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Research paper

Nucleases activities during French bean leaf aging and dark-induced senescence

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ABSTRACT

During leaf senescence resources are managed, with nutrients mobilized from older leaves to new sink tissues. The latter implies a dilemma in terms of resource utilization, the leaf senescence should increase seed quality whereas delay in senescence should improve the seed yield. Increased knowledge about nutrient recycling during leaf senescence could lead to advances in agriculture and improved seed quality. Macromolecules mobilized during leaf senescence include proteins and nucleic acids. Although nucleic acids have been less well studied than protein degradation, they are possible reservoirs of nitrogen and phosphorous. The present study investigated nuclease activities and gene expression patterns of five members of the S1/P1 family in French bean (*Phaseolus vulgaris* L. cv.)Page: 2 during leaf senescence. An in-gel assay was used to detect nuclease activity during natural and dark-induced senescence, with single-stranded DNA (ssDNA) used as a substrate. The results revealed two nucleases (glycoproteins), with molecular masses of 34 and 39 kDa in the senescent leaves. The nuclease activities were higher at a neutral than at an acidic pH. EDTA treatment inhibited the activities of the nucleases, and the addition of zinc resulted in the recovery of these activities. Both the 34 and 39 kDa nucleases were able to use RNA and double-stranded DNA (dsDNA) as substrates, although their activities were low when dsDNA was used as a substrate. In addition, two ribonucleases with molecular masses of 14 and 16 kDa, both of which could only utilize RNA as a substrate, were detected in the senescent leaves. Two members of the S1/P1 family, *PVN2* and *PVN5*, were expressed under the experimental conditions, suggesting that these two genes were involved in senescence. The nuclease activity of the glycoproteins and gene expression were similar under both natural senescence and dark-induced senescence conditions.

1. Introduction

Leaf senescence, the final stage in leaf development, is a highly regulated and complex process which ends with leaf degradation (Woo et al., 2013). The age of the plant, in addition to highly coordinated endogenous and external signalling mechanisms, regulates leaf senescence (Sarwat et al., 2013). The concept of a senescence window evolved to explain how hormones or external factors affect the onset of senescence (Schippers et al., 2015). This concept assumes that senescence involves three phases: a) a growth phase or never-senescent phase; b) a maturation phase, in which the leaf becomes sensitive to internal or external factors and the activation of senescence and c) a senescence phase, in which senescence is induced even under optimal growth conditions (Schippers et al., 2015). External signals that influence senescence include abiotic and biotic environmental factors. The most important abiotic factors are drought, nutrient limitation, extreme temperatures, oxidative stress, UV-B irradiation and ozone (Lim et al., 2007). In perennial plants, foliar senescence is a very effective strategy when unfavourable conditions reduce photosynthetic activity and leaf loss is profitable (Lim et al., 2007). Biotic factors that influence senescence include pathogen infections and competition for light (Lim et al., 2007; Sakamoto and Takami, 2014). A high concentration of carbohydrates, high carbon accessibility and reduced leaf nitrogen content also regulate the onset of senescence (Wingler et al., 2006).

The changes that occur in plant physiology, biochemistry and gene expression during senescence are considered a type of programmed cell death (PCD) (Lopez-Fernandez et al., 2015). PCD progresses in a sequential manner from the cells surrounding the leaves to cells located at the basis (Lim et al., 2007). This sequential process, together with the maintenance of the vascular system until the final phase of senescence, allows efficient mobilization of nutrients derived from the degradation of macromolecules during PCD. Such mobilization of nutrients, which

Abbreviations: PCD, programmed cell death; DAI, days after start imbibition; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA

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are transported via phloem to developing tissues or seeds, is crucial for the development and survival of the plant (Cholewa and Griffith, 2004). Chloroplast degradation is the earliest PCD event in leaves (Lim et al., 2007). Mitochondria and nuclei remain intact until the final phase of PCD to ensure a sufficient energy supply and gene expression (Lim et al., 2007). The disintegration of the chloroplast interrupts photosynthetic activity, thereby reducing anabolism and resulting in the release of 70% of leaf nitrogen and Rubisco (Hörtensteiner and Feller, 2002). The chlorophyll and proteins of chloroplasts are transported to the vacuoles, probably to avoid cell phototoxicity caused by the accumulation of pigments, where complete hydrolysis occurs due to the action of *endo*- and exopeptidases (Christ et al., 2012). Amino acids are released as a result of protein catabolism by the action of transaminases and glutamate dehydrogenase (Masclaux-Daubresse et al., 2010). These then produce ammonium, which is assimilated by glutamine synthetase (Masclaux-Daubresse et al., 2010). Subsequent degradation of membrane lipids results in the release of fatty acids, which are oxidized to produce energy or transformed into α-ketoglutarate by the glyoxylate cycle. This α-ketoglutarate is converted into sugars by glycogenesis or used to mobilize amino acids released after the hydrolysis of proteins (Lim et al., 2007; Sakamoto and Takami, 2014). Once Rubisco has been degraded and catabolism of membrane proteins and lipids has occurred, senescence is irreversible. Thus, the protein and chlorophyll concentration are considered the main markers of leaf senescence (Sato et al., 2007). Genes encoding glutamine synthetase and glutamate dehydrogenase are also used as markers of senescence (Pageau et al., 2006). Permeases, which are involved in the incorporation of amino acids in phloem, are induced during leaf senescence in *Brassica* and *Arabidopsis* (Masclaux-Daubresse et al., 2010).

