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Mini-Rreview

Experimental systems to assess the effects of reactive oxygen species in plant tissues

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Abbreviations: APX, ascorbate peroxidase; CAT, catalase; CuZnSOD, copper zinc superoxide dismutase; H_2O_2, hydrogen peroxide, OH·, hydroxyl radical; ROS, reactive oxygen species; $^1O_2$, singlet oxygen; $O_2^{-\cdot}$, superoxide anion; PCD, programmed cell death

Key words: *Arabidopsis thaliana*, oxidative damage, reactive oxygen species, signaling

Reactive oxygen species (ROS) are continuously produced in several organelles during aerobic metabolism. Furthermore, a wide range of environmental stresses such as chilling, salinity, drought and high light, lead to an elevated production of ROS. ROS can react with biomolecules and cause oxidative damage and even necrosis. Antioxidants and antioxidant-enzymes function to interrupt the cascades of uncontrolled oxidation. On the other hand, ROS influence the expression of genes playing a central role in many signaling pathways. Tools like the exogenous application of oxidative stress-causing agents and the in planta production of ROS in mutants altered in ROS metabolism are increasingly used to assess specific and common responses toward different types of ROS signals. The major challenge is the identification of ROS sensors and signaling components to finally elucidate the molecular mechanisms of oxidative stress response in plants.

Introduction

Reactive oxygen species (ROS) like singlet oxygen, the superoxide anion radical, the hydroxyl radical, and hydrogen peroxide ($^1O_2$, $O_2^{-\cdot}$, OH· and H_2O_2) are generated in chloroplasts, mitochondria and peroxisomes as inevitable by-products of aerobic metabolism. Plant cells evolved non-enzymatic and enzymatic mechanisms to tightly control their basal levels and thus limit their action (Fig. 1). Production of ROS is dramatically enhanced in response to high light, cold, drought, wounding or infection. ROS can cause oxidative damage[1] but they can also act as signaling molecules that influence expression of nuclear-encoded genes which initiate tissue necrosis or acclimation processes.[2-7] Recent studies indicated that the type of ROS or its subcellular production site is critical to provoke specific and selective cellular signals and responses.[8,9] This review aims to summarizing available experimental systems used to assess the effects of ROS in plant tissues, with special focus on non-invasive model systems. For a more detailed description of the metabolism of ROS and their participation in oxidative stress and signal transduction, readers are referred to more comprehensive recent reviews.[10,11]

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Invasive Systems: Exogenous Application of Oxidative Stress-Causing Agents

Methylviologen (MV). The redox-cycling herbicide MV (paraquat) undergoes univalent reduction and transfers electrons to oxygen generating $O_2^{-\cdot}$.[12] During illumination, photosystem I donates electrons to MV ensuring the steady formation of $O_2^{-\cdot}$ in the chloroplasts, thereby inhibiting photosynthesis.[13] Arabidopsis leaves floated in the light in 50 μM MV did not accumulate H_2O_2 and showed unaltered antioxidant machinery by 2 h of treatment. Biochemical and expression profiling analysis of these leaves allowed the identification of $O_2^{-\cdot}$ early-response genes, e.g. upregulation of specific members of transcription factor families and heat shock protein genes.[14] A common GAAAAGTCAAAC motif containing the W-box consensus sequence of WRKY transcription factors was identified in the promoter of genes highly upregulated by $O_2^{-\cdot}$.[14] Moreover, the transcription factors WRKY30 and WRKY48 were shown to respond to intracellularly generated ROS.[14-16] A short and severe stress imposed by $O_2^{-\cdot}$ on Arabidopsis plants indicated a rearrangement of carbon metabolism that reflects a short-term mechanism of survival.[17] Many photosynthetic genes and those encoding enzymes of starch and sucrose biosynthetic pathways were downregulated, while those encoding proteins belonging to the mitochondrial electron transport and enzymes of carbohydrate catabolic pathways were induced. A long-term response to MV application was also observed at the transcriptional level.[9]

Exogenously applied MV requires some time to penetrate the leaves and thus the formation of $O_2^{-\cdot}$ is delayed and a retarded response is provoked in comparison to the *flu* mutant, defective in the regulation of chlorophyll biosynthesis[9] (see below).

