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Oxidative Stress Mechanisms during Flower Senescence

Ajay Arora[1] • V. P. Singh[1] • S. S. Sindhu[1] • D. N. Rao[2] • S. R. Voleti[2*]

[1] Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi-110 012, India
[2] Department of Crop Production, Directorate of Rice Research, Hyderabad-500 030, India

Corresponding author: * **srvoleti@drricar.org**

ABSTRACT

Free radicals and other active derivatives of oxygen are inevitable by-products of biological redox reactions and universal under stress situations. Reactive oxygen species inactivate enzymes and damage important cellular processes and subsequently cell components also. Plants and other organisms have evolved a wide range of mechanisms to contend with this problem by promoting the synthesis of antioxidant molecules and enzymes. The effects of the action of free radicals on membranes include the induction of lipid peroxidation and fatty acid de-esterification. Both ethylene biosynthesis and membrane breakdown, which appear to be closely linked, seem to involve free radicals, although the sequence of events generating these free radicals is still poorly understood. The death of petal cells is preceded by a loss in membrane permeability, partly due to increases in reactive oxygen species that are in turn related to the up-regulation of oxidative enzymes and to a decrease in activity of protective enzymes. It also consists of losses in soluble proteins, nucleic acids caused by the activation of proteinases and nucleases and enzyme-mediated alterations of carbohydrate polymers. Many of the genes for these senescence-associated enzymes have been cloned. In some flowers, the degradative changes of petal cells are initiated by ethylene; in others, abscisic acid may play a role.

Keywords: abscisic acid, antioxidants, ethylene, free radicals, proteases, reactive oxygen species, senescence-associated genes
Abbreviations: **APX**, ascorbate peroxidase; **CAT**, catalase; **H_2O_2**, hydrogen peroxide; **NT**, non-transgenic; **PS**, photosystem; **ROS**, reactive oxygen species; **Rubisco**, Ribulose-1,5-bisphosphate carboxylase; **SOD**, superoxide dismutase

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INTRODUCTION

Living organisms are exposed to different kinds of stresses, which may originate from human activities or natural causes such as air pollution, drought, temperature, light intensities and nutritional limitation. Since plants have limited mechanisms of stress avoidance, they require flexible means of adaptation to changing environmental conditions. A common feature of different stress factors is their potential to increase the production of reactive oxygen species (ROS) in plant tissues. ROS are also generated in plant cells during normal metabolic processes (Fridovich 1995; Alscher *et al*. 1997). The photosynthetic electron transport system is the major source of active oxygen in plant tissues (Asada 1994), having the potential to generate singlet oxygen, $^1O_2$ and superoxide. The production of active oxygen is an unavoidable consequence of the operation of the photosynthetic electron transport chain in an oxygen atmosphere.

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Invited Review

The major oxygen-consuming processes associated with photosynthesis are: (a) the oxygenase reaction of ribulose-1,5-bisphosphate carboxylase (Rubisco), which is the initiating reaction of the photorespiratory pathway, and (b) direct reduction of molecular oxygen by the photosystem I (PSI) electron transport chain. In addition, certain photosystem II (PSII) components are also capable of converting molecular O_2 to high-energy $^1O_2$. A cyanide-insensitive respiratory pathway in chloroplasts that competes for electrons with photosynthetic electron transport (Bennoun 1994) may also reduce oxygen. Oxidative stress is essentially a regulated process, the equilibrium between the oxidative and antioxidative capacities determining the fate of the plant. Under non-stressful conditions the antioxidant defence system provides adequate protection against active oxygen and free radicals (Asada and Takahashi 1987). Both natural and man-made stress situations provoke increased production of toxic oxygen derivatives. In response, the capacity of the antioxidative defence system is increased (Gressel and Salun 1994). But in most situations the response is moderate (Foyer *et al*. 1994). Furthermore, some important sites such as the reaction centre protein of PSII (DI) and the apoplastic space appear to have very little protection against oxidative damage (Lwe *et al*. 1993).

In several flowers pollination appears to initiate the process of senescence followed by an interaction with hormones. Ethylene has long been implicated in the control of the senescence of many cut flower species, but the control of senescence in relation to wild species has received much less attention. The longevity of individual flowers varies greatly from species to species; in some, each flower is open for just a few hours, whilst in others the flower may persist for several weeks, or even months. The functional life of the flower may be terminated by petal wilting, abscission or a colour change of all, or part, of the perianth. In some species pollination appears to reduce floral longevity whilst in others, particularly those species having short-lived flowers, the pattern of flower development and senescence appears unaffected by pollination. Examples of the various pollination-induced strategies shown by plants are presented and the role of ethylene and other potential mediators of senescence in these processes discussed very interestingly by Stead (2004).

Transgenic plants are a means to understand the roles played by enzymes involved in establishing linkages with cellular processes associated with senescence. Studies on transformed plants expressing increased activities of single enzymes of the antioxidative defence system indicate that it is possible to confer a degree of tolerance to stress by these means. In order to better understand the role of antioxidant enzymes in plant stress protection mechanisms, transgenic tobacco (*Nicotiana tabacum* cv. 'Xanthi') plants were developed that overexpress both superoxide dismutase (SOD) and ascorbate peroxidase (APX) in chloroplasts. These plants were evaluated for protection against methyl viologen (MV, paraquat)-mediated oxidative damage both in leaf discs and whole plants. Transgenic plants that express either chloroplast-targeted CuZnSOD (C) or MnSOD (M) and APX (A) were developed (referred to as CA plants and AM plants, respectively). These plant lines were crossed to produce plants that express all three transgenes (CMA plants and AMC plants). These plants had higher total APX and SOD activities than non-transgenic (NT) plants and exhibit novel APX and SOD isoenzymes not detected in NT plants. As expected, transgenic plants that expressed single SODs showed levels of protection from MV that were only slightly improved compared to NT plants. The expression of either SOD isoform along with APX led to increased protection while expression of both SODs and APX provided the highest levels of protection against membrane damage in leaf discs and visual symptoms in whole plants (Kwon *et al*. 2002)

The advent of plant transformation has made it possible to engineer enhanced stress tolerance through antioxidative defense mechanisms. Signal transduction may involve G-proteins (Fujisawa *et al*. 2001), calcium activity changes (Johannes *et al*. 2005), ethylene receptors (Arora *et al*. 2006) and the regulation of protein phosphorylation and dephosphorylation (Fujisawa *et al*. 2001). Variations in floral architecture are of major evolutionary and economic importance, impacting various plant processes such as pollination and gene flow, as well as fruit production and seed dispersal. However, despite the central role of flowers in plant reproduction and agriculture, questions about the origin and diversification of the flower remain fundamental problems in plant biology. Recent studies in plant developmental genetics and genomics have identified dozens of genes with specific roles in flower development in Arabidopsis and other model organisms. Still, many (if not most) genes with critical roles remain undiscovered, largely because of functional redundancy. Because most economically important species are not closely related to model plant species, novel approaches are needed to build upon existing genome projects and transfer knowledge to non-model organisms. Floral genome sequencing project investigates the origin, conservation, and diversification of the genetic architecture of the flower, and develop conceptual and real tools for evolutionary functional genomics in plants. A set of plant 'exemplars' was selected, based on recent phylogenetic studies, to include the basal angiosperm groups where most flower diversity is found, plus key eudicot lineages where many crop species occur. The Floral Genome Project (http://www.floralgenome.org/) will generate large EST datasets, capturing thousands of sequences of genes expressed during early flower development in each species. New sampling approaches will be used to improve the chance of obtaining rare transcripts and of obtaining comparable gene sets from each species. Finished sequencing for up to 300 unique genes per species will allow study of the evolution of key genes and gene families expressed in flowers, and test leading hypotheses about the origin of the flower The Floral Genome Project will examine the site and timing of gene expression for the unique genes detected in each species using a combination of microarray analysis and new methods of high throughput in situ hybridization. Expression patterns will be evaluated for hundreds of genes in each species, and summarized in 3-D virtual reconstructions of developing flowers. This project would generate the first comparative data set of expression patterns for a large number of genes across diverse angiosperms. Floral genome sequencing projects are also providing novel approaches for identifying senescence-associated genes of agricultural importance (Buzgo *et al.* 2005). By devoid of lower interference from the chloroplasts senescence petal systems are best suited to study molecular aspects of organ senescence. The two senescent organs best studied are leaves and flowers. An advantage of flower petals, compared to leaves, as a system for studying organ senescence is that the process is irreversible and has tight developmental control. Within the given species it is possible to predict exactly when a bud will open and how rapidly the petals will senesce. In addition, a number of morphological and physiological changes are evident that allow the process to be readily documented. The efficacy of the floral system as well as the research tools now available make it likely that important information will soon be generated on the molecular mechanisms involved in petal cell death.

There are many review articles on flower senescence on various aspects but very little information on the flower senescence in relation to oxidative stress. This chapter is an attempt to cover the general photoxidative stress mechanism and their relationship with proteolysis and ultimately into petal senescence.

Generation of toxic ROS and associated regulatory mechanisms

Molecular oxygen is produced as a result of the oxidation of water by the photosynthetic electron transport chain. The latter, however, can also use oxygen as an electron acceptor.

In addition, molecular oxygen is assimilated during photorespiration producing phosphoglycollate. Both of these reactions have positive and negative effects. Superoxide, produced by the transport of electrons to oxygen, is not compatible with metabolism and must be eliminated by the antioxidative defence system while recycling of phosphoglycollate to phosphoglycerate (in order to re-enter the Benson–Calvin cycle) results in a considerable loss of assimilated carbon (Zelitch 1990). In addition, large amounts of hydrogen peroxide (H_2O_2) are produced during the oxidation of glycollate in the peroxisomes (Zelitch 1990). Although much of this H_2O_2 is destroyed by catalase (CAT), some chemical decarboxylation of keto acids by H_2O_2 still occurs (Zelitch 1990). Nevertheless, photosynthesis benefits since photorespiration protects the photosynthetic membrane against light-induced damage at times when carbon assimilation is limited (Heber *et al*. 1978).

Formation of singlet oxygen

The chlorophyll pigments associated with the electron transport system are the primary source of $^1O_2$. Singlet oxygen may also arise as a by-product of lipoxygenase activity. Like the hydroxyl radical, $^1O_2$ is highly destructive, reacting with most biological molecules at near diffusion-controlled rates (Knox and Dodge 1985). The lifetime of excited chlorophyll singlet state is short within these aggregates, but varies according to physiological conditions (Cadenas 1989). The excited singlet state of chlorophyll is used for the transfer of energy or electrons. However, there are two other possible routes of de-excitation, radiative decay (fluorescence) and conversion to the triplet chlorophyll state. The latter interacts with oxygen to produce $^1O_2$. There are two strategies for defence against $^1O_2$ in the thylakoid membranes. The first is the regulation of the light-harvesting apparatus to minimize triplet chlorophyll production, and the second is the rapid quenching of both the triplet chlorophyll state and $^1O_2$ by membrane-bound quenchers. Two major processes decrease the lifetime of excited singlet-state chlorophyll; the first is photochemistry and electron transport in the reaction centers and the second process involves thermal dissipation of excess excitation energy that quenches singlet-excited chlorophyll to the ground state (**Fig. 1**). Thermal energy dissipation plays a pivotal role in photoprotection since it limits the rate of reduction of the first stable electron acceptor of PSII (Q_A).

