levels of the different ROS determine the expression of genes for either live or suicide metabolism of leaf cell (Gechev et al. 2006). Among genes expressed for cell death are those encoding enzymes required for the mobilization of valuable leaf components and, in general, for the orchestration of programmed cell death during senescence (SAG, senescence associated genes): an ordered process hardly compatible with the unspecific hazardous damage effects of ROS.

When compared with other cellular signals, the investigation of the role of ROS poses specific challenges related to their generation and scavenging, their translocation among cell compartments and their interactions with other cellular signals. In recent years, improved quantification of specific ROS and indirect evidences based on changes of activities favoring formation or scavenging specific ROS give insights on the involvement of each specific ROS in programmed death of leaf cells. On the basis of their low half-life and

permeability across membranes the primary action of the ROS singlet oxygen, $^1O_2$, and anion radical superoxide, $O_2\bullet^-$, must occur in the same compartment where they are formed, mainly chloroplasts. The other key ROS, hydrogen peroxide, H_2O_2, is by far the most stable and it is formed in, essentially, all cell compartments, although probably chloroplast is the main source of H_2O_2 in all stages of leaf development. H_2O_2 freely crosses membranes and its primary action may occur in cell compartments different from those generating it. There are several proposals for the primary action of the different ROS, that is: for their direct effect generating or modifying other signals in the complex network that regulate cell development, including cell death.

GENERATION AND SCAVENGING OF ROS IN CHLOROPLASTS

In leaves, ROS are mainly generated in chloroplasts that are also the main places that scavenge $^1O_2$ and $O_2\bullet^-$. The contribution of different compartments to scavenging H_2O_2 is uncertain. H_2O_2 may be consumed by different peroxidases (PXs) present in several compartments (among them chloroplasts) that, having high affinity for H_2O_2 (K_m in the range 0.1 to 1 mM), should maintain its low concentration, as long as there is enough supply of electron donors: the other peroxidase substrate, which varies among the different peroxidases. Catalase enzyme decomposes H_2O_2, it is also present in several compartments (but not in chloroplasts) and does not require a second substrate donor of electron. However, the affinity of catalase for H_2O_2 is low (K_m 10 to 100 mM) and can hardly decrease the concentration of H_2O_2 below 1 mM.

To minimize the damaging effects of ROS, the scavenging machineries maintain low steady state concentrations of ROS, compatible with high photosynthetic activity under widely variable and frequently stressing environments. Therefore, levels of $^1O_2$, $O_2\bullet^-$ and H_2O_2 that control cell death are probably low too and their damaging effects do not significantly contribute to the dismantling cell components, at least during early stages of leaf senescence.

The relative level of each ROS depends on relative levels of the diverse activities involved in the generation and the scavenging of the different ROS. Generation of ROS is closely related to activities of the photosynthetic electron transport in chloroplasts. Figure 1 shows the main processes generating $^1O_2$, $O_2\bullet^-$ and H_2O_2 in chloroplasts of illuminated leaves. Most $^1O_2$

is formed in photosystem II (PSII) by transfer of electronic excitation from chlorophyll excited at the triplet state (^{3}Chl*) to molecular oxygen O_2. ^{3}Chl* is formed from chlorophyll excited at the singlet state (Chl*). Accordingly, the fraction of chlorophyll ^{3}Chl* in chloroplasts increases when that of Chl* rises because the absorption of light exceeds the capacity to drain electrons from photosynthetic electron transporters, mainly by assimilation of CO_2. Most $O_2\bullet^-$ is formed in photosystem I (PSI) by one electron transfer from A/Fd_{red} (reduced non-heme iron-sulfur proteins, including ferredoxin) to molecular oxygen O_2 (Mehler reaction). $O_2\bullet^-$ may be also generated in a lesser amount by electron transfer from reduced non-heme iron-sulfur components of the thylakoid Ndh complex (Ndh) and by interaction of $^1O_2$ with different organic and inorganic molecules (not represented in Figure 1). H_2O_2 is mainly generated from $O_2\bullet^-$ by the reaction: $2\ O_2\bullet^- + 2\ H^+ \rightarrow H_2O_2 + O_2$, catalyzed by superoxide dismutase (SOD), which also is the main way for $O_2\bullet^-$ degradation. As pointed, H_2O_2 is consumed in chloroplasts when it oxidizes different substrates in reactions catalyzed by peroxidases.