***Alternaria alternata* toxin (AAL).** The AAL toxin induces programmed cell death (PCD) through interference with the sphingolipid metabolism.[18] Arabidopsis plants infiltrated with nanomolar concentrations of AAL showed an accumulation of H_2O_2 and induced PCD in the light with features resembling the hypersensitive response.[19] Transcription profiling indicated a very early upregulation

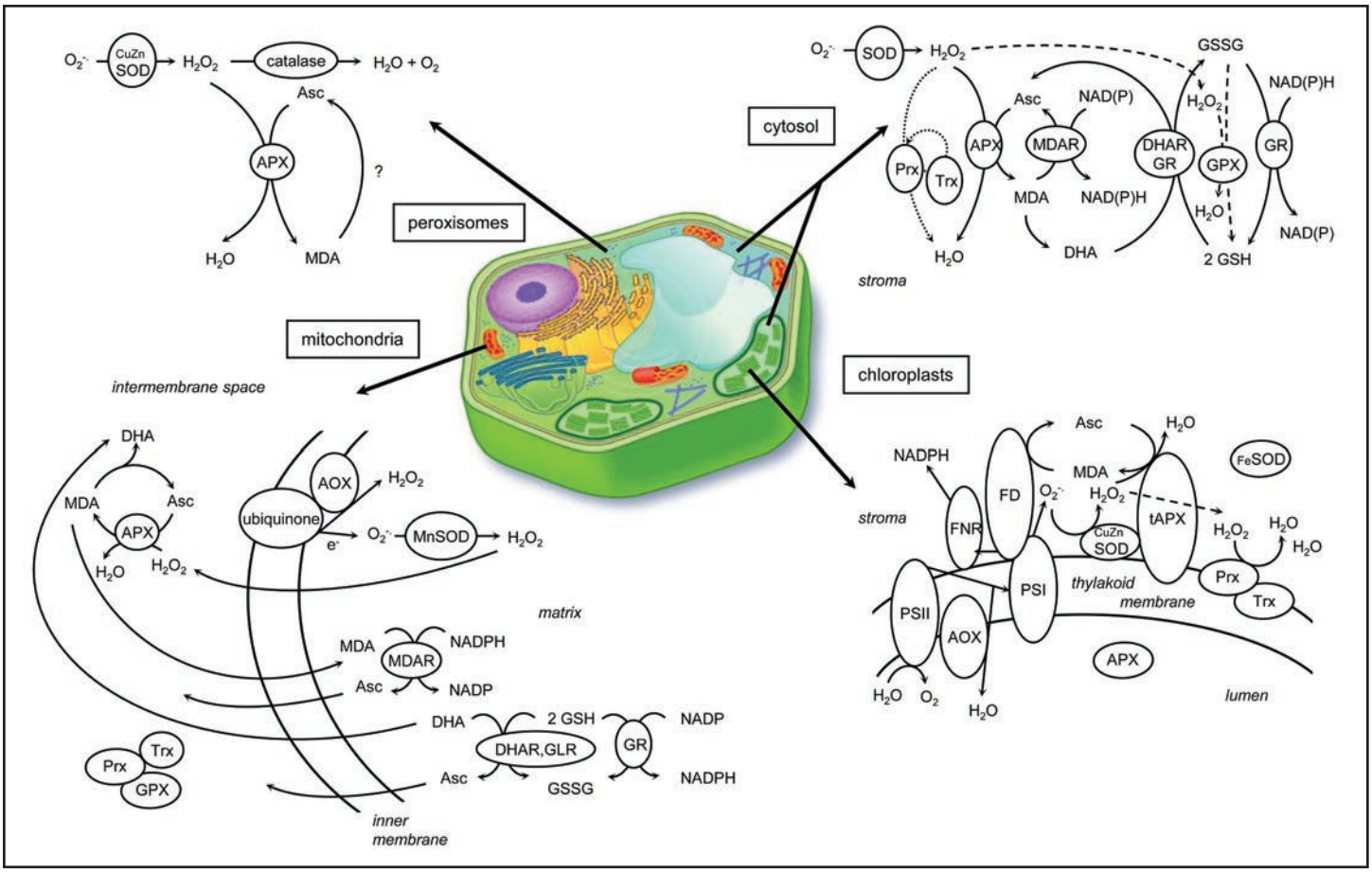

Figure 1. Cellular location of ROS scavenging pathways in plant cells. The GPX and Prx pathways are indicated in dashed and dotted lines in the cytosol and stroma. Asc, ascorbate; AOX, alternative oxidase; APX: ascorbate peroxidase; CuZnSOD, copper-zinc superoxide dismutase; DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FeSOD; iron superoxide dismutase; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, monodehydroascorbate; Prx, Peroxiredoxin; MDAR, MDA reductase; PSI, photosystem I; PSII, photosystem II; tAPX: thylakoid APX; Trx, thioredoxin; Adapted from Mittler et al., 2004.

of transcription factors, proteases and a number of ethylene and ROS-inducible genes potentially involved in transcriptional reprogramming observed at later stages and in the regulation of PCD processes.[9,19]

3-Aminotriazole (AT). The herbicide AT is a catalase inhibitor that causes endogenous accumulation of hydrogen peroxide and is thus used as a system for studying H_2O_2-induced PCD.[20] Microarray analysis of Arabidopsis leaves treated with AT revealed a set of new H_2O_2-responsive genes that were highly regulated in a common fashion during different types of PCD.[20,9] These included an oxoglutarate-dependent dioxygenase and various oxidoreductases, the transcription factors Zat11, WRKY75 and NAM, proteosomal components, a heterologous group of genes with diverse functions, and genes encoding proteins with unknown functions. Physiological analysis of knockout lines of the oxoglutarate-dependent dioxygenase confirmed its role in the cell death network.[20]

Ozone (O_3). O_3 is an atmospheric pollutant that is converted in the apoplast into $O_2^{\cdot-}$ and H_2O_2. When plants are exposed to O_3, a hypersensitive-like oxidative burst is triggered leading to necrotic lesions.[21] It has recently been shown that specific transcription factors, like WRKY70, ERF/AP2, NAM, GT-3a and ERF15 were upregulated in response to exogenously applied O_3.[9]