Superoxide production

Photo-reduction of dioxygen in chloroplasts was first shown by the production of acetaldehyde in the presence of ethanol and CAT and the photo-reduced product was assumed to be hydrogen peroxide (Mehlar 1951). Subsequently, in the 1970s the primary reduced product was identified as the superoxide anion radical. Under most circumstances, the control of electron flow between PSII and PSI regulates the reduction state of the acceptor side of PSI (**Fig. 2**). This means that the redox state of PSI acceptors does not significantly limit electron transport (Harbinson and Hedley 1993). The regulated activation of the Benson–Calvin cycle and control of the rate of electron flow are important factors determining the redox state of the ferredoxin pool (Harbinson *et al*. 1990). This is important because ferredoxin and the electron carriers on the reducing side of PSI have sufficient negative electrochemical potentials to donate electrons to oxygen resulting in the formation of a superoxide radical. There are two sites of superoxide production on the reducing side of PSI (Badger 1985). The majority of O_2 reduction *in vivo* is thought to proceed via reduced ferredoxin (Fd_{red}), which reduces molecular oxygen to the superoxide radical. Hydrogen peroxide (H_2O_2) is then formed through dismutation of superoxide. The latter occurs spontaneously, but the velocity of the reaction is greatly increased by SOD.

Production and scavenging of hydrogen peroxide in chloroplasts

Hydrogen peroxide is produced by the dismutation of superoxide radicals in a reaction mostly catalyzed by SOD (Asada *et al*. 1984). In leaf cells, CAT is exclusively localized in peroxisomes and has not been found in chloroplasts. The hydrogen peroxide in chloroplasts is scavenged by a peroxidase reaction using the photo-reductant produced in the thylakoid as the electron donor (Asada and Badger

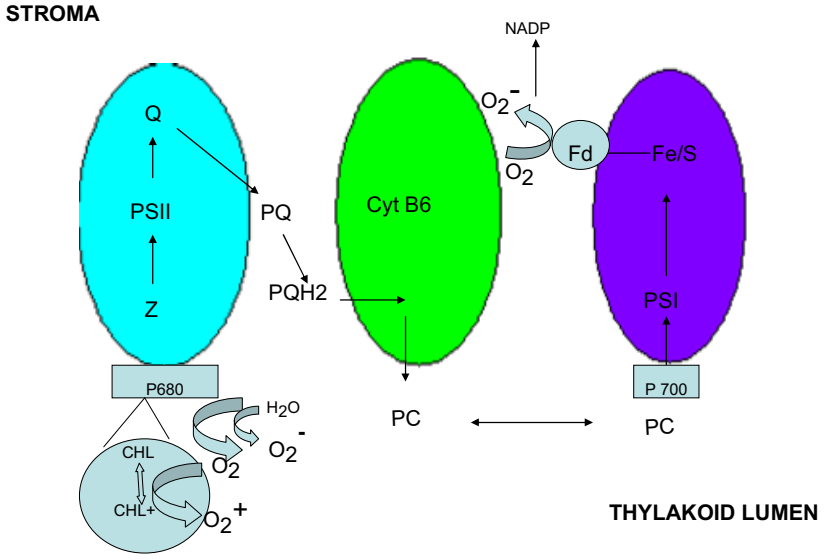

Fig. 1 Production of superoxide radical and singlet oxygen in chloroplast at the site of PSI and PSII. (Modified from Arora *et al*. 2002).

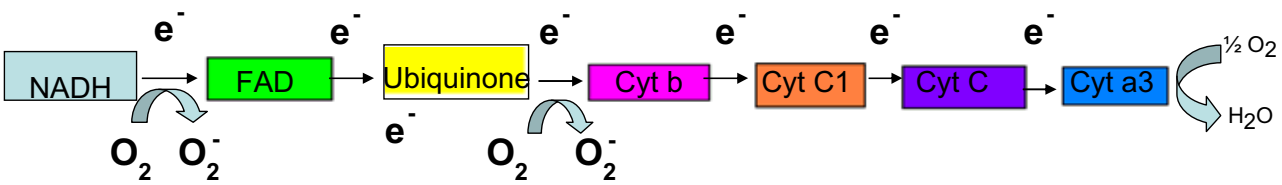

Fig. 2 Sites of superoxide radical formation in mitochondrial electron transfer system. (Modified from Arora *et al*. 2002).

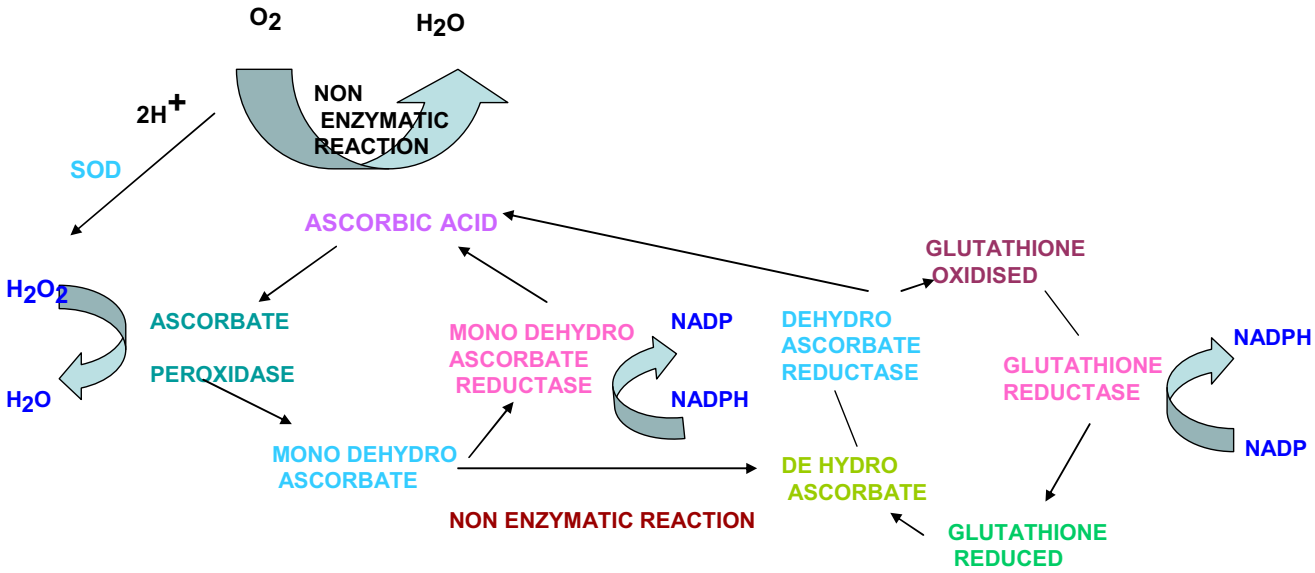

Fig. 3 Asada–Halliwell pathway of hydrogen peroxide scavenging and ascorbic acid regeneration involving various antioxidant enzymes. (Modified from Arora *et al*. 2002).

1984). Thus, diffusion of hydrogen peroxide from chloroplasts to peroxisomes and its scavenging by CAT are very unlikely to occur. The electron donor for the peroxidase reaction has been identified as ascorbate (Foyer and Halliwell 1976) (**Fig. 3**).

Superoxide generation in plant mitochondria

Several pathways of oxygen consumption are potentially available to isolated plant mitochondria or submitochondrial particles (Rich and Bonner 1978). These can be identified as follows:

1. Oxygen consumption via cytochrome oxidase to produce water, a process which accounts for more than 95% oxygen consumption in normal, cyanide sensitive mitochondria.

2. Direct reduction of oxygen to superoxide anions in the flavoprotein region of NADH dehydrogenase segment of the respiratory chain. The component responsible is likely to be the flavoprotein (of either internal or external dehydrogenase) or perhaps an iron–sulphur centre. The process may be identified by its insensitivity to KCN, antimycin A and salicylhydroxamic acid and by the sensitivity of the assayed epinephrine oxidation rate to superoxide dismutase.

3. Oxygen reduction to superoxide anions in the ubiquinone–cytochrome region of the respiratory chain. The process may be identified by its insensitivity to salicylhydroxamic acid and antimycin A, its sensitivity to KCN and the sensitivity of the assayed rate to superoxide dismutase. In these schemes, fully-reduced ubiquinone donates an electron to cytochrome C1 and leaves an unstable, highly reducing semiquinone species, which would normally reduce cytochrome b_{566}. It is presumably this unstable semiquinone or a closely interacting species which reduces the oxygen to superoxide anion, since only a species at this site would have enough reducing potential for the reaction. (The oxygen–superoxide couple has an Em at pH 7 of around – 330 mV).

Hydroxyl radical: The most reactive oxidant in cells

Hydrogen peroxide and superoxide radical by themselves are relatively less damaging, but they can form species damaging the essential cellular components such as hydroxyl radicals, that can initiate lipid peroxidation and also attack DNA, proteins and many small molecules. In the presence of trace amounts of iron ion, superoxide and hydrogen peroxide will form the destructive hydroxyl radical, and initiate the oxidation of organic substrates. Metal ions such as Cu^{+}, Cu^{2+} can replace Fe^{2+}, Fe^{3+} in these reactions.

Oxidation of organic substrates by hydroxyl radical

Oxidation of organic substrates may proceed by two possible reactions: (1) addition of a hydroxyl ion to an organic molecule, or (2) abstraction of a hydrogen atom from it. In the addition reaction the hydroxyl ion add to organic substrate forming a hydroxylated product, which is further oxidized by Fe^{3+} ion, O_2 or other agents to a stable oxidized product. The hydroxylated product can also dismutate to form cross-linked products.

In the abstraction reaction, the hydroxyl radical oxidizes the organic substrate by forming water and an organic radical. The latter product has single unpaired electron and thus can react with oxygen in triplet ground state. The addition of triplet oxygen to the organic radical can lead to the formation of a peroxy-radical, which can readily abstract hydrogen from another organic molecule leading to the formation of a second organic radical. This chain reaction is far more damaging than any other reaction catalyzed by ROS (Arora *et al*. 2002).

This hydrogen abstraction reaction of hydroxyl radical is best demonstrated by lipid peroxidation of linolenic acid in cell membranes (Frankel 1985). The lipid peroxides (ROOH) are unstable in the presence of Fe^{2+} or other reduced metal ions (such as Cu^{+}), as they participate in a Fenton reaction leading to the formation of reactive alkoxy radical. This alkoxy radical is as damaging as the hydroxyl radical, thus starting a cascade of oxidative reactions.