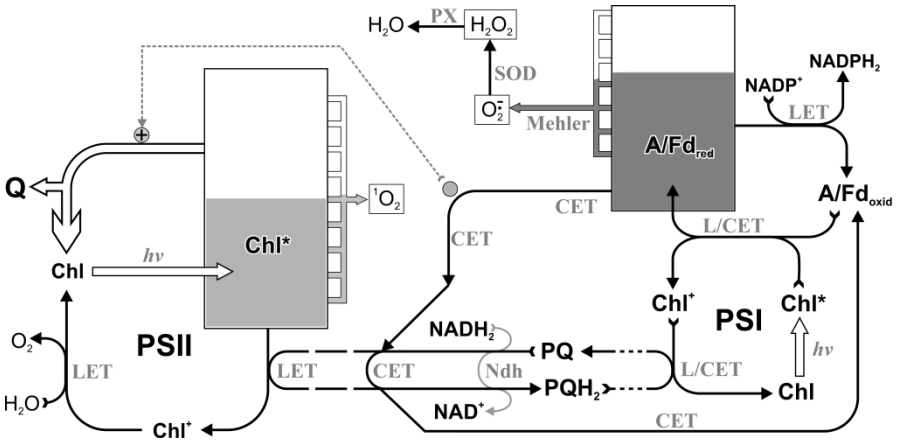

Figure 1. Generation of ROS in chloroplasts. Non-conventional representation of the photosynthetic electron transport to highlight processes generating ROS. Electron transport from H_2O to $NADP^+$ is depicted by arrows marked with LET (linear electron transport) connecting water oxidation (down left), photosystem II (**PSII**), photosystem I (**PSI**) and the final $NADP^+$ reduction step (up right). Specific steps of cyclic electron transport around **PSI** are marked as CET and common steps of linear and cyclic electron transport are marked as L/CET. A/Fd, non-heme-iron sulfur proteins (including ferredoxin); Ndh, the thylakoid NADH dehydrogenase complex; PX, peroxidase; **Q**, energy dissipated as heat. SOD, superoxide dismutase. Other lettering and symbols are described in the text.

Levels of Chl* in PSII and of A/Fd_{red} in PSI (represented in Figure 1 by partially filled tanks with vertically disposed leakage exits) play key roles for the generation of, respectively, $^1O_2$ and $O_2\bullet^-$. The higher levels of Chl* and A/Fd_{red}, higher are the rates of generation of, respectively, $^1O_2$ and $O_2\bullet^-$. The level of Chl* in PSII increases with the intensity of light, which excites Chl to Chl* by absorption of one photon ($h\nu$), and decreases by Chl* oxidation to Chl^+ (loss of the excited electron, which is transferred to plastoquinone PQ, A/Fd_{red} and $NADP^+$ through different steps of the linear electron transport LET chain), by emission of fluorescent light, and by heat dissipation (Q) after transfer of excitation to zeaxanthin formed in the xanthophyll cycle (Eskling et al. 2001). The last process is stimulated by high light intensity and cyclic electron transport (CET) through thylakoid polarization. The rate of electron transfer from Chl* of PSII through LET essentially equals its use in the reduction of CO_2 (through NADPH) plus, to a much lower extent, the reduction of nitrate and the Mehler reaction. When light intensity exceeds the capacity of the Benson-Calvin cycle to drain electrons, Chl* and A/Fd_{red} increase and the production of $^1O_2$ and $O_2\bullet^-$ rise. In these conditions, subsequent photoinhibition of PSII and activation of heat dissipation Q contribute to alleviate the generation of $^1O_2$ and $O_2\bullet^-$. In addition, increase of the SOD activity contributes to maintain low levels of $O_2\bullet^-$.