Harpin. Harpin is proteinaceous bacterial elicitor secreted by plant pathogens.[22] Treatment of Arabidopsis suspension cells with harpin was used as a system to induce ROS formation.[23] Harpin was found to initiate two signaling pathways, one leading to an increased generation of H_2O_2 and the expression of phenalalanine-ammonia lyase and glutathione S-transferase and the other leading to increased glutathione S-transferase and anthranilate synthase expression, independent of H_2O_2.

H_2O_2. Exogenously applied H_2O_2 confirmed its role as a cell death trigger and showed that high H_2O_2 concentrations can cause necrosis instead of PCD.[23,24] The application of H_2O_2 to Arabidopsis plants and cultured Arabidopsis cells allowed comprehensive analyses of transcriptional gene networks.[25-27] The treatment of Arabidopsis seedlings with H_2O_2 showed that many non-redundant expressed sequence tags are regulated by H_2O_2, most of them related to cell rescue, signaling and defense processes, indicating that they have multiple roles in plant responses to stress.[26] The treatment of Arabidopsis cells with H_2O_2 was also used as a model system to investigate the impact of oxidative stress on mitochondrial function.[28] This treatment lowered the O_2 consumption by mitochondria and the abundance of several key mitochondrial proteins (e.g., aconitase, the E2 subunit of the 2-oxoglutarate dehydrogenase,

fumarase, succinyl CoA ligase, subunits of the complex I and the ATP synthase complex). Using 2D-gel electrophoresis coupled to MS/MS, a mitochondrial protease was identified, which is responsible for the degradation of oxidatively damaged proteins.[28]

Menadione. Arabidopsis cells incubated with sub-lethal doses of this redox active quinone that generates intracellular $O_2^{\cdot-}$ showed a decreased cell growth rate with unaffected cell viability.[28] The effect of oxidative stress on mitochondrial function was found to be similar to that caused by the application of H_2O_2. A peroxiredoxin as part of the thioredoxin system was identified by which mitochondria can detoxify H_2O_2. A second protein induced by menadione was a mitochondrial disulfide isomerase, which may participate in the redox regulation of the alternative oxidase (AOX). Other mitochondrial proteins identified include a calreticulin, a glyceraldehydehyde-3-phosphate dehydrogenase and a glutathione S-transferase. Moreover, menadione resulted in the degradation of sensitive protein components as also described for H_2O_2.[28]