FREE RADICALS DURING SENESCENCE

Membrane breakdown and ethylene biosynthesis, which appear to be closely linked, seem to involve free radicals (Carlos *et al*. 1996). *In vitro* studies have suggested that the conversion of ACC to ethylene may involve a peroxidation reaction (Legge and Thompson 1983). Studies performed with *Dianthus caryophyllus* indicate that senescence can be slowed by retarding peroxidation by neutralizing free radicals. Moreover, inhibition of ethylene bursts slows peroxidation and prolongs the life of cut carnations, suggesting a relationship between free radical generation and ethylene production (Borochov *et al*. 1997). Sylvestre *et al*. (1989)

showed that during petal development in cut carnation, ethylene content increases simultaneously with peroxidation and the activities of SOD and CAT decrease from the initial stage to blooming. McRae *et al*. (1982) demonstrated more precisely the role of superoxide anion in this reaction. Baker *et al*. (1977) reported that the vase-life of carnations was increased by the use of sodium benzoate (a free-radical scavenger) at a concentration of 10^{-3} M, and that the outburst of ethylene was inhibited. According to Mayak *et al*. (1983), the microsomal membranes of carnation petals produce an increasing amount of superoxide radicals during senescence, and this increase parallels the decrease in membrane fluidity. The addition of a free-radical scavenger, propyl gallate at a concentration of 10^{-2} M, prevents change in the fluidity of the microsomal membranes. The superoxide anions contribute to breakdown of phospholipids and the fatty acids released may then be peroxidized (Mayak *et al*. 1983). This phenomenon leads to the rigidification of the membrane in senescent tissues. The following hypothesis concerning the sequence of events (and in particular those believed to affect membrane integrity) has been put forward by Mayak *et al*. (1983). Initially, a transformation of the lipids leads to membrane breakdown. Free radicals are then produced by peroxidation, and these free radicals promote the burst of ethylene. The effect of the rise in ethylene is, therefore, to accelerate the senescence. This hypothesis is in agreement with the work of Mayak and Adam (1984), who suggested that ethylene synthesis requires membrane deterioration so that ACC, a polar molecule, may approach the ethylene forming enzyme, the membrane enzyme that transforms ACC into ethylene. Aerobic respiration, which is strongly inhibited by cyanide (CN^-) and azide (N_3^-) ions, is found to continue even when cytochrome oxidase is blocked by these inhibitors in certain organs and/or tissues, due to the existence of an alternate short branch in the electron transport pathway at the first step involving ubiquinone. This provides a means for continued oxidation of NADH and operation of the TCA cycle. The alternate pathway is highly significant in the respiratory climacteric of ripening fruit and leads to the production of hydrogen peroxide and superoxide, which in turn enhance the oxidation and breakdown of membrane, necessary activities in the ripening process.

Oxidative stress and gene expression

When oxidative stress occurs, cells function to counteract the resulting oxidant effects of oxidative stress, with antioxidant defense enzymes being induced by changes in the levels of H_2O_2 or $O_2\bullet^-$, leading to the activation or silencing of genes encoding defensive enzymes, transcription factors and structural proteins (Dalton *et al*. 1999). ROS have also been proposed to function as second messengers independent of oxidative stress and to signal such cellular fates as cell proliferation, necrosis, and apoptosis. Although various observations have led to the suggestion that cells have the means to sense ROS and to induce specific responses (Scandalios 1997), the underlying mechanisms are still not fully understood. Regulators of oxidative stress responses are currently best characterized in bacteria (Storz and Imlay 1999), but progress is now also being made towards understanding such regulators in higher eukaryotes (Dalton *et al*. 1999; Guan *et al*. 2000). The transcriptional network that responds to ROS in eukaryotes is only now being unraveled but the prokaryotic system is quite well understood. Seventeen years ago, it was shown by two-dimensional gel electrophoresis that the expression of approximately 30 proteins was induced in bacteria by H_2O_2. Of these 30 proteins, 12 were maximally induced within 10 minutes and the other 18 between 10 and 30 minutes (Christman *et al*. 1985). Subsequent work led to the discovery of the OxyR regulatory protein, which was shown to regulate the expression of 9 of the 12 rapidly induced proteins. The tetrameric OxyR protein is a member of the LysR family of transcription activators and exists in two forms, reduced and oxidized; the latter is the only form able to activate transcription. Further studies led to the identification of a number of OxyR-activated genes (Storz and Zheng 2000). Similarly, the superoxide response or SoxRS regulatory proteins were found to regulate expression of $O_2\bullet^-$-responsive proteins in bacteria (Wu and Weiss 1991). Regulation of the *soxRS* regulon occurs by conversion of SoxR protein to an active form, which enhances *soxS* transcription. The enhanced levels of SoxS, in turn, activate expression of the regulon (Wu and Weiss 1991). In addition to SoxR and OxyR, several other transcriptional regulators modulate the expression of antioxidant genes in bacteria, indicative of the complexity and connectivity of overlapping regulatory networks (Storz and Imlay 1999). There are no apparent homologs of OxyR, SoxR, or SoxS in eukaryotes, but a number of other transcription factors have been found to play a role in regulating eukaryotic antioxidant genes. In yeast, transcriptional regulators of antioxidant genes include ACE1, MAC1, YAP1, YAP2, HAP1, and HAP2/3/4 (Ruis and Schuller 1995). In higher eukaryotes, oxidative stress responses are more complex and are modulated by several different regulators. In mammalian systems, nuclear factor κB (NFκB) and activator protein-1 (AP-1) are involved in regulating the oxidative stress response. The antioxidant responsive element (ARE) is present in the promoter region of genes encoding mammalian glutathione *S*-transferase (GST), metallothionein-I and manganese SOD (MnSOD), and is responsible for the induction of these genes in response to oxidants (Scandalios 2001). The promoters of antioxidant genes in higher plants also contain the ARE and the NFκB and AP-1 binding sites (Scandalios 1997). The role of these factors is not unique to activation of antioxidant genes, however, NFκB, in particular, is known to play central roles in regulating cellular responses to stresses other than oxidants, as well as regulating normal growth and metabolism. There is substantial evidence to suggest that a variety of biotic and abiotic stresses induce H_2O_2, which serves as a common factor in regulating various signaling pathways (Scandalios 1997; Somssich 1998). Similar stresses also activate mitogen-activated protein (MAP) kinases, with kinetics that either precede or parallel H_2O_2 production, suggesting that MAP kinases may be among the many converging points in the defense-signaling network (Zhang and Klessig 2001). In addition, exogenous application of several plant hormones and toxins has been shown to induce synthesis of $O_2\bullet^-$ and H_2O_2, leading to differential induction of some antioxidant genes and isozymes (Ruis and Schuller 1995; Scandalios 1997; Guan *et al*. 2000). Thus, from a utilitarian viewpoint, the identification of all genes and proteins regulated by H_2O_2 is an important step toward treatments that might confer tolerance to multiple, but interrelated, stresses. In addition to induction or repression of antioxidant defense genes, ROS are known to similarly affect expression of a variety of other genes involved in different signaling pathways in microbes (Storz and Imlay 1999), yeast (Causton *et al*. 2001), plants (Desikan *et al*. 2000), and animals (Finkel and Holbrook 2000).

Gene expression on a genomic scale

Efforts to identify ROS-responsive genes on a global scale were limited until the advent of microarray-based gene-expression analysis (Schena *et al*. 1995): DNA microarrays are now being used to comprehensively examine gene expression networks during oxidative stress. Reports on the stress responses of *Escherichia coli* (Zheng *et al*. 2001), yeast (Gasch *et al*. 2000), and higher plants (Desikan *et al*. 2001) have now provided significant progress in surveying gene expression in response to H_2O_2. The transcriptional profile of *E. coli* cells exposed to H_2O_2 was examined with a DNA microarray composed of 4169 *E. coli* open reading frames (Zheng *et al*. 2001). Gene expression was measured in isogenic wild-type and *oxyR*-deletion mutants (Δ*oxyR*) to confirm that the H_2O_2-response regulator OxyR activates most of the H_2O_2-inducible genes. There was a very rapid

and strong induction of a set of OxyR-regulated genes in the wild-type but not in the Δ*oxyR* strain, providing an internal validation of the experiment and confirmation of the induction of the oxidative stress genes identified by other means (Christman *et al*. 1985). In addition, several new H_2O_2-inducible genes were identified: some are members of the OxyR regulon and some are induced by an OxyR-independent mechanism. These findings indicate that other H_2O_2 sensors and regulators are present in *E. coli* (Zheng *et al*. 2001). Several genes that are known to be repressed by OxyR were found to be significantly expressed in the Δ*oxyR* mutant. Overall, the mRNA of 140 genes in the wild-type and 167 genes in the Δ*oxyR* strain were significantly induced after H_2O_2 treatment. It was also found that the superoxide response transcription factor gene *soxS* was induced by H_2O_2, indicating an overlap with other regulatory pathways. Also highly induced by H_2O_2 in both wild-type and Δ*oxy*R cells were two genes known to be members of the SoxRS regulon, *Fpr* and *sodA* (encoding NADH-ferredoxin oxidoreductase and manganese-superoxide dismutase, respectively). The microarray data revealed an overlap between the oxidative stress and heat-shock and 'SOS' DNA-damage responses (Zheng *et al*. 2001). Thus, the results from *E. coli* microarrays clearly indicate that the activities of transcription factors in addition to OxyR and SoxRS are likely to be modulated by oxidative stress.

In addition to bacteria, the transcriptional profile in response to oxidative stress has also been characterized in eukaryotes. In one broad-ranging study, the expression profile was examined in *Saccharomyces cerevisiae* cells exposed to H_2O_2, in addition to other stresses, and the global set of genes induced or repressed by each environmental signal was identified (Gasch *et al*. 2000; Causton *et al*. 2001). The results indicated that about two-thirds of the genome is involved in the response to environmental changes and the response to oxidative stress involves about one-third of the yeast genome, with the maximal effects on gene expression occurring slightly later relative to other stresses examined during similar time courses. Most of the transcriptome returns to pre-stress levels within 2 hours of exposure to H_2O_2 (Gasch *et al*. 2000). Genes that are repressed for approximately 1 hour after exposure to H_2O_2 are only transiently repressed in other stress time courses. Thus, genes encoding the translation apparatus and its regulators are remarkably coordinated in the responses to each environmental change, although the dynamics of each response are different. The expression programs following H_2O_2 or $O_2^{\bullet -}$ treatment were essentially identical, despite the fact that different ROS are involved. There was strong induction of genes known to be involved in detoxification of both H_2O_2 and $O_2^{\bullet -}$, such as CAT, superoxide dismutase, and glutathione peroxidase, as well as genes involved in oxidative and reductive reactions (for example, thioredoxin, glutathione reductase, and glutaredoxin). The genes most strongly induced in response to H_2O_2 and $O_2^{\bullet -}$ were dependent on the transcription factor Yap1 for their induction. Genes that are moderately induced by ROS and other signals are regulated by different transcription factors, depending on the conditions, and their response may be governed by different upstream signaling pathways (Gasch *et al*. 2000).