Therefore, Mehler reaction, SOD and PX activities are key players to maintain low levels of $O_2\bullet^-$. They also affect indirectly the level of $^1O_2$. In effect, as pointed out above, heat dissipation requires CET to maintain the polarized thylakoid membrane. When the absorption of light exceeds the electron drain toward the assimilation of CO_2, the ratio: reduced forms to oxidized forms of transporters of LET and CET becomes high, which decreases the rate of CET whose maximum requires balanced (poised) levels of reduced and oxidized forms of transporters. Low rate of CET, by over-reduction of transporters, decreases heat dissipation of the Chl* of PSII as indicated and, hence, facilitates over-production of $^1O_2$. Therefore, generation of $O_2\bullet^-$ by Mehler reaction alleviates over-reduction of transporters and restores maximum rates of CET allowing efficient heat dissipation of the excess of light energy and low production of $^1O_2$. In this way, the production of $O_2\bullet^-$ by Mehler reaction decreases the production of the more dangerous $^1O_2$. SOD and PX are necessary to quench the level of excess $O_2\bullet^-$ and H_2O_2 produced. Among PXs, plastoquinol peroxidase oxidizes reduced PQ with H_2O_2 (Zapata et al. 1998; Casano et al. 2000). Therefore, it consumes H_2O_2 and drains electrons, contributing to alleviate over-reduction of the photosynthetic electron transport chain.

Several stressing conditions, transitorily or long-term, increase the excess of light absorption over the capacity to drain electrons from the photosynthetic electron transport chain. The sudden increase of light intensity is the most obvious. In addition, lowering temperature (which decreases the rate of enzymes of the Benson-Calvin cycle) and water deficit (which closes stomata and decreases the availability of CO_2) are frequent stresses where maintaining efficient CET transport (Segura and Quiles 2015) is correlated with the control of levels of different ROS.

Low rate of the CET can also be produced by the opposite condition: a low ratio of the reduced to the oxidized forms of transporters. This is a frequent circumstance under rapidly fluctuating light intensities (sun fleck). During a period under high light intensity, photoinhibiton of PSII and active heat dissipation depresses the capacity of PSII to send electrons to LET. Depression become dramatic when light intensity suddenly and strongly decreases. Then, transporters would become over-oxidized and the rate of CET and polarization of the thylakoid membrane lowers. Recovery from photoinhibition and decrease of dissipating zeaxanthin may require between several seconds and minutes: too long for efficient photosynthesis, especially at high concentrations of CO_2 (Martín et al. 2015). To restore the balanced redox state of transporters and the consequently high rate of CET, the transitory low supply of electrons from PSII is supplemented by the thylakoid Ndh complex (see Figure 1) that transfers electrons from NADH to PQ. Therefore, under usual fluctuating lights, leaves meet stresses of alternating high and low light intensities where the photosynthetic electron transport chain alternates steps of electron drain by Mehler reaction, SOD and PX with steps of electron filling by the Ndh complex. Jointly, although not simultaneously, the successive reactions conform the chlororespiratory electron transport chain (Casano et al. 2000; Joët et al. 2002; Nixon and Rich 2007):

(Ndh complex)

$$2\ NADH + 2\ H^+ + 2\ PQ \text{ ----------} \rightarrow 2\ NAD^+ + 2\ PQH_2$$

LET

$$2\ A/Fd_{oxid} + PQH_2 \text{ ----------} \rightarrow 2\ A/Fd_{red} + PQ + 2H^+$$

Mehler reaction

$$2\ A/Fd_{red} + 2\ O_2 \text{ ----------} \rightarrow 2\ A/Fd_{oxid} + 2\ O_2^{\bullet -}$$

$$2\ O_2\bullet^- + 2\ H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

$$H_2O_2 + PQH_2 \xrightarrow{\text{PX}} 2\ H_2O + PQ$$

Which results in the global reaction:

$$2\ NADH + 2\ H^+ + O_2 \longrightarrow 2\ NAD^+ + 2\ H_2O$$

that transports electrons from NADH to oxygen to maintain the poised redox level of electron transporters and, hence, appropriate rates of CET.