Recently, metabolic changes caused by oxidative stresses were first described.[29] Menadione-treated Arabidopsis cells indicated that oxidative stress had profound effects on central metabolic pathways, principally causing an inhibition of the tricarboxylic acid cycle and amino acid metabolism. This was accompanied by a subsequent backing up of glycolysis and an activation of the oxidative pentose phosphate pathway. Microarray analysis showed a switch from anabolic to catabolic metabolism indicative for a metabolic reconfiguration to bypass damaged steps.[29]

It is worth noting, that this treatment and the external H_2O_2 application are both non-specific oxidative stresses that act throughout the cell and trigger general responses.

Non-Invasive Model Systems: Use of Mutants Altered in ROS Metabolism

The specific effects of metabolically generated ROS within a particular subcellular compartment can be assessed using transgenic Arabidopsis with altered levels of specific ROS-scavenging enzymes such as the chloroplastic copper zinc-superoxide dismutase (CuZnSOD), cytosolic and thylakoid ascorbate oxidases (APX), peroxisomal catalase (CAT) and the mitochondrial AOX (Fig. 1).[16,30-35] Some of these plants were used as model systems to investigate transcriptomic variations triggered by a particular type of ROS.

In the following, different strategies are summarized which were used to modulate ROS levels in planta and to assess corresponding responses.

Thylakoid CuZnSOD. Studies using knock-down Arabidopsis plants with a reduced expression of the thylakoid-attached CuZnSOD due to a T-DNA insertion in the promoter provided evidence for the importance of the water-water cycle in protecting the photosynthetic apparatus from photooxidative damage.[31] Plants grown at moderate light intensities were impaired in growth and showed reduced chloroplast size, chlorophyll content and photosynthetic activity, a phenotype that could be reverted by growth at low light intensities. Microarray analysis of plants grown under controlled conditions revealed changes in transcript levels consistent with an acclimation response to light and indicated that anthocyanin biosynthetic transcripts were up-regulated in these plants.[9,31]

Cytosolic APX. Cytosolic *apx1* T-DNA mutants showed an accumulation of H_2O_2, suppression of growth, lower rates of photosynthesis but no alteration of transcripts encoding H_2O_2-scavenging enzymes under optimal conditions of growth.[30] On the contrary, during light stress, the expression of catalase, peroxidases and several transcripts encoding signal transduction proteins (especially heat shock proteins) was elevated pointing at novel components involved in H_2O_2-sensing. Moreover, the oxidation and degradation of chloroplastic proteins in these plants suggested that the cytosolic APX might be essential for the protection of chloroplasts during light stress.[30-34]

Thylakoid APX. Arabidopsis lines overexpressing the thylakoid APX (tAPX) were phenotypically indistinguishable from the wild-type under normal growth conditions. Seedlings did not show an enhanced protection against photoinhibition caused by high light or chilling but an increased resistance to MV-induced photoxidative stress.[33] Moreover, after treatment with sodium nitroprusside (SNP, leading to the formation of nitric oxide), these plants accumulated lower levels of H_2O_2 and were more resistant to nitric oxide-induced cell death.[33] On the other hand, tobacco lines overexpressing tAPX showed increased tolerance to chilling and MV-treatment suggesting that tAPX is a limiting factor of antioxidative systems under photo-oxidative stress in chloroplasts.[36] These results suggested that Arabidopsis and tobacco tAPXs might have non-overlapping roles in plant defense mechanisms. Indeed, while tobacco plants with reduced tAPX did not grow to maturity,[36] antisense Arabidopsis plants with a reduced level of *tAPX* expression showed no obvious phenotype under normal conditions but showed enhanced damage upon treatment with SNP.[37] The extent of cell death induced by SNP is inversely correlated with APX-scavenging of H_2O_2. Taken together, these results highlighted the role of Arabidopsis tAPX in the fine modulation of H_2O_2 levels for signaling.

