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Research article

Altered apoplastic ascorbate redox state in tobacco plants via ascorbate oxidase overexpression results in delayed dark-induced senescence in detached leaves

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ABSTRACT

Ascorbate oxidase (AO) is an apoplastic enzyme that uses oxygen to catalyse the oxidation of ascorbate (AA) to dehydroascorbate (DHA) via the unstable radical monodehydroascorbate (MDHA). Here, we report that transgenic tobacco plants (*Nicotiana tabacum* L. cv. *Xanthi*) with an *in vivo* lowered apoplastic AA redox state through increased AO expression demonstrate signs of delayed dark-induced senescence compared with wild-type plants, as shown by chlorophyll loss assay. *In situ* localization of hydrogen peroxide (H_2O_2) suggests that, although transgenic plants have higher constitutive levels of H_2O_2 under normal growth conditions, imposed dark-induced senescence results in smaller induction levels of H_2O_2, an observation which correlates with increased antioxidant enzyme activities and an induction in the expression of AA recycling genes compared with that in wild-type plants. Our current findings, combined with previous studies which showed the contribution of AO in the regulation of AA redox state, suggest that the reduction in AA redox state in the leaf apoplast of these transgenic plants results in an increase in the endogenous levels of H_2O_2, which provides a form of 'acquired tolerance' to oxidative stress imposed by dark-induced senescence.

1. Introduction

Senescence is a normal and even essential feature of the post-mitotic phase of the plant cell life cycle and is directly linked to declining viability [1]. Despite the importance of senescence, current knowledge of the regulatory mechanisms of this process is still rather limited. Leaf senescence, which is the most extensively studied senescence process in plants, occurs as part of the normal developmental program and is controlled by a wide array of external and internal factors such as age, plant hormone levels and environmental stress factors [2]. Upon the onset of senescence, shifts in gene expression lead to the degradation of macromolecules such as chlorophyll and nucleic acids, followed by the reallocation of nutrients, the breakdown of organelles and, ultimately, cell death [3]. Such molecule degradation is partly the result of a strong enhancement in the generation of reactive oxygen species (ROS), which also play an important signalling role during leaf senescence [4]. However, plants have developed a complex network of enzymatic and non-enzymatic low-molecular-weight antioxidative components acting in different cellular compartments in order to regulate ROS homoeostasis [5].

Ascorbate (AA) is the most abundant antioxidant in plants and serves as the major contributor to cell redox state [6]. It is also considered to be the most abundant antioxidant in the apoplast, comprising up to 5% of the leaf's ascorbate pool [7]. Apoplastic AA is thought to represent the first line of defence against potentially damaging external oxidants, and may play an important role in mediating response to stresses generating an enhanced oxidative burden [8]. In the apoplast, ascorbate oxidase (AO; a glycoprotein belonging to the family of blue copper oxidase enzymes) oxidises AA to the unstable radical monodehydroascorbate (MDHA), which rapidly disproportionates to yield dehydroascorbate (DHA) and AA [9]. Alternatively, MDHA is probably reduced by a plasma-membrane cytochrome *b* system [10].

Ascorbate oxidase expression is regulated by oxidative stress and hormones [11,12], with AO activity being proportional to light intensity [11,13]. In addition, AO transcripts are highly expressed in roots and young fruits [12,14], while transgenic tomato plants with

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suppressed *AO* expression exhibited increased AA accumulation in fruit [15] as well as increased fruit yield under conditions where assimilate became limiting for wild-type plants such as when leaves were removed [16]. Interestingly, AO was recently attributed a new role as high AO expression was shown to act as a possible strategy to down-regulate oxygen diffusion in root nodules containing nitrogen-fixing bacteria as well as during symbiosis with arbuscular mycorrhizal fungi [17].

Another important function of AO is the regulation of AA redox status, which is crucial in controlling plant response to environmental conditions as plants can sense shifts in the amount and redox state of AA [18] and this may be important in the perception of potentially stressful situations as well as in the modulation of compensatory defence responses [19]. For example, Sanmartin et al. [20] showed that tobacco plants overexpressing AO are characterized by the oxidation of both the apoplastic and symplastic AA pool, while similar lowering of apoplastic AA redox state was reported by Pignocchi and colleagues [11]. The apoplast may play an important role in mediating responses to abiotic stresses because the initial events most likely occur at the apoplasm–plasmalemma interface [8]. In line with these suggestions, transgenic tobacco plants with lower apoplastic AA redox state via AO overexpression appear to show increased sensitivity to ozone [20] and to various agents imposing oxidative stress which correlated with the suppression of genes involved in AA recycling [21]. Contrarily, suppression of *AO* expression resulted in increased tolerance to salinity stress [22]. Interestingly, AO overexpressing tobacco plants exhibited elevated endogenous levels of hydrogen peroxide (H_2O_2) and a decline in H_2O_2-scavenging enzymatic activity, resulting in partial stomatal closure and subsequent reduced rates of water loss in detached leaves [23].