Accordingly, levels and activities of the chloroplast Ndh complex, SOD and PX increase under a variety of stresses (Bowler et al. 1992; Casano et al. 1994; Martín et al.1996; Casano et al.1999, 2001; Martín et al. 2004; Serrot et al. 2008; Paredes and Quiles 2013; Segura and Quiles 2015).

SWITCHING OF ROS METABOLISM TO LEAF SENESCENCE

Appropriate balance of activities maintains low levels of $^1O_2$, $O_2\bullet^-$ and H_2O_2 to cope with different stress conditions, reduces damage by ROS to a minimum and induces defensive adaptation of the leaves against stressing agents. Several evidences point specifically to H_2O_2 as a signal in the induction of the defense responses against stress (Mubarakshina et al. 2010; Wiciarz et al. 2015). Certainly, longer half-life, mobility in the cell and lower toxicity make H_2O_2 more appropriate than $^1O_2$ and $O_2\bullet^-$ to address adaptation-surviving responses. The situation must be different if leaf survival does not yet provide evolutionary advantages for the plant. In other words: if the leaf should senesce and die.

In effect, although the Ndh complex and PX increases similarly under stress along leaf development since birth to senescence, the induction of chloroplastic SODs (Cu/Zn and Fe SODs) by photo-oxidative stress is impaired early during leaf senescence (Casano et al. 1994; Kurepa et al. 1997; Casano et al. 1999; Abarca et al. 2001a,b; Prochazkova et al. 2001; Ohe et al. 2005). The first consequence is an increase of $O_2\bullet^-$ and a decrease of H_2O_2 early in senescence. Further increases of poorly scavenged $O_2\bullet^-$ occur due to enhanced Mehler reactions at the level of non-heme iron-sulfur proteins of the increased Ndh complex and of the A/Fd that become over reduced by the

feeding of electrons through the Ndh complex and by the low electron drain at the level of plastoquinol peroxidase due to the low concentration of the substrate H_2O_2. The increased over-reduction of transporters leads to progressive increases of the production of $O_2•^-$ which, complemented by falling induction of SOD, decreases the ratio of H_2O_2 to $O_2•^-$. In addition, not only $O_2•^-$ increases, over-reduced transporters hinder the transfer of electrons from the Chl* of PSII to LET, which facilitates its transition to ^{3}Chl* (chloropyll excited at the triplet state) that transfers excitation to O_2 enhancing the generation of more $^1O_2$. The generation of $^1O_2$ is further favored because over-reduction decreases CET, which impairs the dissipation as heat of the light energy absorbed to form Chl*. Therefore, when compared with young leaves, changes of enzyme activities increase levels of the ROS $O_2•^-$ and $^1O_2$ in respect to H_2O_2 in response to mild stress in early senescent leaves. The changes are auto-catalytically amplified by the functional properties of the photosynthetic machinery, cancel the defense response mediated by H_2O_2 against stress and lead to senescence processes mediated by $O_2•^-$ and/or $^1O_2$. In contrast to young leaves, in senescent leaves, mild stress shoots uncontrolled increases of $^1O_2$ and $O_2•^-$. Accordingly, Krieger-Liszkay et al. (2015) observed that senescing flag leaves of two varieties of barley show increased levels of $^1O_2$ or $O_2•^-$.