Recently, it has also been demonstrated that H_2O_2 activates the Sty1 (stress-activated MAP kinase) pathway in *Schizosaccharomyces pombe* in a dose-dependent manner, via two sensing mechanisms (Quin *et al*. 2002). At low H_2O_2 levels, Sty1 is regulated by a two-component signaling pathway that feeds into either of the two – Wak1 or Win1 – stress-activated MAP kinase kinase kinases upstream of Sty1. In contrast, at high H_2O_2 levels, Sty1 activation is controlled mainly by an independent two-component mechanism, requiring the function of both Wak1 and Win1. In addition, the individual bZip transcription factors Pap1 and Atf1 were found to function within a limited range of H_2O_2 concentrations: Pap1 activates target genes at low H_2O_2 concentration, whereas Atf1 controls transcriptional responses to high H_2O_2, with some minor overlap. Some apparent cross-talk among Sty1, Atf1, and Pap1 has been detected (Nguyen *et al*. 2000). Thus, *S. pombe* deploys a combination of stress-responsive regulatory proteins to gauge and trigger the appropriate transcriptional response to increasing H_2O_2 concentrations (Quin *et al*. 2002). This yeast mounts two separate responses to oxidative stress: an adaptive response to low-level H_2O_2 exposure that protects it from subsequent exposures to higher H_2O_2 levels, and an acute response that allows the cell to survive a sudden, potentially lethal dose of H_2O_2. Our recent work also showed that ethylene signal transduction pathway affected by the oxidative stress but the complete mechanism has yet to be deciphered (Arora *et al*. 2006).

The oxidative-stress response has also recently been characterized in an organism more complex than yeast and bacteria. A recent large-scale cDNA microarray analysis of the *Arabidopsis* transcriptome during oxidative stress identified 175 non-redundant expressed sequence tags (ESTs) from a sample of 11,000 that are regulated by H_2O_2. Of these, 62 are repressed and 113 are induced; and RNA blots showed that some of the H_2O_2-regulated genes are also modulated by other signals known to involve oxidative stress (Desikan *et al*. 2001). Furthermore, a substantial number of these genes have predicted functions in defense responses, cell signaling, transcription, and cell rescue (from environmental insults and developmental arrest), underscoring the pleiotropic effects of H_2O_2 in the response of plants to stress. Overall, the microarray used was estimated to represent only about 30% of the *Arabidopsis* genome, depending on redundancy, and 1% to 2% of the genes represented in the array are affected by H_2O_2-imposed oxidative stress (Desikan *et al*. 2001), which is comparable to the situation in yeast (Gasch *et al*. 2000). Of the 175 genes identified as H_2O_2-responsive, most have no obvious direct role in oxidative stress but may be linked to oxidative stress indirectly, as a consequence of other biotic and abiotic stresses, explaining their sensitivity to H_2O_2. Among the genes induced by H_2O_2 were genes encoding transcription factors, suggesting that they may mediate downstream H_2O_2 responses consistent with genomic studies in other species. Also, expression of the MAP kinases in *Arabidopsis* is induced by oxidative stress, as in other organisms, which in turn can mediate the induction of oxidative stress-responsive genes (Kovtun *et al*. 2000).

PETAL SENESCENCE

All cut flowers are destined to die, and the challenge for postharvest researchers is to slow the processes controlling flower death to enable cut flowers to reach distant markets with a display life that will ensure their sale and display, and return custom of the buyers. A thorough understanding of the processes that lead to cell death of floral tissues is integral to achieving this goal.

Postharvest performance of cut flowers is affected by the developmental stage of a flower at harvest, pro-senescence signals that originate from specific tissues within the flower (e.g., pollination-induced petal senescence), and stress-related metabolism (in response to temperature, wounding, nutrient starvation). Cut flower stems are removed from a source of nutrients, undergo water restrictions and may be held at undesirable temperatures in the dark for days prior to sale. Plant hormones, membrane stability, water availability, cellular proteolysis and carbohydrate metabolism act in concert to determine the differential rate of senescence for each floral organ. Currently, flowers can be grouped into several categories based on postharvest technologies that can extend their vase life (e.g., sensitivity to ethylene, chilling sensitivity, leafy stems, multiple/single flowers per stem, woody stems).

Flower petals are ideal tissues for cell death studies as they are short lived, the tissue is relatively homogenous, chemical manipulation can be applied without substantial wounding (i.e., feeding through the vascular tissue), and the process of flower senescence has been shown to be a gene-

tically programmed event (Eason *et al*. 2002). To date, most genetic analyses of floral senescence have focused on changes that occur in mature flowers just prior to wilting or colour change. However, senescence of one floral organ (e.g., petal) is part of a developmental continuum in the flower, preceded by tissue differentiation, growth and maturation of the petal, followed by growth and development of seeds, and coordinated by plant hormones. Cell-death processes are thought to be regulated by anti- and pro-death proteins, which may be expressed throughout the life of the flower, providing for the most part a highly regulated homeostatic balance. Future genetic analyses of floral senescence are likely to identify the proteins that function to maintain a non-senescent 'youthful' state, and the 'pro-senescence' proteins which function to progress cell death.

In petals of cut flowers undergoing senescence, protein content falls, protease activity increases, lipid fluidity in the membranes declines, and respiration rate increases (van Doorn and Stead 1997; reviewed in Teixeira da Silva 2006). Aging of petals is accompanied by a morphological, biochemical and biophysical deterioration. Senescing carnation flowers exhibit a climacteric-like rise in ethylene production and exposure of carnation flowers to exogenous ethylene induces in-rolling of petals, triggering ethylene synthesis, and inducing chemical and physical changes in microsomal membrane lipids of senescing petals (Bartoli *et al*. 1996). In chrysanthemum, which is nonclimacteric, ethylene does not play a role in flower senescence, with only minor changes in protein content and the proportion of major polypeptides being observed (Williams *et al*. 1995), explaining the long post-harvest life of chrysanthemum. Conditions inhibiting the action of, i.e. by the supply of silver salt, sodium benzoate or boric acid, or the synthesis of ethylene, i.e. by the supply of aminooxyacetic acid (AOA), prolong the vase-life of carnations (Serrano *et al*. 2001); an invertase inhibitor, apparently synthesized in wilting petals of a number of flowers (*Ipomoea*, alstroemeria, carnation, dahlia, gladiolus, petunia and rose) affects the senescence of petals by blocking sucrose hydrolysis to glucose and fructose in the senescing tissue, which may control the translocation of sucrose from wilted petals to other organs of the flower. Petal abscission in rose petals is not affected by the water status unless the plants reach a low water potential early on during vase life, neither inhibited by low light intensity nor affected by the Pr/Pfr ratio (van Doorn *et al*. 1996).

The termination of a flower involves at least two, sometimes overlapping, mechanisms. In one, the perianth abscises before the majority of its cells initiate a cell death program (van Doorn and Stead 1997). Abscission may occur before or during the mobilization of food reserves to other parts of the plant. Alternatively, the petals may be more persistent, so that cell deterioration and food remobilization occur while the petals are still part of the flower. The overall pattern of floral senescence varies widely between plant genera; therefore, a number of senescence parameters have been used to group plants into somewhat arbitrary categories. One distinction that is often made is the relative response of flowers to ethylene, resulting in the recognition of "ethylene-sensitive" (e.g. Orchidaceae, Campanulaceae, and Cruciferae) and "ethylene-insensitive" (e.g. Iridaceae, Liliaceae, and Amaryllidaceae; Woltering and van Doorn 1988; van Doorn and Stead 1994) systems.

The maintenance of cellular integrity and subcellular compartmentation is integral to cell function. However, during senescence of both ethylene-sensitive and –insensitive flowers, marked changes occur in the biochemical and biophysical properties of the cell membranes. These result from losses of membrane phospholipids, increases in neutral lipids, increases in sterol to phospholipid ratio, and increases in the saturation:unsaturation index of fatty acids (Lesham 1992; Thompson *et al*. 1998). Membrane polyunsaturated fatty acids are prone to oxidation either by enzymatic means (LOX, lipoxygenase) or through autoxidative events (non-enzyme catalyzed). In a number of floral tissues, such as carnation (*Dianthus caryophyllus*; Fobel *et al*. 1987), daylily (*Hemerocallis hybrid*; Panavas and Rubinstein 1998), and rose (*Rosa hybrid*; Fukuchi-Mizutani *et al*. 2000), LOX activity increases before the onset of electrolyte leakage (a marker of loss of membrane semipermeability). Increase in lipid peroxidation, usually estimated as TBARS, accompanies the increase in LOX activity and the products of peroxidation are considered to perturb membrane function, in part, at least, by causing increased membrane rigidification (Thompson *et al*. 1998). Examples exist of both ethylene-sensitive LOX-mediated peroxidation (e.g. carnation and rose) and ethylene-insensitive LOX-mediated peroxidation, e.g. day-lily (Panavas and Rubinstein 1998) and *Gladiolus* hybrid (Woltering and van Doorn 1988; Peary and Prince 1990). However, daylily flowers have a particularly short life span of around 24 h (Lay-Yee *et al*. 1992); therefore these flowers might be anomalous with respect to other longer lived ethylene-insensitive floral systems.

Oxidative stress and flower senescence

Flowers are the structures responsible for sexual reproduction, and thus play a crucial role in the perpetuation of the earth's most dominant group of plants. Many flowers have evolved elaborate, complex corollas which serve to entice the pollinators that aid in reproduction, but the larger and more attractive the petals may be, the more resources are needed for their preservation (Ashman and Schoen 1997) and the more likely the chances are for infection (Shykoff *et al*. 1996). Indeed, flowers having the showiest petals often have the shortest life times (Ashman and Schoen 1994). The importance for the plant of strictly regulating the death of its flowers makes it obvious that a sensitive, tightly controlled program for cell death must exist.

ROS have been linked to stress-induced and normal death of animal and plant cells, including petal cells (Borochov and Woodson 1989). In fact, ion leakage of daylily (*Hemerocallis* spp.) petals does not occur under non-lethal conditions of hypoxia (Panavas and Rubinstein 1998), suggesting that the oxygen is required for reactions leading to ROS as has been proposed for leaf senescence of tobacco (Mittler *et al*. 1996). ROS may also be a by product of LOX activity (Siedow 1991), increases during senescence of carnation and daylily petals (Panavas and Rubinstein 1998). LOX (EC 1.13.11.12) occurs widely in higher plants and catalyzes the dioxygenation of polyunsaturated fatty acids containing one or more *cis*,*cis*-1,4-pentadiene structure(s). The primary products of LOX-catalyzed reactions, fatty acid hydroxyperoxides, are further metabolized into molecules functioning in many biological processes, such as plant growth regulators, signal transduction molecules (e.g., jasmonates), and compounds responsible for green odor (hexanals). Many physiological and biochemical changes, including ethylene production and degradation of cellular membranes, occur during flower senescence (Porat *et al.* 1995) Furthermore, the ROS-gene-rating herbicide paraquat hastens the appearance of ion leakage in whole carnation flowers (Bartoli *et al*. 1996).