Catalase. Antisense tobacco plants with reduced levels of CAT1 (the most abundant isoform in leaves) were used to study several aspects of H_2O_2 signaling from locally and systemically acquired resistance to the induction of an active cell death program.[38,39] These plants exhibited no visible phenotype when grown at low light conditions but showed cell death in clusters of perivenial palisade cells after exposition to high light.[38,6] This irreversible cell death process was shown to involve an oxidative burst triggered by the accumulation of photorespiratory H_2O_2.[6] Transcriptional changes monitored during exposition to high light indicated a rapid upregulation of signal transduction components involved in stress resistance and genes associated with cell death, and a downregulation of nuclear-encoded photosynthetic genes.[40] Moreover, pretreatment of these plants with high light induced resistance to pathogen infection (Chamnongpol 1996) and cell death caused by an additional exposure to high light,[38] clearly indicating the induction of a defense response at lower H_2O_2 concentrations.

On the other hand, antisense Arabidopsis plants with decreased levels of CAT2 activity (the most prominent leaf isoform, At1g20630) displayed an increased sensitivity towards ozone and photorespiratory H_2O_2-induced cell-death.[32] These plants allowed to assess H_2O_2-dependent and -independent high light-triggered transcriptional responses during a sustained H_2O_2-stress over time.[16,32] Of major importance was the identification of small heat shock proteins, transcription factors and regulatory genes involved in H_2O_2 transcriptional gene networks. Moreover, the accumulation of H_2O_2 was shown to negatively impinge on high light-stress induction of transcripts within the anthocyanin biosynthetic pathway, which

correlated with a lack of anthocyanin accumulation in *cat2* mutant plants during long-term high light exposure.

AOX. The mitochondrial AOX transfers electrons from the ubiquinone pool directly to oxygen without energy conservation and may function to prevent the formation of ROS by burning off an excess of energy in form of heat.[41,42] AOX antisense cultured tobacco cells produced more ROS than wild-type cells, while AOX-overexpressing cells produced less ROS.[43] These studies were extended to Arabidopsis plants overexpressing sense and antisense AOX constructs under the control of the CaMV 35S promoter.[35] In intact leaves and roots of antisense AOX lines, enhanced levels of ROS and oxidative damage were observed when the cytochrome pathway was chemically inhibited, whereas an increased AOX activity was sufficient to prevent ROS-related oxidative damage.[35] A transcriptome analysis under normal growth conditions provided only limited evidence for oxidative stress being chronically sustained in antisense AOX plants and, if at all, the stress appeared to be restricted to chloroplasts. On the other hand, when grown at low temperature, antisense AOX plants showed a reduced leaf area and smaller rosettes, while AOX overexpressors presented larger leaf areas and rosette sizes.[44] These phenotypic differences were not the result of major alterations in the tissue redox state because changes in levels of lipid peroxidation products and the expression of genes encoding antioxidant and electron transfer chain redox enzymes did not correspond with the shoot phenotypes.[44] These results demonstrate that AOX plays a role in shoot acclimation to low temperature in Arabidopsis and functions to prevent excess ROS formation in whole tissues in response to environmental stress conditions.

The FLU protein. FLU is a nuclear-encoded chloroplast protein that plays a key role during the negative feedback control of chlorophyll biosynthesis.[45] The inactivation of this protein in the conditional fluorescent Arabidopsis *flu* mutant leads to an accumulation of protochlorophyllide in the dark. Upon irradiation, protochlorophyllide transfers light energy to ground-state O_2 and thus, the *flu* mutant represents an experimental tool to induce the release of $^1O_2$ within the plastids after a dark-to-light shift. After reillumination, *flu* mutant plants stopped growing and developed necrotic lesions. In this condition, early stress response genes were activated that were different from those induced by $O_2^{-\cdot}$ and H_2O_2 generated, for example, by treatment with MV.[4,9] Specifically induced transcripts in the *flu* mutant are ethylene-responsive elements-binding proteins, indicative for ethylene signaling.[9] Recently, by varying the length of the dark period, conditions were established that either endorse the cytotoxicity of $^1O_2$ and allow non-enzymatic lipid peroxidation or support its signaling role involving oxylipin synthesis.[46,47]