Low levels of SOD are associated with senescence in animals (Orr and Sohal 1994) and plants (Bowler et al. 1992; Casano et al. 1994, 1999; Abarca et al. 2001b), and a question on the control of leaf cell death by ROS refers to the failure to express the nuclear genes for chloroplastic SODs at advanced developmental stages of the leaf. Genes for chloroplastic SODs are commonly expressed during chloroplast development and (similarly to genes encoding proteins of enzymes of Benson-Calvin cycle, light-harvesting complexes, photosystems and photosynthetic electron transport) their expression is stimulated by light, which agrees with a functional role of chloroplastic SODs closely associated to photosynthesis. Accordingly, there are several TATA and AGATAA sequences up-stream of SOD genes that are involved in the binding of the transcriptional complex and promoter sequences specific for photosynthetic genes, as GT-elements (GGTTAA) involved in the expression of genes for the photosynthetic machinery (Zhou 1999). In addition, the expression of SOD genes increases when leaves are subjected to photo-oxidative stress. That is, when excess of light or inhibitors of the photosynthetic electron transport increases the formation of $O_2•^-$ due to transfer of electrons from over-reduced A/Fd to oxygen. This recalls the protective function of SOD against stress by scavenging $O_2•^-$. Less is known

about factors responsible for the failure to induce SOD in senescent leaves. One probable clue may be provided by the comparison of the up-stream sequences of the Cu/Zn SOD genes in Arabidopsis ecotypes Ler and Cvi. The Cvi ecotype shows delayed senescence (Luquez et al. 2006) and a distinctive Cu/Zn SOD that remains inducible by photo-oxidative stress at advanced plant age (Abarca et al. 2001b). In respect to the standard Ler ecotype, upstream of Cu/Zn SOD gene, Cvi has a 20-base deletion immediately down-stream of one AGATAA and two TATA box motifs (position −257 to −238) (Sabater and Martín 2013a,b). The sequence deleted in Cvi includes the AACTAA motif recognized by some MYB transcriptional factors that, upon binding it inhibits the transcription of downstream genes (Jin et al. 2000). It is tempting to suggest that in the Ler ecotype of Arabidopsis, and in general in normal plants, one MYB factor specifically accumulates at appropriate advanced development of the leaf, binds to the AACTAA motif and inhibits the transcription of the Cu/Zn SOD gene. Future progress in understanding the decline of SOD, which leads to leaf senescence, is hindered by the complex network of signals (that include a high number of MYB factors) controlling gene expression during plant development.

The other key feature in the control of programmed cell death of leaves by ROS is the autocatalytic increase of the Ndh complex and PX despite the falling induction of chloroplastic SOD. Some investigations with the Ndh complex provide insights to this question and the signal cascade down $^1O_2$ in programmed cell death.

SIGNAL CASCADE DOWNSTREAM ROS

H_2O_2 (frequently generated in chloroplasts) increases in the cytoplasm in the systemic and hyper-sensible defense responses (Levine 1999; Van Breusegem et al. 2001; Overmyer et al. 2003) and acts as a signal of transduction cascades, probably involving mitogen-activated protein kinases, which induce in the nucleus the expression of genes protecting against different stresses (Piñas and Strand 2008). Cytosolic and chloroplastic 2-cysteine peroxiredoxins can further modulate the level of H_2O_2 in response to stress (Dietz 2003; Muthuramalingam et al. 2009), but the protein that firstly interacts with H_2O_2 in network signaling has not yet been identified.