Nucleic acid degradation is another cellular change characteristic of leaf senescence (Lim et al., 2007). During nucleic acid degradation, RNA levels rapidly decline. Degradation occurs in two phases. In the first phase, rRNAs from chloroplast and cytoplasm are degraded, whereas cytoplasmic mRNA and tRNA are degraded in the second phase (Lim et al., 2007). The decrease in the level of RNA is accompanied by an increase in the activity of ribonucleases (Lim et al., 2007). As senescence progresses, controlled collapse of the vacuole takes place, leading to degradation of the nucleus. Condensation of nuclear chromatin by endonucleolytic hydrolysis of nuclear DNA is a characteristic of cells undergoing PCD, resulting in a laddering structure of DNA (Domínguez and Cejudo, 2012). The degradation of nucleic acids of nuclei or organelles during PCD leads to the release of nucleotides, bases of purines and pyrimidines, all of which could be mobilized and transported to sink organs, where they provide a source of nitrogen and phosphate. Little is known about the degradation process of nucleic acids in plants (Sakamoto and Takami, 2014). Improved knowledge of nutrient remobilization could have agronomic implications. For example, maximizing nutrient remobilization from senescing leaves would aid crop production efficiency by minimizing the use of unsustainable and contaminating fertilizers in agriculture. The use of nitrate as fertilizer is responsible for a high percentage of greenhouse gas, which poses an environmental risk (Have et al., 2017). Extensive use of phosphorous in agriculture is also a problem, as rock phosphate reserves are limited (Veneklaas et al., 2012; Stigter and Plaxton, 2015).

Nucleases are classified as endonucleases or exonucleases according to their enzymatic properties, although most nucleases identified so far are endonucleases (Sakamoto and Takami, 2014). Endonucleases are categorized according to their catalytic divalent cations as zinc (Zn)-dependent (type I) or calcium-dependent (type II), depending on the optimum pH for their activity or their similarity to S1 nucleases or staphylococcal nucleases, (Sakamoto and Takami, 2014). In French bean, the S1 family is composed of five members, two of which are induced during cotyledon senescence (Lambert et al., 2016). We previously demonstrated the induction of a Zn-dependent nuclease in French bean seedlings, with maximal activity at an acidic pH (Lambert et al., 2014). This activity was coincident with the accumulation of ureides (Quiles et al., 2009) and the induction of nucleotidases (Cabello-Díaz et al., 2012, 2015). Based on the aforementioned findings, we postulated that nucleic acids might serve as nutrient storage compounds during seedling development (Lambert et al., 2014, 2016). Ureides could be used to mobilize some of these nutrients. Ureides occur ubiquitously in plants as an intermediary metabolite of purines, which can be derived from nucleotides synthesised *de novo* or produced by nucleic acid degradation. In tropical legumes, ureides are the predominant form of nitrogen in xylem of plants under nitrogen fixation conditions (Smith and Atkins, 2002). In non-legume plants, purine catabolism is thought to function as a route for nitrogen recycling and remobilization, although this has not been demonstrated experimentally. The role of ureides and amino acids in mobilization from source to sink tissues was reviewed recently (Tegeder, 2014). Several studies revealed that ureides accumulated in plants subjected to various types of abiotic stress possibly playing a role in protecting plants against the effects of reactive oxygen species (Brychkova et al., 2008; Irani and Todd, 2016; Malik et al., 2016).

Light plays a crucial role in leaf senescence, with shading of leaves leading to senescence. Improved knowledge of processes that occur in dark-induced senescence can be expected to have agronomic benefits, as senescence affects crop yield and post-harvest shelf life (Liebsch and Keech, 2016). Previous research showed that the induction of senescence by light deprivation was the result of carbon starvation in leaves due to reduced photosynthesis (Liebsch and Keech, 2016). The same study demonstrated that the absence of photosynthesis-independent light signals suppressed senescence and that phytochromes and phytochrome-interacting factors acted as direct and indirect regulators of genes induced during senescence (Liebsch and Keech, 2016). A complete transcriptome analysis of *Arabidopsis* revealed differences in gene expression between natural leaf aging and dark-induced senescence, both in detached leaves and in individual leaves attached to the whole plant (van der Graaff et al., 2006).

The aim of the present study was to analyse the nuclease activities during leaf senescence in French bean (*Phaseolus vulgaris* L. cv.), as well as the expression patterns of five members of the S1/P1 family. In addition, we compared nuclease gene expression and activity in natural leaf aging with dark-induced senescence. Furthermore, we compared two mechanisms of dark-induced senescence in detached leaves and individual leaves attached to the whole plant.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of the French bean (*Phaseolus vulgaris* L. cv. Great Northern) were sterilized and germinated as indicated in an earlier study (Lambert et al., 2016). Seedlings were sown in vermiculte:perlite (3:1, w/w)-containing pots (diameter: 16 cm; height: 18 cm) three days after the start of imbibition (DAI). The plants were cultured in a growth chamber and watered every three days with 4 times diluted culture medium (Harper and Gibson, 1984) and enriched with 10 mM KNO_3 as a nitrogen source. In the natural senescence experiments, plant material was collected at 25, 30, 35, 38, 42 and 45 days after start of imbibition, frozen in liquid nitrogen and stored at −80 °C until further analysis. In the dark-induced senescence experiments, the plant material was treated as described in Section 2.2.

2.2. Dark-induced senescence

Senescence was induced by subjecting the first trifoliate leaf of French bean plants to total darkness using two different experimental approaches, as described below:

In the first approach, leaves were excised from plants at 25 DAI using a sterile blade, placed in 120 mm petri dishes (one leaf per plate) containing three wet paper discs at the base and covered by a fourth