GDP-mannose pyrophosphorylase. L-ascorbate is a powerful reducing agent found in millimolar concentrations in plant tissues and playing an important role in scavenging of free radicals. The Arabidopsis mutant *vtc1* exhibits reduced levels of ascorbate due to a mutation in the GDP-mannose pyrophosphorylase, involved in the L-ascorbate biosynthetic pathway.[48,49] These plants were used to assess the role of ascorbate as antioxidant and to elucidate its role in signal transduction pathways. ROS detoxification is impaired in *vtc1* exposed to ozone, sulfur dioxide and ultraviolet B irradiation, indicating that ascorbate is implicated in the defense against these environmental stresses.[48] Infection with virulent bacteria (*Pseudomonas syringae* or *Peronospora parasitica*) indicated that *vtc1* is more resistant to these pathogens and that pathogenesis-related genes are induced in these plants.[50,51] Transcript analyses also indicated that growth and development are constrained in vtc1 by a constitutive upregulation of ABA-associated pathways.[50,52] In addition, the *vtc1* mutant exhibits elevated levels of some senescence-associated gene transcripts as well as enhanced salicylic acid levels.[51] These results suggested that low ascorbate is causing *vtc1* to enter prematurely at least some stages of senescence.

Tocopherol cyclase and homogentisate phytyl transferase. Vitamine E is the collective term for tocopherols and tocotrienols, which have the ability to terminate chain reactions of polyunsaturated fatty acids free radicals generated by lipid oxidation. The Arabidopsis mutants *vte1* and *vte2*, deficient in tocopherol cyclase and homogentisate phytyl transferase, respectively, showed a complete loss of tocopherols.[53,54] These mutants were used to demonstrate that the antioxidant function of tocopherols in vivo are masked by compensatory protective mechanisms. By crossing the *vte1* mutant with the zeaxanthin-deficient *npq1* or the PsbS-deficient *npq4* mutants[55,56] it was shown that tocopherols fulfill two different functions in chloroplasts at the two major sites of $^1O_2$ production, protection of photosystem II against photoinactivation as well as of thylakoids membranes against lipid peroxidation.[54] The *vte-npq* mutants showed high levels of $^1O_2$-mediated lipid peroxidation and was recently used as a genetic model system to demonstrate that $^1O_2$ plays a major destructive role in photo-oxidative damage.[47]

Glycolate oxidase. To assess the effects of metabolically generated H_2O_2 in chloroplasts, transgenic Arabidopsis plants were generated in which the peroxisomal GO was targeted to chloroplasts.[15] GO overexpressing plants (GO plants) accumulated H_2O_2, showed retarded development, yellowish rosettes and impaired photosynthetic performance at moderate light intensities, while this phenotype disappeared at low light. A response to oxidative stress was installed in GO plants, with increased expression of the small heat shock protein (Hsp17.6B-CI or sHSP), the transcription factor WRKY30, the chloroplast-localized ferritin and enhanced foliar APX and CuZnSOD activities. GO plants developed oxidative stress lesions and showed repression of anthocyanin biosynthetic genes under photorespiratory conditions. These plants are unique in that H_2O_2 is generated inside the chloroplast even in the absence of biotic or abiotic stress or oxidants. Thus, GO plants represent a novel tool to study the action of H_2O_2 as a signaling molecule in chloroplasts as well as the consequences of oxidative stress from a holistic approach.[15]

Concluding Remarks

To date, it is not known to which extent the chemical specificity of the ROS species and the cellular compartment of their release may contribute the multiplicity of responses that occur in plants. Stress treatments and external application of oxidative stress-causing agents may activate additional signal transduction pathways that may complicate the analysis of ROS-signal transduction pathways. Some responses of plants to elevated in planta levels of ROS in the absence of abiotic or biotic stresses were investigated by using non-invasive model systems like mutants altered in the ROS scavenging machinery or the *flu* mutant. Moreover, plants expressing GO in the chloroplasts were recently proposed to represent an ideal system to study the effects of H_2O_2 directly in chloroplasts because H_2O_2

accumulation in this organelle can be induced by exposing the plants to different ambient conditions.

A common feature of plants that accumulate ROS is the inhibition of growth and flowering time.[15,30,31] This response resembles stress tolerance strategies used by plants exposed to adverse environmental conditions,[57] where plants may pass into a state of conservative metabolic activity, similar to hibernation in mammals. Until now, no direct link between ROS production and the inhibition of growth has been elucidated opening a challenging area for future studies.

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