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Short communication

Changes in lipid peroxidation in stay-green leaves of tobacco with senescence-induced synthesis of cytokinins

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ABSTRACT

The involvement of reactive oxygen species (ROS) in the progress of leaf senescence has long been suggested, but there are contrasting results to either support or deny the positive correlation between the senescence progression and the level of ROS-triggered lipid peroxidation. The inconsistency among reported results can partly be attributed to the poor specificity of the most commonly employed colorimetric assay and changes in the ratio of dry weight/fresh weight during leaf senescence. In this study we determined the end-product of lipid peroxidation malondialdehyde (MDA) by GS-MS, and analyzed its changes during senescence of tobacco leaves as calculated on dry weight basis. In leaves of the wild type plants the MDA level did not change during senescence. In the mutant P_{SAG12}::*IPT* leaves stayed green because of the elevated synthesis of cytokinins, but the MDA level was much higher in comparison to WT when leaves of the same age were compared. These results clearly show that lipid peroxidation is not associated with leaf senescence, at least in tobacco. This GS-MS method can be used to judge the involvement of lipid peroxidation in senescence in other species.

1. Introduction

The main function of leaves is to perform photosynthesis and to provide assimilates for growth of plant. Leaves can also be considered as storage organs because they represent a big investment in the organic matter which is partly recycled during senescence. Before the death of a leaf an intensive decomposition of cellular proteins, lipids and carbohydrates takes place. The recovered nutrients are reallocated to the other, still growing parts of plant or to storage tissues (Gregersen et al., 2013). The fatty acids liberated from membranes after lipolytic reactions are subjected to peroxidation initiated by a multiple isoforms of lipoxygenase or reactive oxygen species (ROS). The most abundant final product of ROS-triggered lipid peroxidation is malondialdehyde (MDA) and from this reason it is commonly used as a cellular stress marker (Bhattacharjee, 2014).

Accumulating evidences suggest, that the senescence program is promoted by an age- or stress-dependent disturbance in the production and scavenging of ROS. As documented in leaves of the annual *Pisum sativum*, leaf senescence was correlated with an increase in ROS and with a gradual decline in antioxidants (Vanacker et al., 2006). Such a trend was also observed in perennial plant *Cistus clusii,* when leaves of different age were compared (Munne-Bosch and Alegre, 2002). During the late stages of seasonal leaf senescence of birch and oak Berger et al. (2001) detected high amounts of hydroxy fatty acids originating from lipid peroxidation. Also, an increased concentration of H_2O_2 was demonstrated in concert with a decline of activities of catalase, ascorbate peroxidase and dehydroascorbate reductase, as well as ascorbate and glutathione content during senescence of *Pisum* and *Arabidopsis* leaves (Vanacker et al., 2006; Zimmermann and Zentgraff, 2005; Zimmermann et al., 2006). In agreement with that, several senescence-associated genes and senescence regulators, such as a transcription factor *ORS1* and *ATAF1*, appeared to be H_2O_2-regulated (Balazadeh et al., 2011; Garapati et al., 2015). By these and other studies, ROS have been considered as trigger of the senescence program (Wang et al., 2013a; Zimmermann and Zentgraff, 2005).

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However, several other studies indicate that the correlation between senescence and the level of ROS leading to oxidative damage is not as obvious. Vanacker et al. (2006) showed that the increase of H_2O_2 concentration in senescing pea leaves was not necessarily linked with increased oxidative damage reflected by peroxidation of membrane lipids. Moreover, no clear correlation between the level of ROS and the extent of lipid peroxidation was noted for senescent leaves of barley (Jajić et al., 2015) except at the last stage, when the highest levels of ROS and lipid peroxidation were detected. Moreover, a non-uniform pattern of senescence of the tobacco leaf (with delayed senescence of veins) was rather negatively correlated with the spatial distribution of ROS (Niewiadomska et al., 2009). A comparison of several plant species made by Wang et al. (2013b) did not reveal any clear rule regarding the extent of MDA in senescing versus non-senescing leaves. These results rather indicate that an increased concentration of certain ROS may even protect against senescence by triggering defense responses.

It is proposed that for a substantial part of leaf senescence an excessive ROS formation has to be prevented to enable an efficient remobilization of nutrients and to avoid a premature cell death (Hörtensteiner and Kräutler, 2011). This is achieved by a removal of chlorophyll and its photo-reactive catabolites. Such strategy would prevent oxidative damage of leaf tissue assuming that it is correlated with the level of ROS. On the other hand, oxidative processes are necessary for degradation of cellular components (Vanacker et al., 2006).

A goal of this study was to evaluate the extent of lipid peroxidation induced by ROS during leaf senescence. To verify this we used the two types of leaf senescence. The first type was represented by a naturally-aging and yellowing leaves of tobacco, and the second by leaves which retain the chlorophyll until the late stages of leaf development (so called 'stay-green' phenotype). For stay-green phenotype we chose a transgenic P_{SAG12}::*IPT* plants in which a chlorophyll degradation is prevented due to the senescence-induced auto-regulated synthesis of cytokinins (Gan and Amasino, 1995). The concentration of MDA, reflecting the extend of lipid peroxidation, was evaluated by employing a highly specific and sensitive GC-MS-based method.