Highly oxygen radicals can be formed from H_2O_2 during various oxidase reactions (Halliwell 1989). In daylily petals, exogenous H_2O_2 increase along with Thiobarbituric acid reactive substances (TBARS), which is the measurement of lipid peroxidation, before flower opening. Hydrogen peroxide also increases in the petals after treatments that accelerate senescence and it decreases when senescence is retarded by the antioxidant sodium benzoate (Panavas and Rubinstein 1998).

Since higher levels of endogenous H_2O_2 would help stimulate senescence, it is important to determine the activity of enzymes that serve to regulate levels of H_2O_2. One well-known reaction involves reduction of H_2O_2 by ascorbate peroxidase (APX) and the consequent oxidation of ascorbate to dehydroascorbate (Asada 1992). In carnation petals, APX actually increases during senescence (Bartoli *et al*. 1995), but in daylily petals, APX activity decreases sharply,

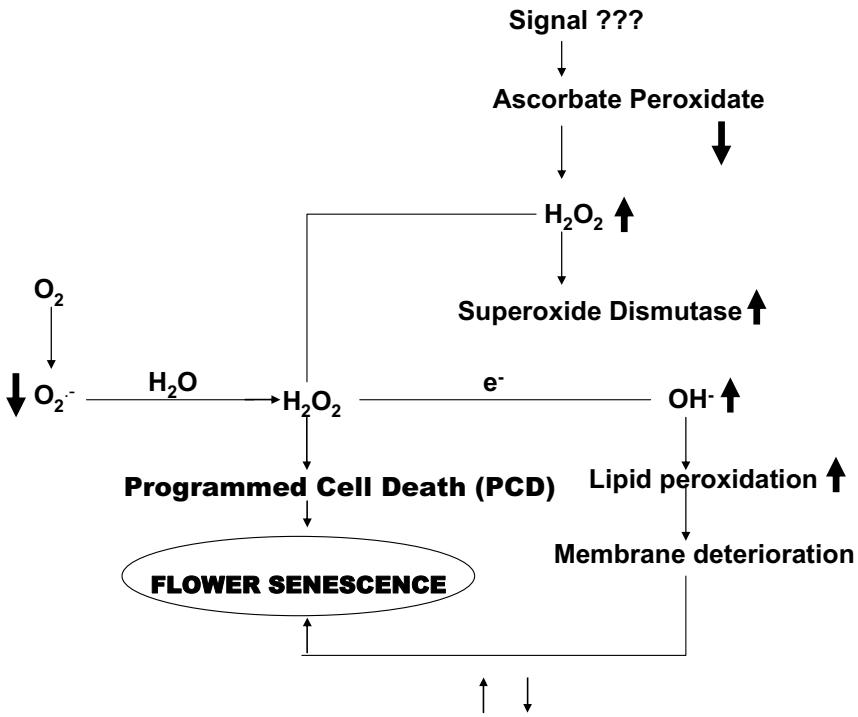

Fig. 4 Proposed model of senescence mechanism in gladiolus tepal with special reference to antioxidant enzymes activity. Symbols,↑, ↓, mean increase and decrease, respectively. (Modified from Hossain *et al*. 2006).

especially as the flower opens, which is about 18 h before cell deterioration becomes obvious (Panavas and Rubinstein 1998).

Hossain *et al*. (2006) studied the flower senescence in gladiolus in terms of antioxidant enzymes (superoxide dismutase, SOD; APX; glutathione reductase, GR) activities and membrane integrity. Membrane integrity was studied by measuring lipid peroxidation (TBARS) content and membrane stability index (MSI) percentage and concluded that an increase in endogenous H_2O_2 level during senescence may be the result of a programmed down-regulation of APX enzyme activity, which seems to be the prerequisite factor for initiating senescence process in gladiolus tepal (**Fig. 4**).

Ezhilmati *et al*. (2007) studied the effect of 5-sulfosalicylic acid (5-SSA) on the vase life of cut flowers of *Gladiolus grandiflora*. Spikes kept in vase solution containing 5-SSA exhibited lower respiration rates, lipid peroxidation and LOX activity, and higher membrane stability, soluble protein concentration, and activity of SOD and CAT. Results suggest that 5-SSA increases vase life by increasing the ROS scavenging activity of the gladiolus cut flowers.

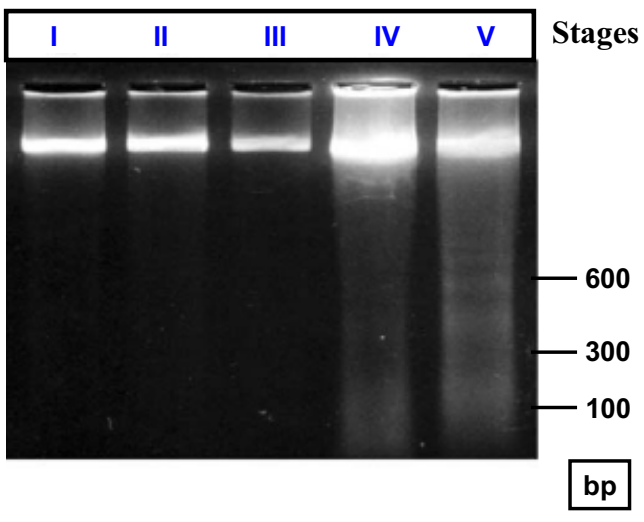

Fig. 5 DNA fragmentation detected in petals from each stage of gladiolus. (Modified from Arora and Singh 2006)

We also showed in our preliminary investigation that polyols regulate flower senescence by delaying programmed cell death (PCD) in *Gladiolus* (**Fig. 5**; Arora and Singh 2006). Further studies are required with more evidences like TUNEL assay, nuclear fragmentation and *in situ* hybridization to confirm that polyols regulates the flower senescence by delaying the PCD in gladiolus flowers.

Another enzyme that can lower H_2O_2 level is CAT, which converts H_2O_2 to H_2O and O_2 (Scandalios 1993).

Biochemical and molecular changes

Along with structural alterations measurements of physical and biochemical properties of the membranes indicate that important changes occur before the leakage of nutrients is evident (Lesham *et al*. 1986; Thompson 1988; Paliyath and Droillard 1992; Thompson *et al*. 1997). For example, the fluidity of membranes from a variety of different flower petals decreases, usually before senescence becomes obvious (Paliyath and Droillard 1992; Thompson *et al*. 1997). There is also a lateral phase separation of lipids in the membrane bilayer detected in carnations by X-ray diffraction (Faragher *et al*. 1987) and in roses by ^{2}H-NMR (Itzhaki *et al*. 1995), resulting in the formation of gel phase lipid. This, in turn, probably leads to the loss of water from the cells of many types of flower petals during the later stages of senescence. Evidence is accumulating that the phase changes are related, at least in part, to the inability of the membrane to remove metabolites by a blebbing of lipid-protein particles that occurs during normal membrane turnover (Thompson *et al*. 1997). The cause for this impairment is not very clear and under investigation, and its outcome may have important implications for understanding PCD in petals as well as in other systems.

Biochemical changes at the membrane include simultaneous declines in all classes of phospholipids (PL) and increases in neutral lipids (Paliyath and Droiliard 1992). The sterol/PL ratio also increases, which may contribute to the decreased fluidity mentioned above. The reasons for these alterations in PL content are related in part to greater activities of phospholipases and acylhydrolases, which would not only lower PL, but would also lead to increases in neutral lipids (Paliyath and Droillard 1992). In rose petals, however, where 50% of the total membrane PL is phosphatidyl choline (PC), a study of COP-choline phosphotransferase, the enzyme catalyzing the ultimate step in PC biosynthesis, in-

dicates that the amount of enzyme and its V_{max} decrease during senescence (Itzhaki *et al*. 1998). In daylily, too, the data suggest that phospholipid synthesis is blocked early in senescence (Bieleski and Reid 1992). An mRNA coding for a carboxyphosphoenolpyruvate/phosphoenolpyruvate mutase is up-regulated in carnation petals during senescence; the enzyme could be involved in membrane turnover (Wang *et al*. 1993). Thus, both a down-regulation of enzymes responsible for PL synthesis and up-regulation of hydrolytic enzymes may cause the membrane breakdown that is an important component of PCD.

Another senescence-associated event that leads to a loss of membrane permeability is the oxidation of existing membrane components. For example, lipid peroxidation as measured by TBARS increases during senescence in carnation (Sylvestri *et al*. 1989; Bartoli *et al*. 1995), daylily (Panavas and Rubinstein 1998) and gladiolus petals (Ezhilmati *et al*. 2007). Peroxidation may occur in part by the action of lipoxygenase, which oxidizes fatty acids liberated from membranes (Siedow 1991). LOX activity increases before senescence becomes obvious in carnation petals (Sylvestre *et al*. 1989), and in daylily, the specific activity of LOX increased before the flowers even open, which is about the same time that increases of TBARS occur (Panavas and Rubinstein 1998).

In orchid species, *Phalaenopsis* hybrid cv. 'Herbert Hager', however, even though linoleic and linolenic acids, two substrates of LOX, promoted senescence, no increases in LOX specific activity were detected over time, and inhibitors of LOX were without effect (Porat *et al*. 1995). So, even though the peroxidation of lipids that may result from LOX activity precedes loss of membrane integrity, the activity of lipid oxidases other than LOX may be limiting factors for membrane degradation in petals of these species of orchid.

Two cDNA clones from daylily have been sequenced whose gene products may play a role in oxidizing membrane lipids and whose message levels are up-regulated prior to or during senescence (Panavas *et al*. 1999). One clone, detected mainly in petals, shows the greatest similarity to an in-chain fatty acid hydroxylase bound to cytochrome P450, The translated product of this mRNA may modify fatty acids, leading to their degradation (Cabelio-Hurtado *et al*. 1998). The other clone is highly similar to an allene oxide synthase, which converts fatty acid hydroperoxides to allene epoxides, and eventually results in molecules that may have signaling capabilities (Song and Brash 1991). The increase in peroxidized lipids already described makes it likely that substrates would be available for this enzyme. No data are available as to whether these messages are translated in day lily petals in proportion to their abundance.

Another enzyme that can lower H_2O_2 levels is CAT, which converts H_2O_2 to H_2O and O_2 (Scandalios 1993). Overexpression of the gene for this enzyme protects leaves against ROS (Zelitch *et al*. 1991) and CAT-deficient plants are more sensitive to a variety of stresses (Willekins *et al*. 1997). In carnation, CAT activity increases during flower aging and then remains unchanged (Bartoli *et al*. 1995), but in daylily, the specific activity of the only form of CAT detected on activity gels decreases steadily from about 6 h before flower opening. When senescence is induced prematurely, CAT activity decreases earlier (Panavas and Rubinstein 1998).

