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Increase in reactive oxygen species (ROS) and in senescence-associated gene transcript (*SAG*) levels during dark-induced senescence of *Pelargonium* cuttings, and the effect of gibberellic acid

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Abstract

Dark-induced senescence in the leaves of *Pelargonium* cuttings was manifested in chlorophyll breakdown, and an increase in reactive oxygen species (ROS) levels followed by a subsequent induction of two senescence-associated gene (*SAG*) transcripts: senescence-related transcription factor *PeWRKY*6-1 and cystein protease homolog *PeSAG*12-1. Glutathione applied at the onset of ROS increase, reduced ROS accumulation and prevented the increase in *PeSAG*12-1 expression. These results suggest that in darkness *PeSAG*12-1 and maybe *PeWRKY*6-1 are induced by increases in ROS levels. Since *PeWRKY*6-1 expression increased concomitantly with that of *PeSAG*12-1, it most likely does not function as a senescence inducer.

Application of gibberellic acid (GA_3) to *Pelargonium* cuttings before the dark treatment prevented chlorophyll breakdown, ROS increase and *PeWRKY*6-1 and *PeSAG*12-1 accumulation. GA_3 also decreased ROS levels when it was applied during the dark period at the onset of ROS accumulation, but not when applied after high ROS levels were evident. The pattern of GA_3 suppression of ROS levels positively correlated with its inhibitory effect on chlorophyll breakdown. However, GA_3 application after ROS accumulation inhibited *PeWRKY*6-1 and *PeSAG*12-1 gene expressions, despite high ROS levels in the tissue. Taken together, our results suggest that GA_3 acts to inhibit leaf senescence of *Pelargonium*, probably not only by reducing ROS levels, but also by interfering with senescence regulation, through an as yet unknown mechanism.

Keywords: *Pelargonium* cuttings; Senescence-associated gene; Reactive oxygen species

1. Introduction

Dark-induced senescence occurs during storage and shipment of many agricultural products, including *Pelargonium* cuttings. Although not much is known about the mechanisms of dark-induced senescence, it shares some similarities with natural senescence [1–4].

Natural senescence is a genetically determined cell death process, characterized by chlorophyll breakdown and up-regulation of many senescence-associated genes (*SAG*s), which encode for signal molecules and transcription factors as well as for enzymes involved in the catabolism of macromolecules such as RNA, proteins and membrane lipids, and in the translocation of nutrients [3–6]. Recent studies, searching for senescence inducers, have suggested that transcription factors might induce senescence [4,7,8]. Members of the *WRKY* gene family could fulfill such a function [8]. Family members are defined by the DNA binding domain, WRKY, which is a 60-amino-acid domain with the conserved WRKYGQK motif at the N-terminal end that binds to a W box motif of target genes. The expression levels of *WRKY*4, *WRKY*6 and *WRKY*7 [8,9], and *WRKY*53 [10] increased during senescence, which supports

Abbreviations: GSH, glutathione; H_2DCF-DA, 2,7-dihydrodichlorofluorescein diacetate; EGO, eukaryote gene ortholog; ROS, reactive oxygen species; *SAG*, senescence-associated gene

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the notion that these transcription factors may be involved in senescence induction and/or progression. The importance of *WRKY*6 for senescence induction was highlighted by the expression of *SAG*s in WRKY6-overexpressing transgenic lines and, especially, by the discovery of senescence induced receptor-like kinase (SIRK) as a WRKY6 target [11].

Dark-induced senescence, like natural senescence, is characterized by chlorophyll breakdown and increase in catabolic processes [4,12]. The expression of cystein protease (*SAG*12) was increased during dark-induced senescence of mature leaves, as in natural senescence [13]. Recently, it was shown that the expression of *WRKY* family members, including *WRKY*6, increased at an early stage of the dark-induced senescence of a whole *Arabidopsis* plant [12]. In addition, several transcription factors that are activated during senescence are also activated by other stresses, possibly by increase in reactive oxygen species (ROSs) [8].

The chain of events controlling dark-induced senescence has not been studied and is not well understood. ROSs have been implicated in deterioration processes that occur during both natural and dark-induced senescence [14,15]. Lipid peroxidation products, resulting from ROS attack, were reported to increase gradually during natural senescence in tobacco leaves [16] and maize flag leaves [17], and also during dark-induced senescence of watercress and parsley leaves [18]. Treatments with antioxidants inhibited the natural senescence of wheat coleoptiles [19] and the dark-induced senescence of watercress [20] and *Pelargonium* cuttings [21].