2. Materials and methods

2.1. Plant material

Seeds of *Nicotiana tabacum* L. cv. Wisconsin (wild type, WT) and homozygous transgenic P_{SAG12}::*IPT* plants were obtained from Dr Richard Amasino, University of Wisconsin, USA. For analysis of leaf senescence plants were grown in a greenhouse supplied with additional illumination by HS2000 lamps to 15 h per day at an average intensity of 450 μmol quanta m^{-2} s^{-1}. The temperature in the light period varied in the range from 20 to 30 °C, while during the dark period the temperature was adjusted to 18 °C. The phenotype of the transgenic plants was comparable to previously reported by Gan and Amasino (1995). Leaf fragments (interveinal parts from the middle part of a leaf blade) representative for a different developmental stages, were collected from plants after four months of growth, immediately frozen in liquid nitrogen and kept at −80 °C for further analysis.

Developmental stages of WT leaves were recognized by an eye observation of leaf color and arrangement of leaves on a stem, such as: young (Y) – not fully developed leaves, mature (M) – fully developed with no signs of senescence, and three stages of senescence – S1 leaves with green color prevailing, S2 leaves with yellow color prevailing and S3 with no green color visible. For P_{SAG12}::*IPT* plants leaves corresponding to WT (the same position on stem) were collected (suppl. Fig. 1).

For HL treatment WT tobacco plants were grown in the climatic chamber in conditions of 15 h day at an average intensity of 300 μmol quanta m^{-2} s^{-1}. Eight weeks after sowing discs were excised from interveinal parts of the mature leaves and subjected to: 300, 1000 or 2000 μmol quanta m^{-2} s^{-1} for 1 h. High irradiance was obtained with LED light source SL3500-W-B attached to the light controller LC100 (Photon Systems Instruments, Brno, Czech Republic).

2.2. MDA determination

Sample preparation and detection of MDA was performed essentially after Cighetti et al. (1999) with some modifications. Frozen leaves (100 mg) were ground in liquid nitrogen and lyophilized. Leaf powder was mixed with 50 μL 0.05% (w/v) butylated hydroxytoluene and 1.5 mL 0.1 M HCl and vortexed for 2 min. After centrifugation (10 min at 6000 rpm) supernatant (1 mL) was collected. Subsequently, 100 μL of 13.8 mM phenylhydrazine hydrochloride (PH, $C_6H_5NHNH_2 \cdot HCl$) was added, vortexed for 1 min and heated at 75 °C for 30 min. After cooling to RT, 100 μL of pure chloroform was added, samples were sonicated for 1 min, vortexed for 2 min and allowed to phase separation. After centrifugation (10 min at 6000 rpm) chloroform layer was carefully collected (50 μL), quickly evaporated under nitrogen stream, sealed and stored. Immediately before measurement, 30 μL of chloroform was added to the vials and gas chromatography-mass spectrometry (GC-MS) analyses were carried out on a 7890 A GC system equipped with 7692 A ALS auto-sampler coupled to a 7000 MS/Triple Quad mass spectrometric detector (Agilent Technologies, Palo Alto, USA). The chromatographic capillary column HP-5MS was used. The mass spectrometer was manually tuned using perfluorotributylamine with the masses *m/z* 69.219 and 502. Helium was the carrier gas at a constant flow rate of 1 mL min^{-1}. The operating parameters for the GC were as follows: injector temperature 250 °C, source temperature 280 °C, MS transfer line 300 °C and quadrupoles temperature 150 °C. The initial GC oven temperature was 80 °C (1.2 min hold) and increased at a rate of 25 °C min^{-1} to 250 °C (2 min hold). Injections were done in the split mode with the split ratio 10:1. The organic layer (3 μL) was injected. Standard electron impact conditions were used (70 eV). For Collision induced dissociation (CID), ultra-high purity nitrogen was used (flow rate a 1.5 mL min^{-1}) and 25 V as collision cell voltage. To eliminate the metastable helium species, helium gas (2.25 mL min^{-1}) was used as a quench gas. The MDA-PH derivative was detected and quantified using multiple reaction monitoring mode (MRM). Four transitions were used for the detection and quantification of MDA-PH derivative: 144.1 → 143.1, 144.1 → 117 (qualifiers) and 144.1 → 90, 144.1 → 77 (quantifiers). The chromatographic data were analyzed by Agilent Technologies MassHunter data analysis reporting software ver.B07 SP2. Calibration was done with 100 mg of 1,1,3,3-tetraethoxypropane (MDA-precursor) dissolved in 1 mM HCl. The intensity of MDA-PH peak was correlated with the concentration of 1,1,3,3-tetraethoxypropane (Havaux et al., 2005). Recovery of MDA amounted to 88.76%.

2.3. Determination of chlorophyll and soluble proteins

Chlorophyll was extracted and determined in 80% acetone with the addition of $MgCl_2$, according to Lichtenthaler and Buschmann (2001). Leaf soluble proteins were extracted in 0.1 M phosphate buffer pH 7.0 supplemented with protease inhibitor cocktail (Sigma-Aldrich), according to Wiciarz et al. (2015). Protein concentration was determined using Roti – Nanoquant Protein quantitation assay (Carl Roth).