SOD may also protect against buildup of ROS, but because the product of SOD is H_2O_2, an effective scavenging system must be present if cell damage is to be avoided. SOD decreases in activity in carnation petals (Sylvestre *et al*. 1989) and increases in daylily (Panavas and Rubinstein 1998), although in both cases the changes occur rather late in the progression of senescence. Since both CAT and APX decrease when daylily flowers open, the H_2O_2 build-up observed in these petals may in part be a result of increased SOD activity. Thus, in daylily, at past, SOD may be hastening cell death.

Peroxidase activity uses H_2O_2 as a substrate for several reactions and its specific activity increases in both carnation (Bartoli *et al*. 1995) and daylily (Panavas and Rubinstein 1998) during senescence. The transcript for an enzyme that detoxifies peroxidized lipids, GST (Meyer *et al*. 1991), and the corresponding enzyme activity (Sylvestri 1989) is also up-regulated in carnation petals. These data suggest, however, that POX and GST activities are responding to conditions of oxidative stress that result from H_2O_2 accumulations, and are thus not directly responsible for cell death.

Because lipid metabolism and ROS are so important to PCD, one must consider a role for peroxisomes. These organelles are the site of lipid breakdown and can produce O_2^- and H_2O_2, especially if protective enzymes are absent. Furthermore, as these organelles are converted to glyoxysomes, a rapid oxidation of lipids would ensue. There is an increase in numbers of peroxisomes during senescence of carnation petals (del Rio *et al*. 1996). A cDNA up-regulated during orchid petal senescence has been cloned that appears to code for a peroxisomal acyl-CoA oxidase (Do and Huang 1997). This particular enzyme may not be a cause of PCD, but it could serve to oxidize the fatty acids resulting from lipid breakdown at the membranes.

Natural antioxidants, such as ascorbate, glutathione and α-tocopherol, also are present in flower petals. All of these substances decline in carnation petals, although α-tocopherol decreases only after visual symptoms of senescence appear (Bartoli *et al*. 1997). In day lily, ascorbate drops by 50% even before the flowers open (Panavas and Rubinstein 1998). Inhibiting senescence of carnation petals with an ethylene synthesis inhibitor maintained higher levels of α-tocopherol and glutathione, and both antioxidants decreased when senescence was accelerated by ethylene or paraquat (Bartoli *et al*. 1996). Thus, with some exceptions (Bartoli *et al*. 1997), α-tocopherol levels are correlated with changes occurring later in senescence, but in both carnation and daylily, the decrease in ascorbate precedes many of the parameters associated with senescence. However, it is not possible to determine the involvement of antioxidants or the protective enzymes mentioned above in PCD until more information becomes available about their localization in the cell and the degree of increase or decrease in activity that is needed to affect a particular cellular process.

Membrane proteins may also play a role in petal senescence. Membrane protein and the content of thiol groups decline during aging of carnation petals (Borochov and Woodson 1989). Furthermore, in older compared to non-senescent petals, there is a large decrease in vanadate-sensitive ATPase activity, presumably the plasma membrane H^+ pump. But smaller decreases also occur in activities of cytochrome c oxidase, cytochrome c reductase and the nitrate-sensitive ATPase, which is likely the tonoplast H^+ pump (Beja-Tal and Borochov 1994). Taken as a whole, the data indicate that the membrane proteins mediating transport and redox reactions decrease in activity during aging. The question remains, however, if these decreases in activity are causally related to PCD or are just a result of previously triggered degradative processes.

Genetic modification to improve postharvest performance

To date, use of gene transfer technology to delay flower senescence has highlighted the need for tightly regulated transgene expression to avoid affecting other non-target developmental processes, particularly in the modification of plant hormone levels (e.g., poor rooting and lower disease resistance in ethylene-insensitive plants (Clark *et al*. 1999; Shaw *et al*. 2002). Thus, the need for tissue-specific promoters is paramount for exploiting this avenue of crop development in commercially important cultivars. Alternatively, modifying the expression of metabolic genes may produce satisfactory postharvest improvements without the need to alter hormone biosynthesis or perception, which may have pleiotrophic effects. Pollen sterility in flowers innately

lowers pollen-induced senescence signals and may make currently unsuitable flowers suitable for cut flower cropping without needing anti-ethylene treatments. The use of traditional breeding to select for genetic improvement of vase life may progress more rapidly as genetic markers for 'long life' are identified and as gene transfer technologies provide a way to improve the postharvest characteristics of crops with low genetic diversity. This theme is dealt with in greater detail in a comprehensive series of reviews (Teixeira da Silva 2006a).

PROTEOLYSIS AND FLOWER SENESCENCE

Proteolysis is an indispensable process in all living organisms. A continual turnover of proteins removes functionally impaired proteins (due to biosynthetic errors, improper folding, thermal denaturation, oxidative damage), which if left unchecked may restrict metabolic activities and jeopardize a cell's integrity. Proteases also recycle essential amino acids, and are important in the recovery of valuable nutrients. Proteases regulate metabolic pathways and developmental programs by affecting the rapid turnover of rate-limiting enzymes, and key regulatory proteins (Clarke 2005). Proteolytic cleavage is thought to play a significant role in the senescence of flowers because expression of protease genes is one of the earliest senescence-related gene changes to be identified (Eason *et al*. 2002). In plants, protein degradation linked to different developmental stages, such as germination, differentiation and morphogenesis, senescence, and PCD, has been reported (Huffaker 1990; Vierstra 1996; Beers *et al*. 2000; Arora 2007a). On the other hand, there are an increasing number of references that report in certain circumstances, proteolysis is also associated to oxidative stress promoted by ROS (Solomon *et al*. 1999). At cellular level, proteolytic processes commonly take place as a consequence of a regulated turnover of most cell components, including organelle biogenesis and autophagy (Klionski and Emr 2000). During organelle biogenesis, translocation of preproteins to their respective target compartments, i.e. mitochondria, chloroplasts, endoplasmic reticulum, etc., often requires the cleavage of a signal peptide. Once the polypeptide crosses the membrane, it is folded to form the final mature protein. In this event, chaperones are present on both sides of the membrane, thus facilitating the whole processing of proteins. This mechanism, which has been well studied in mitochondria and chloroplasts, is less understood in peroxisomes, where proteolysis is necessary for the import of some proteins like thiolase, acyl-CoA oxidase, and malate dehydrogenase (Gietl 1996; Hayashi *et al*. 1998). Abnormal/misfolded and mistargeted polypeptides occurring by mistakes during the translation are also proteolytically degraded. These errors might be a consequence of mutations not only in those polypeptides but also in the set of enzymes/proteins involved in their synthesis from transcription to the final processing in their target organelles (Adam 1996; Vierstra 1996).

Protein degradation in plants is a complex process involving a multitude of proteolytic pathways that can be carried out in different cell compartments. The presence of proteolytic activity has been reported in several cell loci, such as vacuoles, chloroplasts, cell wall, microsomes, mitochondria, cytosol, and the Golgi apparatus (Distefano *et al*. 1997; Buchanan and Gruissen 2000).

Among the functions assigned to proteolysis are: (a) the removal of abnormal/misfolded, modified, and mistargeted proteins; (b) the supply of amino acids needed to make new proteins; (c) contribution to the maturation of zymogens and peptide hormones by limited cleavages; (d) the control of metabolism and homeostasis by reducing the abundance of key enzymes and regulatory proteins; and (e) the cleavage of targeting signals from proteins prior to their final integration into organelles (Vierstra 1996). These molecular mechanisms form a part of more sophisticated global processes related to plant growth and development. Thus, the role of proteases in some developmental stages, such as germination, morphogenesis and cell biogenesis, senescence, and PCD, is essential.

Most proteases act either on the interior of peptide chains (endopeptidases, EP) or on their termini (exopeptidases). Exopeptidases have been differentiated according to their substrate specificity as aminopeptidases (AP), which are able to cleave peptides at the N-terminus, and carboxypeptidases (CP), which degrade peptides at the C-terminus (Dalling 1986; Huffaker 1990).

EP is classified according to their catalytic mechanism, which implies specificity in the enzyme active sites. It has been suggested that the term EP should be used synonymously with proteinase. In plants, four classes of endopeptidases have been described by Huffaker (1990): serine proteinases (EC 3.4.21), cysteine-proteinases (EC 3.4.22), aspartic-proteinases (EC 3.4.23), and metallo-proteinases (EC 3.4.24).

Several senescence-induced cDNAs have been sequenced whose identities suggest close homology to cloned proteinase genes. For example, a cysteine proteinase was cloned from carnation by amplifying a specific cDNA by PCR (Jones *et al*. 1995). RNA gel blot analyses indicate that the transcript increases after the pollination-induced burst of ethylene production that leads to senescence. Furthermore, appearance of the transcript is stimulated by adding ethylene to pre-senescent petals, and the ethylene action inhibitor NBD prevents the appearance of the transcript (Jones *et al*. 1995).

Differential screening of a cDNA library yields two cDNAs from daylily petals whose derived amino acid sequences show a strong homology to cysteine proteinases (Valpuesta *et al*. 1995; Guerrero *et al*. 1998). They appear to have an ER retention signal and an ERFNIN sequence that is consistent with activation by cleavage (Guerrero *et al*. 1998). Northern blot analysis indicates that message level have two peaks, one at 12 h and one at 19 h after flower opening. These transcripts were also found in leaves, but the mRNAs decreased at senescence (Guerrero *et al*. 1998).

Certain cysteine proteinases, collectively called caspases, are enzymes leading to apoptosis in animal cells, regardless of the initiating factor or the nature of the signal transduction pathway (Cryns and Yuan 1998). Caspase-like activity is detected in tobacco leaves and caspase-specific inhibitors prevent the hypersensitive response (del Pozo and Lam 1998). A cDNA for a putative aspartic proteinase with several family members has been cloned from daylily petals (Panavas *et al*. 1999). The steady state level of the message as determined by the RNase protection assay increases steadily from flower opening and the message is present only at much smaller levels in roots and leaves; in the latter, there is no correlation with senescence. Perhaps aspartic proteinases, whose message levels also increase in senescing leaves (Buchanan-Wollaston 1997; Griffiths *et al*. 1997), are involved in enzyme precursor processing as they are in some other plant systems (Mutlu and Gal 1999). However, it remains to be shown if aspartic proteinase activity increases during petal senescence.

Protein degradation and oxidative stress in plants

In addition to the involvement of proteases in the biological processes described, protein degradation also occurs under conditions that induce oxidative stress. In fact, a number of reports have shown that cells exhibit increased rates of proteolysis following exposure to oxidative stress-inducing agents (Pacifici *et al*. 1989; Grune *et al*. 1997). The working hypothesis for these reports implies that intracellular proteins are oxidatively modified by free radicals and/or related oxidants, and these modified proteins are selectively recognized and preferentially degraded by intracellular proteolytic enzymes (Grune *et al*. 1997). This model has been proved to be valid in all eukaryotic organisms, including plants (Solomon *et al*. 1999).