High level of H_2O_2 increases the expression of the *ndh* genes encoding the thylakoid Ndh complex in an action depending on protein kinases (Casano et al. 2001; Lascano et al. 2003). The increased activity of the Ndh complex may

explain the stimulation of CET by H_2O_2 providing the link between environmental stress, metabolism, and redox regulation of CET in higher plants (Stranda et al. 2015). However, the increase of Ndh during senescence is not mediated by increases of H_2O_2 and, probably, other ROS-derived signals induce processes related to programmed cell death into and outside of chloroplasts. As pointed above, increase of PX and decrease of SOD suggest that the production of H_2O_2 decreases early in senescence and, accordingly, bundle sheaths in the maize do not accumulate H_2O_2 during programmed cell death (Huang and Braun 2010). Accordingly, an increase of the ratio of $^1O_2$ plus $O_2\bullet^-$ to H_2O_2 marks the transition from a response of defense against stress to a response of programmed cell death in leaves (Sabater and Martín 2013a,b).

Low half-life and permeation through membranes suggest that the immediate effect of $^1O_2$ and $O_2\bullet^-$ in the signal cascade that shoots programmed cell death must occur within the chloroplasts. $O_2\bullet^-$ could initiate a signal chain by oxidation of cysteine, free and forming part of proteins, but successive steps (if any) leading to programmed cell death are unknown. Alternatively, unspecific oxidation by $O_2\bullet^-$ damages components of the photosynthetic machinery, increasing the production of $^1O_2$ by transfer of excitation from ^{3}Chl* to oxygen because Chl* can barely transfer electrons to damaged transporters. Therefore, direct or non-directly, $^1O_2$ would be the key signal that leads the cell to death. One of the best-known effects of $^1O_2$ is that it attacks polyunsaturated fatty acids. Several evidences suggest that this may be the distinctive starting point leading to specific expression of genes for programmed cell death. When the linoleic acid components of membrane lipids react with $^1O_2$ they form 13-hydroperoxy linoleic acid, which is transformed in chloroplasts to the oxylipin (9S,13S)-12-oxo-phytodienoic acid. Oxylipins exit chloroplasts and (9S,13S)-12-oxo-phytodienoic acid is transformed to jasmonic acid in peroxisomes, which is methylated to methyl jasmonate in cytosol (Creelman and Mulpuri 2002; Wasternack 2007).

Jasmonic acid and methyl jasmonate are known mediators of the responses to stress (Laloi and Havaux 2015), where there is over-production of $^1O_2$ (Wagner et al. 2004), and stimulate the expression of the senescence-associated genes (SAG) (Creelman and Mulpuri 2002), among them probably MYB transcription factors (Chen et al. 2002; Buchanan-Wollaston et al. 2005). In addition, jasmonic acid and methyl jasmonate increase the levels of the thylakoid Ndh complex (Cuello et al. 1995) and of chloroplast lipoxygenase (Bachmann et al. 2002). These two actions seem key to complete a first turn of the spiral rise of $^1O_2$ and $O_2\bullet^-$ because they are not accompanied

by a rise of the concentration of H_2O_2 due to the low SOD activity. The progressively misbalanced levels of the different ROS probably determine the programmed cell death (Sabater and Martín 2013a,b). In effect, as explained above, the increase of the thylakoid Ndh complex raises the reduction level of electron transporters and the rate of the Mehler reaction, which, without increase of SOD, further raises the level of $O_2•^-$. In addition, lipoxygenase catalyzes the oxidation of free linoleic acid with non-excited molecular oxygen (O_2) to form 13-hydroperoxy linoleic acid (Schaller et al. 2005) later transformed to jasmonic acid and methyl jasmonate as indicated. This determines an autocatalytic increase of the $^1O_2$ and jasmonates (mainly jasmonic acid and methyl jasmonate) signals that lead to the death of the cell. Significantly, jasmonic acid increases the production of $^1O_2$ (Guo et al. 2010) and accelerates senescence (Wasternack 2007). The possibility exists that the gene encoding a MYB factor involved in the negative control of chloroplastic SOD expression is one of the SAG jasmonate-mediated induced by $^1O_2$. If true, a suggestive model would emerge where a few signals and transcription factors initiate the autocatalytic path mediated by ROS and lead to leaf cell death.