In the present study, transgenic tobacco plants over-expressing cucumber AO were used to probe the effect of altered apoplastic AA redox state on the capacity exhibited by plant tissue to detoxify the reactive oxidative burden created by dark-induced senescence. Transgenic AO over-expressing tobacco plants suffered reduced damage as indicated by chlorophyll breakdown, suggesting that they show delayed senescence. No distinct signs of programmed cell death were detected in any of the samples, although Trypan Blue staining revealed a higher capacity for WT plants subjected to dark-induced senescence to fix the dye compared with AO transgenic plants. The delayed entry of AO transgenic plants to dark-induced senescence correlated with lower induction levels of H_2O_2, increased activity of antioxidant enzymes and an induction in the expression of AA recycling genes compared with that in wild-type plants.

2. Results

2.1. Effect of dark-induced senescence on chlorophyll content

The effect of dark-induced senescence in wild-type and AO over-expressing transgenic tobacco plants was examined by estimating chlorophyll breakdown levels in treated leaf discs. Macroscopic observation revealed darker green colouration (in web version) of transgenic tobacco leaf discs compared with WT controls (Fig. 1A), accompanied by significantly lower chlorophyll loss levels (Fig. 1B), thus suggesting a delayed entrance in dark-induced senescence.

2.2. Programmed cell death assays

The induction of programmed cell death (PCD) by dark-induced senescence was studied using different approaches. Genomic DNA integrity was examined, as DNA fragmentation is a common indication of programmed cell death occurring in senescing tissue. No distinct DNA laddering was observed in any of the samples (Fig. 2), which is indicative of the absence of PCD symptoms. Staining with Trypan Blue dye was also used as a marker of cell death. Wild-type plants subjected to dark-induced senescence showed a higher capacity to fix the dye in comparison with AO transgenic plants (Fig. 3B), as further evidenced following macroscopic observation (Fig. 3A). These results suggest that dark-induced senescence could cause damage in leaf cells which could lead to cell death although it cannot be concluded that dark-induced senescence leads to PCD.

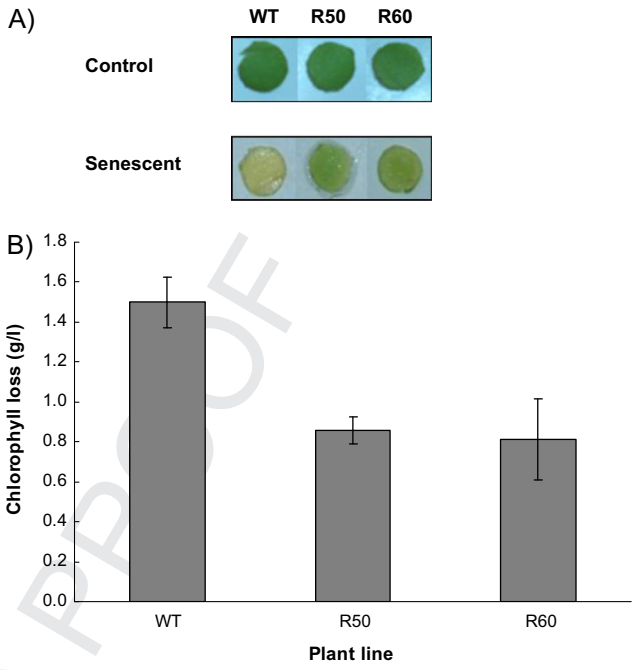

Fig. 1. Effect of dark-induced senescence on WT and R50, R60 transgenic lines. (A) leaf discs following 6 d incubation on dH_2O in the dark. (B) chlorophyll loss to indicate damage levels in dark-induced senescent leaf discs in comparison with control samples. Bars represent SE ($n = 3$).

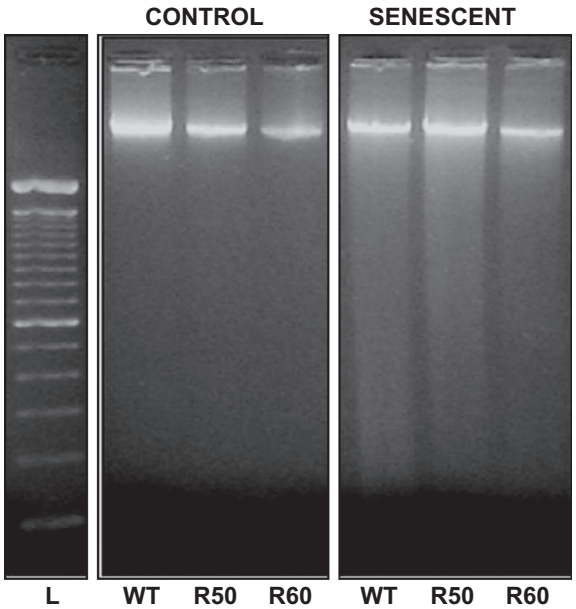

Fig. 2. DNA laddering of wild-type and AO over-expressing leaves subjected to dark-induced senescence. Three μg genomic DNA was extracted and electrophoresed on a 2% (w/v) agarose gel.

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