The protein modification promoted by oxidative stress is characterized by the production of carbonyl groups in the

molecule (Levine *et al*. 1994; Reinheckel *et al*. 1998). Basal levels of carbonyl groups are detected in plants, as a result of their generation as byproducts of normal physiological processes. However, increases in carbonyl contents have been observed in maize seedlings after chilling-induced oxidative stress (Prasad 1996; Kingston-Smith and Foyer 2000), in senescing nodules from pea and bean (Matamoros *et al*. 1999), in isolated chloroplasts exposed to oxygen radical-generating systems (Stieger and Feller 1997), in thylakoid proteins from water-stressed leaves of wheat (Tambussi *et al*. 2000), and in leaves of pea plants grown under toxic Cd concentrations (Romero-Puertas *et al*. 2002). In the case of Cd, it was found that this metal induced oxidative stress in pea plants (Sandalio *et al*. 2001), and some peptides, such as Rubisco, glutathione reductase, manganese superoxide dismutase, and CAT, were specifically oxidized by treatment with Cd. It was also found that Cd enhanced the proteolytic activity, and by using specific antibodies, it was demonstrated that the oxidized proteins were more efficiently digested by proteases (Romero-Puertas *et al*. 2002). Lascano and colleagues (1998) also reported that exposing chloroplastic glutathione reductase to an ·OH generating system brought about the breakdown of the enzyme by a sulfhydryl- and metal-containing protease. Works by other workers have associated the expression of different proteases and/or their proteolytic activity to conditions that usually induce oxidative stress, i.e. anoxia (Subbaiah *et al*. 2000), drought and temperature stress (Stroeher and Maclagan 1997), and the pathogen-promoting hypersensitive response (Yano *et al*. 1999). However, the specific combination oxidative stress–proteolysis was not directly addressed.

Different evidence obtained substantiated the idea that PCD and senescence (Quirino *et al*. 2000) are the physiological archetypes where controlled proteolysis is linked to the oxidative stress generated by ROS. PCD is one of the biological processes most thoroughly studied nowadays. It is generally accepted that ROS trigger PCD and antioxidants inhibit mechanisms leading to apoptosis (Jabs 1999; Solomon *et al*. 1999). During apoptosis, protein degradation also takes place in a modulated way (Beers *et al*. 2000; Fukuda 1997). In recent years, several proteases (Cys-EPs and Ser-EPs) have been shown to be involved in plant PCD (Arora and Ezura 2003; Arora and Singh 2004).

Flower senescence has been postulated to be a genetically regulated process, controlled by internal and external signals, in which proteases play a key role (Buchanan-Wollaston 1997). Moreover, the ability of cells to switch from one developmental state to another or to adapt to new environmental conditions often requires the rapid dismantlement of existing regulatory networks through proteolysis (Vierstra 1996).

At the subcellular level, most of the information available on the combined action of oxidative stress plus proteolysis has been obtained from studies on chloroplasts. Thus, Ishida and colleagues (Ishida *et al*. 1998) reported that, in wheat chloroplasts, the large subunit (LSU) of Rubisco is broken down by ROS into 37- and 16-kDa polypeptides. Similar results were also found in pea plants (Roulin and Feller 1998). In this plant species, the degradation of chloroplastic phosphoglycolate phosphatase, glutamine synthetase, and other enzymes took place as well (Stieger and Feller 1997). These studies also revealed that the degradation of the stroma proteins was light dependent, although some light-independent degradation may also occur (Roulin and Feller 1998). The fact that proteins that become nonfunctional due to interactions with oxygen species are further degraded by proteolysis is particularly significant in the thylakoid membranes. In these membranes, enzymes operate in a highly oxidizing environment and are susceptible to structure and function impairments (Lindahl *et al*. 2000). Within the thylakoid membrane, photosystem II (PSII) is the most susceptible component to oxidative damage. The degradation of the PSII D1 protein has been found to be carried out by the metallo-peptidase FtSH in a reaction that requires GTP (Lindahl *et al*. 2000).

Plant peroxisomes and proteases

In plants, the presence of proteolytic activity has been reported in several cell compartments, such as vacuoles, chloroplasts, the cell wall, microsomes, mitochondria, cytosol, and the Golgi apparatus (Buchanan *et al*. 1997; Distefano *et al*. 1997). However, the demonstration of the presence of proteolytic activity in plant peroxisomes is recent, and the information in this field is still scarce. Peroxisomes are subcellular respiratory organelles, containing, as basic enzymatic constituents, CAT and H_2O_2-producing flavin oxidases (del Río *et al*. 2002). These organelles have an essentially oxidative type of metabolism, and it has become increasingly clear that they carry out vital functions in plant cells and play an important role in the generation of signal molecules (del Río *et al*. 2002).

Several metabolic processes support the idea that some proteolytic activity must exist in peroxisomes. The conversion of glyoxysomes into leaf peroxisomes during the germination of seeds, as well as the opposite process converting leaf peroxisomes into glyoxysomes, implies the degradation of the organelle's pre-existing proteins (Nishimura *et al*. 1996). During the translocation of some polypeptides into the peroxisome, a proteolytic cleavage of the N-terminus takes place (Olsen and Harada 1995; Mullen *et al*. 2001). This partial proteolysis has been observed in *in vitro* import assays with some peroxisomal proteins, such as thiolase, MDH, and acyl-CoA oxidase, whose molecular mass inside the organelle is lower than that of the translation product (Gietl 1996; Hayashi *et al*. 1998). The cleaved peptidic sequence corresponds to the peroxisomal targeting signal located at the N-terminus (PTS2), and the process is accomplished in a way similar to that reported for mitochondrial and chloroplastic proteins (Bar-Peled *et al*. 1996). On the other hand, it has been reported that plant peroxisomes contain the xanthine oxidoreductase system (del Río *et al*. 2002). Xanthine oxidoreductase is an FAD-, molybdenum-, iron-, and sulfur-containing hydroxylase that have been found as two interconvertible forms (D and O). The conversion of form D (xanthine dehydrogenase, XDH) into form O (xanthine oxidase, XOD) is carried out by either reversible or irreversible pathways. In the irreversible step, XDH is converted into XOD by a proteolytic cleavage (Nishino 1994; Distefano *et al*. 1999). In peroxisomes from senescent pea leaves, a much higher increase of XOD activity is observed compared to XDH activity (Pastori and del Río 1997). This suggests that the conversion of XDH into XOD seems to occur inside peroxisomes, as has been observed in mitochondria (Saksela *et al*. 1999).

The presence of EP activity in peroxisomes has been investigated in pea leaves. In assays with peroxisomes from young leaves in the presence of azocasein, a specific substrate for endoproteases, a low EP activity of approximately 167 nKat mg^{-1} proteins was measured. By contrast, an up to six-fold higher endoproteolytic activity was observed in senescent leaves (Distefano *et al*. 1997). Through analysis by SDS–PAGE in gelatin-containing gels, three EP isozymes were detected in peroxisomes from young leaves, which were designated EP2, EP4, and EP5. However, four additional isoenzymes (EP1, EP3, EP6, and EP7) were detected in senescent plants (Distefano *et al*. 1997). The endopeptidases were characterized by using different class-specific inhibitors. All proteases were neutral, and the serine-proteinases (EP1, EP3, and EP4) represented about 70% of the total EP activity of peroxisomes from senescent leaves. Besides, these serine-proteinases showed a notable thermal stability, not being inhibited by incubation at 50°C (Distefano *et al*. 1997).

Role of peroxisomal proteases in oxidative stress situations

The higher proteolytic activity found in peroxisomes from

senescent leaves suggested a role for peroxisomal EPs at that developmental stage. Senescence in plants is associated to several metabolic events similar to those that occur at the subcellular level during oxidative stress situations. In particular, plant peroxisomes were postulated to play an activated oxygen-mediated function during natural senescence (Pastori and del Río 1997). In order to know how peroxisomal endopeptidases participate in the processes occurring during senescence, the degradation of peroxisomal and nonperoxisomal proteins by the endoproteases of purified peroxisomes from senescent pea leaves was investigated (Distefano *et al*. 1999). It was found that most peroxisomal proteins were endoproteolytically degraded, and this was specifically demonstrated in the case of CAT, glycolate oxidase, and glucose-6-phosphate dehydrogenase. Peroxisomal proteases were also able to cleave nonperoxisomal proteins like Rubisco and urease (Distefano *et al*. 1999). These results indicate that proteases from plant peroxisomes might play an important role in the turnover of peroxisomal proteins during senescence. This means that the organelle's own proteolytic machinery could participate in the mechanism of conversion of leaf peroxisomes into glyoxysomes, which is known to occur in senescent tissues (Distefano *et al*. 1999). Also, peroxisomal EPs might be involved in the turnover of proteins located in other cell compartments during advanced stages of senescence, when deterioration of membranes and leakage of the organelles' soluble fraction takes place. In experiments carried out *in vitro*, it was demonstrated that bacterial XDH was converted into XOD in the presence of peroxisomes from senescent leaves (Distefano *et al*. 1999). These data suggest that peroxisomal EPs could also participate in regulatory mechanisms that do not necessarily imply full degradation of proteins.

On the other hand, it was reported that 50 μM CdCl2 produced an enhancement of the hydrogen peroxide concentration in peroxisomes and also an increase in the activity of some antioxidative enzymes of the ascorbate–glutathione cycle and NADP-dependent dehydrogenases located in these cell organelles (Romero-Puertas *et al*. 1999). Furthermore, in western blotting assays using an antibody against dinitrophenylhydrazone (DNPH), which recognizes oxidatively modified proteins, a higher level of oxidized proteins was found in peroxisomes from Cd-treated plants (Romero-Puertas *et al*. 2002). Under the same experimental conditions, Cd induced senescence symptoms in leaf peroxisomes, with an enhancement of the endogenous proteolytic activity and the activity of the glyoxylate cycle enzymes malate synthase and isocitrate lyase (McCarthy *et al*. 2001). Taken together, these results indicate that the peroxisomal proteases could participate in the metabolic changes produced by Cd, by degrading the oxidized proteins, and possibly also in the transition of leaf peroxisomes into glyoxysomes, which is known to occur during senescence (Nishimura *et al*. 1996; del Río *et al*. 1998).