The increase of the thylakoid Ndh complex is another key aspect of the autocatalytic travel to cell death. Accordingly, leaves of transgenic tobacco defective in the Ndh complex show delayed senescence when compared with normal plants containing full functional Ndh complex (Zapata et al. 2005) and the expression of *ndh* genes and the level of the Ndh complex increases during leaf senescence and fruit ripening (Martín et al. 1996; Casano et al. 1999, 2000; Lascano et al. 2003; Nashilevitz et al. 2010; Nilo et al. 2012; Serrot et al. 2012). In this respect, the longevity (more than 30 years) of the needles of many conifers may be related to the absence of *ndh* genes in their plastid DNA sequenced so far (Wu et al. 2011).

CONCLUSION

Many questions remain on the molecular mechanisms through which ROS controls cell death. The identification of the transcription factors involved is one aspect of the complexity of the network of signals controlling cell development through the expression of specific genes and by affecting the activity of enzymes and protein in general. Several hormonal and environmental factors that affect leaf senescence may be involved in the loss of sensitivity of the expression of chloroplast SOD genes to photo-oxidative

stress and in the successive autocatalytic events. Further investigations must solve the fragmentary knowledge of the signal factors and their interactions, where ROS are important but not the only players. However, as deduced from experimental results and plausible hypothesis, the actual knowledge of the control of leaf senescence by ROS provides valuable guides for investigation ahead.

A plant invests most of its assets (usually nutrients) to do efficient conversion of light energy into chemical energy of biomass in photosynthesis. The intensity of light and other factors, such as water availability and temperature, strongly varies with time. Therefore, the selection of the photosynthetic machinery traits during evolution have confronted a trade-off between the specialization for a narrow variety of light-environments to optimize photosynthesis or the use of a wide variety of light-environments where more light is available for energy conversion although with low yield. The results of trade-off issues greatly vary at the level of leaves among different plants, but they all lay between defenses against stress conditions to perform photosynthesis and die to recover nutrients in seeds (monocarpic senescence), in upper new leaves (senescence of basal shadowed leaves) or for new leaves in the following year (autumnal senescence). Not surprising, at least for the involvement of ROS in defense and death, programmed cell death uses a subtle modification of the mechanism through which ROS direct the response of defense against stress.

Most abiotic stresses provoke photo-oxidative stress in leaves in which over-reduction of photosynthetic electron transporters generates ROS. Leaves have enzyme and non-enzyme systems to diminish the production of ROS and scavenge them. The different ROS, by still not completely known mechanisms but where H_2O_2 seems to play a predominant role, act as signals that activate feed-back responses to alleviate the stress and decrease the level of ROS. Increased activities of chlororespiratory electron transport are one of the protective responses. Chlororespiration transforms the highly reactive ROS $O_2^{\bullet -}$ in the mild ROS H_2O_2 and, maintaining active CET, decreases the production of the also highly reactive ROS $^1O_2$. The thylakoid Ndh complex completes the chlororespiratory chain to maintain active CET in the transition to non-stressing conditions. This is particularly important for very rapid transitions (in less than one seconds) as in sun-fleck, where the Ndh complex is required for rapid recovery of photosynthesis rates (Martín et al. 2015). Coordinated induction of the Ndh complex, SOD and PX ensures low levels and appropriate ratios of ROS to alleviate damages and induce defense responses. For still poorly known mechanisms, at specific developmental

stages and age of the leaves, coordination broke down by failing the induction of SOD. Then, mild stress is sufficient to start a response where the ratio $O_2\bullet^-$ plus $^1O_2$ to H_2O_2 feed-forward increases, preventing defense responses and leading to death through mechanisms involving lipoxygenase, jasmonates and different SAG (Sabater and Martín 2013a,b; Laloi and Havaux 2015). In other words: the program of cell leaf senescence enters into action with small changes of a program (defense against stress) designed for the normal functioning of the cell under variable environments.

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