In *Pelargonium* leaves, dark-induced senescence is manifested by reduced contents of starch, chlorophyll, total proteins and carbohydrates [22,23]. Gibberellic acid (GA_3), applied before dark treatment exposure, inhibited senescence of *Pelargonium* cuttings [22,24]. However, GA_3 did not prevent a reduction in carbohydrate levels [22].

Gibberellic acid also delayed leaf senescence in several species [25] such as *Taraxacum* [26], *Rumex* [27], *Hedra-Helix* [28], *Nasturtium* [29], lettuce [30], several herbs [31], Easter lily [32], hybrid lilies [33], and *Alstromeria* [34–36].

The mechanisms by which GA_3 delays dark-induced senescence are quite intriguing, because in contrast to its effect on leaves, it induced cell death in barley aleurone—an effect that involved down-regulation of ROS-scavenging enzymes, with subsequent ROS accumulation [37].

Dark-induced senescence of *Alstromeria* leaves was correlated with a decrease in non-13-hydroxylated gibberellins (GA_1, GA_4 and GA_9) [36], as was found also for general GA activity during dark-induced senescence in lettuce [30]. The effects of GA_3 on carbohydrate levels and on the photosynthetic activity during the dark-induced senescence of *Alstromeria* leaves were investigated [34,35]: GA_3 did not prevent a rapid fall in carbohydrate levels in *Alstromeria* leaves held in darkness, nor did it prevent the reduction of photosynthetic activity [34], despite its prevention of chlorophyll breakdown. Thus, the studies on *Alstromeria* suggest that gibberellins do not prevent the dismantling of the chloroplasts. As of now, the mode of action of gibberellin is still not clear and, moreover, the effect of GA_3 on ROS in leaves has not been investigated.

In the present study the effects of GA_3, applied either prior to or during dark treatment, on ROS levels have been examined by using the cell-permeating, radical-sensitive dye 2,7-dihydrodichlorofluoroscein diacetate (H_2DCF-DA). At the same time the effects of GA_3 on chlorophyll content and on the expression levels of *PeWRKY*6-1 and *PeSAG*12-1 were examined. The expression of these *SAG*s was determined by real-time PCR analysis, which has been proven to be a sensitive and reliable method for gene quantitation, especially of individual genes within a family [38]. The results show that GA_3 acts to inhibit leaf senescence of *Pelargonium*, probably not only by reducing ROS levels, but also by interfering with senescence regulation, through an as yet unknown mechanism.

2. Materials and methods

2.1. Plant material and treatments

All experiments were performed on freshly harvested *Pelargonium* (*Pelargonium hortorum* cv. 'Kira') cuttings that comprised two apparent internodes with two large leaves, two small leaves and an apical meristem. The cuttings were darkened at 21 °C in a moist atmosphere of 99% RH. All measurements were performed on leaves from the bottom of the cutting, on consecutive days during the dark treatment, or as indicated.

The cuttings were sprayed before or during the dark period with solutions containing gibberellic acid (GA_3) or glutathione (Sigma, Israel). In most experiments the GA_3 concentration was $(1–5) \times 10^{-5}$ M, as indicated. GSH was used at 10 mM, which had been found to be the optimal concentration for ROS reduction, in preliminary experiments with concentrations ranging from 1 to 50 mM. All materials were applied with 0.01% Triton X100 as a surfactant, and the controls contained Triton only. During the dark period materials were applied to *Pelargonium* cuttings under a green safelight.

2.2. Chlorophyll determination

Chlorophyll was extracted from five randomly cut discs from each *Pelargonium* cutting (each disc was 5 mm in diameter and weighed about 10 mg). The discs were boiled for 30 min in 1 ml of 80% (v/v) ethanol. Chlorophyll was determined in 0.4 ml of the extract that had been transferred to 5 ml of acetone, by measuring absorbance at 645 nm for Chl a and at 663 nm for Chl b, according to [20]. Each data point represents an average of four or five cuttings and is expressed in micrograms per disc, to avoid the effect of weight loss during senescence.

2.3. Cloning of PeWRKY6-1 and PeSAG12-1 and quantification of their expression

*PeWRKY*6-1 and *PeSAG*12-1 genes were cloned from *Pelargonium* cuttings by RT-PCR, with degenerate primers