Ubiquitin-proteosome pathway

The ubiquitin-proteosome pathway is a major cytoplasmic proteolytic system for the degradation of short-lived and abnormal proteins (Vierstra 1993). In plants, changes in the level of ubiquitinated proteins have been associated with cell death during the development of vascular tissue (Bachmeier *et al*. 1990; Stephenson *et al*. 1996; Woffenden *et al*. 1998) as well as during senescence of leaves (Garbarino *et al*. 1995; Pinedo *et al*. 1996). In immunoblots of daylily petals, protein-ubiquitin conjugates were detected, some of which increased and some decreased during senescence. RNA gel blots revealed several ubiquitin transcripts that increased, decreased or stayed unchanged during flower opening and senescence (Courtney *et al*. 1994). Analysis in daylily of enzymatic components of the ubiquitin proteosome pathway (ubiquitin-activating enzyme, ubiquitin-conjugating enzymes, a subunit of the multiubiquitin binding protein) show detectable but steadily decreasing quantities during senescence when expressed on a per petal basis (Stephenson and Rubinstein 1998). Because proteinase activity increases during senescence, but banding patterns on PAGE gels remain sharp and well defined, the ubiquitin-proteosome system may be efficiently breaking down the cleaved proteins to amino acids rather than initiating senescence. However, inhibitors of proteosome activity prevent cell death leading to tracheid formation (Woffenden *et al*. 1998) and delay the ion leakage associated with senescence in daylily petals (Stephenson and Rubinstein 1998). This implies that there may be a causal role for the ubiquitin-proteosome system in petal senescence.

SUMMARY, CONCLUSIONS AND FUTURE RESEARCH

Petal senescence provides a facile system for studying PCD of plant organs. In many cases isolated petals behave similarly to those on the plant, and the process occurs relatively quickly, synchronously and uniformly throughout the tissue. The most extensively investigated flowers; however, are generally not the species that lend themselves readily to genetic manipulation, mutagenesis, or transformation. But it is likely that more tractable systems such as *Arabidopsis*, tomato or tobacco can also be employed to further analyze the role of particular pathways in petal PCD.

The fact that carbohydrates delays petal senescence demonstrates that the synthesis of particular suicide proteins orchestrates the cell death program. It is necessary, however, to show that these proteins and their products are not just correlated with PCD, but actually play a crucial role. For example, particular protein may serve only to mobilize materials during transport out during cell, or a substance may be produced as a result of a perceived stress associated with senescence, and there is little direct effect on those processes most closely related to cell death. After a causal role is established, one must then determine if the substance acts alone or in concert with others.

With this in mind, several enzymatic reactions have been identified in petals that may directly affect senescence. These reactions result in the oxidation of membrane components and the breakdown of proteins and nucleic acids. Genes for several enzymes that mediate these events have been cloned and the mRNAs may be up-regulated prior to, or during, senescence. If the higher steady-state message level is a result of transcriptional regulation, techniques exist to examine the regulatory regions of these genes for consensus sequences that may indicate the factor(s) that enhances transcription. Other regulatory mechanisms that may be investigated include senescence-associated transcription factors, and a role for protease inhibitor genes (Solomon *et al*. 1999). Characterization of genes that are down-regulated during senescence should also yield useful information. For example, expression of the DAD gene, which appears to protect cells against cell death, decreases in pea and gladiolus petals during senescence (Orzaez and Granell 1997; Yamada *et al*. 2004).

The endogenous and environmental triggers that initiate suicide genes are also being identified. Senescence in many flowers is controlled in part by ethylene, but whereas other factors may also play a role, one must determine for the particular flower if only the production of ethylene is a limiting factor for senescence or if the sensitivity to ethylene is also important (**Fig. 6**; Arora 2005). An enhanced evolution of ethylene may be related to higher levels of the mRNAs for the enzymes responsible for synthesis of this hormone. The up-regulation of the ethylene-binding protein also implicates this process in an increased sensitivity to ethylene. The crucial question remains, however, as to how these messages are regulated (Arora 2005).

There are also flowers in several families that are insensitive to ethylene. Even though ABA may be the senescence-inducing factor, many species should be tested before a generalization can be made. Furthermore, it is important to show that depletion of ABA delays senescence in these flowers. There may also be ABA-responsive sequences on

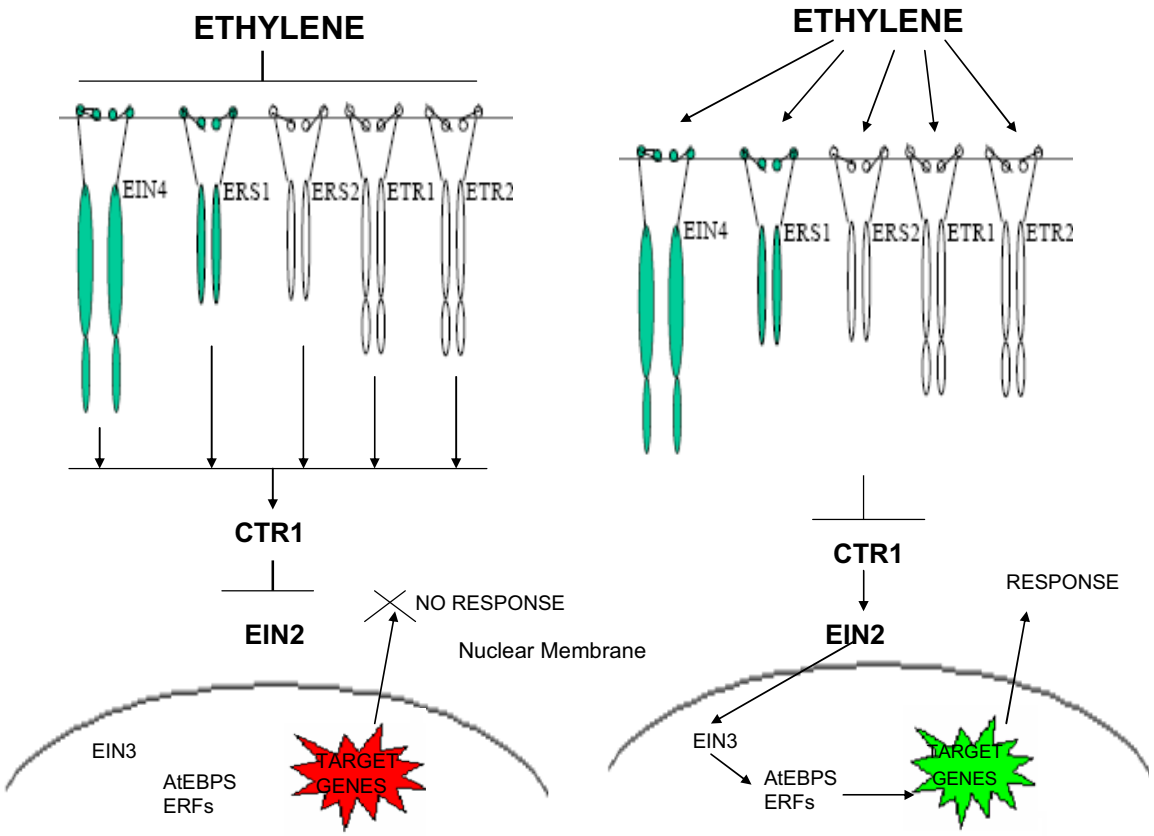

Fig. 6 Schematic diagram of the pathway of ethylene signal transduction in *Arabidopsis*. In the receptor inhibition model, receptor isoforms signal to CTR1 in the absence of ethylene. Ethylene inhibits this signalling and CTR1 is inactivated. Then, EIN2, EIN3 and EILs are activated and ethylene responses occur. Mutants that have lost ethylene-binding ability would continue to activate CTR1 in the presence of ethylene and thus suppress response pathways downstream from CTR1. (Modified from Arora 2005).

the regulatory regions of the genes that directly or indirectly cause PCD.

Finally, pollination, injury, dehydration and light are environmental triggers of petal senescence that may act first on other parts of the flower. The transportable signal, which may be ACC or ethylene, leads to elevation of ethylene evolution (or ABA production in the case of water stress) in the petals. Different mechanisms may exist for different species.

An area that is ripe for exploration is the identification of components of the signal transduction chain resulting in PCD due to oxidative stress. Various factors and processes known to be important for developmental processes, including PCD, have been implicated in petal senescence, such as G-protein activation, inositol phosphate metabolism, alterations in cytosolic free calcium, and phosphorylation/dephosphorylation reactions. However, events such as calcium level changes need to be investigated on the cell level in living material, and genes involved in producing signal transduction components such as kinases and phosphatases must be identified and their regulation studied. It is important to keep in mind that different species may have different signal transduction systems.

The impact of characterizing PCD in petals allows an understanding of a process crucial to the survival of plants and other plant organs as well as providing economic benefits. The ease of working with flowers and the exciting advancements made in techniques at both the molecular and cell levels should lead to the identification of particular mutants, the cloning of more suicide genes and the production of transformed plants, all of which make it very likely that great strides will be made in the next few years.

At present, one of the most studied aspects of peroxisomes biology is the biogenesis of these organelles. Peroxisomal proteins (PTS) are synthesized in free polyribosomes, with a targeting signal that drives proteins to their organelle. Much is known about the different targeting signals of PTS and the required cofactors for the translocation inside the organelle (Olsen and Harada 1995; Mullen *et al.* 2001). Two main PTSs have been reported so far. PTS1 resides in the C-terminus with a tripeptide and PTS2 is in the N-terminus and is usually cleaved upon the translocation of polypeptides through the membrane. This process possibly implies the involvement of an aminopeptidase. The occurrence of a Leu-AP in peroxisomes suggests that it might be involved in the processing of imported precursor polypeptides in peroxisomes. The purification of the peroxisomal Leu-AP, and its further characterization and assays with its specific substrates, will help in clarifying the mechanisms of processing of polypeptides till their mature form during the translocation to the peroxisome. On the other hand, the study of the combined mechanism of oxidation plus proteolytic degradation of proteins under certain conditions is gaining relevance in plant biology. Senescence and metal toxicity by cadmium is representative of an oxidative stress taking place together with an enhanced proteolytic activity (Sandalio *et al.* 2001; Romero-Puertas *et al.* 2002). In these experimental conditions, a parallel behavior was observed in the metabolism of leaf peroxisomes. The molecular and biochemical characterization of peroxisomal proteases will allow the comparison of their properties with those available in databanks. It will provide information on their catalytic mechanism and their putative substrates. Studies on the role of peroxisomal proteases in oxidative stress situations can be accomplished by the incubation of enzymes with different cell components and specifically in petal tissues. This is particularly relevant in the light of recent results in *Arabidopsis* plants that demonstrated that oxidative stress induced *PEX* genes involved in the peroxisome biogenesis (Lopez-Huertas *et al.* 2000). The control of proteolysis will be also of interest in terms of increasing plant productivity. This control could be established by using specific inhibitors. Plants are known to contain important levels of protease inhibitors that regulate enzyme activity.

This strategy of manipulating senescence could overlap other strategies such as that of breeding or genetic engineering to improve crop yields by keeping leaves photosynthetically active for longer.

Today, much attention is being devoted to the investigation of PCD in plants, a physiological stage characterized by a notable oxidative stress and the activation of several proteolytic pathways. At the subcellular level, there is still little information on the events that occur in PCD processes in plants. Taking into account that senescence is a type of PCD, the cellular and molecular study of the possible involvement of peroxisomal proteases in apoptosis during flower senescence could provide deeper insights into this important physiological process.

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