Journal of Plant Physiology 212 (2017) 29–44

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Original article

Analysis of nodule senescence in pea (*Pisum sativum* L.) using laser microdissection, real-time PCR, and ACC immunolocalization

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ARTICLE INFO

Article history:
Received 15 August 2016
Received in revised form
23 December 2016
Accepted 2 January 2017
Available online 17 February 2017

Keywords:
Rhizobium-legume symbiosis
Nodule
Cysteine protease
Ethylene
Abscisic acid
Gibberellins

ABSTRACT

A delay in the senescence of symbiotic nodules could prolong active nitrogen fixation, resulting in improved crop yield and a reduced need for chemical fertilizers. The molecular genetic mechanisms underlying nodule senescence have not been extensively studied with a view to breeding varieties with delayed nodule senescence. In such studies, plant mutants with the phenotype of premature degradation of symbiotic structures are useful models to elucidate the genetic basis of nodule senescence. Using a dataset from transcriptome analysis of *Medicago truncatula* Gaertn. nodules and previous studies on pea (*Pisum sativum* L.) nodules, we developed a set of molecular markers based on genes that are known to be activated during nodule senescence. These genes encode cysteine proteases, a thiol protease, a bZIP transcription factor, enzymes involved in the biosynthesis of ethylene (*ACS2* for ACC synthase and *ACO1* for ACC oxidase) and ABA (*AO3* for aldehyde oxidase), and an enzyme involved in catabolism of gibberellins (GA 2-oxidase). We analyzed the transcript levels of these genes in the nodules of two pea wild-types (cv. Sparkle and line Sprint-2) and two mutant lines, one showing premature nodule senescence (E135F (*sym13*)) and one showing no morphological signs of symbiotic structure degradation (Sprint-2Fix$^-$ (*sym31*)). Real-time PCR analyses revealed that all of the selected genes showed increased transcript levels during nodule aging in all phenotypes. Remarkably, at 4 weeks after inoculation (WAI), the transcript levels of all analyzed genes were significantly higher in the early senescent nodules of the mutant line E135F (*sym13*) and in nodules of the mutant Sprint-2Fix$^-$ (*sym31*) than in the active nitrogen-fixing nodules of wild-types. In contrast, the transcript levels of the same genes of both wild-types were significantly increased only at 6 WAI. We evaluated the expression of selected markers in the different histological nodule zones of pea cv. Sparkle and its mutant line E135F (*sym13*) by laser capture microdissection analysis. Finally, we analyzed ACC by immunolocalization in the nodules of both wild-type pea and their mutants. Together, the results indicate that nodule senescence is a general plant response to nodule ineffectiveness.

1. Introduction

Symbiotic nodules develop as a result of the interaction between legumes and soil bacteria that are collectively known as rhizobia. Rhizobia trigger root hair deformations and curling and can penetrate inside the root tissue through a specialized structure known as the infection thread (Brewin, 2004). Simultaneously, cortical cell divisions are reactivated, and a nodule primordium forms (Timmers et al., 1999). The infection thread penetrates the nodule primordium, forming specialized unwalled outgrowths known as infection droplets, from which rhizobia are released into the host cell cytoplasm, enclosed by the peribacteroid (symbiosome) membrane (Brewin, 2004). After release, the bacteria differentiate into bacterioids, a specialized form for nitrogen fixation. The symbiosome is the structure that consists of bacteroids enclosed by the peribacteroid membrane (Brewin, 2004). There are two main types of nodule ontogenesis: determinate and indeterminate. In determinate nodules, meristematic activity is transient and cells of the central tissue are at similar developmental stages. In indeterminate nodules, meristematic activity is persistent and infection threads continually colonize cells leaving the meristem. Thus, in indeterminate nodules there are different stages of differentiation, and

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http://dx.doi.org/10.1016/j.jplph.2017.01.012
0176-1617/

the central tissue can be subdivided into the meristem, infection, nitrogen fixation, and senescence zones (Guinel, 2009).

Senescence is the final stage of symbiotic nodule development in which cells of both symbiotic partners decay. During the process of senescence, the color of the nodule changes from pink to green. At the ultrastructural level, there are alterations in the symbiosomes and the organelles, notably the electron density of the cytoplasm decreases. As a result of the collapse of the host cells and symbiosomes, numerous vesicles and membrane ghosts are observed (Borisov et al., 1997; Morzhina et al., 2000). Functionally, senescence is a remobilization of nutrients from the nodule to growing plant organs (Puppo et al., 2005; Serova and Tsyganov, 2014), and protein degradation is probably the most important process occurring, as it allows the recycling of nitrogen and other substances. Therefore, many protease genes are up-regulated during senescence, especially those encoding the most active proteases, the cysteine and threonine proteases (Pladys et al., 1991; Granell et al., 1992; Kardailsky and Brewin, 1996; Van de Velde et al., 2006; Guerra et al., 2010). The transcriptome analysis of *Medicago truncatula* Gaertn. nodules of different ages revealed three groups of genes corresponding to three subsequent stages of nodule aging: (i) early stage (absence of visible signs of senescence), (ii) stage I (the destruction of bacteroids) and (iii) stage II (the degradation of bacteroids and host cells) (Van de Velde et al., 2006). The regulatory genes active in the onset of senescence were combined in the first cluster. Genes in the second and third clusters are involved in the stages accompanied by the death of bacteroids and the degradation of the infected cells, respectively. Both of these clusters contain potential regulatory and signal transduction genes and genes encoding representatives of the five protease families. The induction of genes that encode proteins involved in the transport of a wide range of molecules (ATP-binding proteins and specific phosphate, amino acids and metal ion transmitters) suggests that the degradation of macromolecules and the mobilization functions are closely related. These catabolic events related to transport processes convert a nodule from a carbon sink to a source of nutrients for the plant (Van de Velde et al., 2006; Guerra et al., 2010).

Nodule senescence is regulated transcriptionally, and three transcription factors involved in this process have been identified to date. The expression of *MtATB2*, which encodes a bZIP transcription factor, was shown to increase in senescing nodules of *M. truncatula*. *MtATB2* transcription is regulated by sucrose and light, and MtATB2 is involved in regulating the transcription of genes related to amino acid metabolism (D'haeseleer et al., 2010). Another transcription factor, MtNAC969, which represses genes that are activated in roots under salt stress, is also involved in nodule senescence, because RNA silencing of this gene led to early nodule senescence (De Zélicourt et al., 2012). The transcription factor MtNAC920, which is activated by ethylene, up-regulates a cysteine protease gene, *MtCP2*, the overexpression of which triggered nodule senescence (Karmarkar, 2014). Nodule senescence is also regulated by host plant hormones. Ethylene activates nodule senescence, as illustrated by the up-regulation of ethylene response factors (ERFs) and genes encoding the ethylene biosynthesis enzymes S-adenosylmethionine (SAM) synthetase and 1-aminocyclopropane-1-carboxylate (ACC) oxidase (Van de Velde et al., 2006) during nodule senescence. As shown recently, ethylene may act as a second messenger in the regulation of symbiosome development and nodule senescence (Karmarkar, 2014). Abscisic acid (ABA) biosynthesis is also triggered during nodule senescence, alongside a decrease in both the ascorbate-glutathione antioxidant pool and the carbon-to-nitrogen ratio. In pea nodules, the ABA content is relatively high during the first two weeks, but then it reaches a plateau, and increases again during the final developmental stages (Charbonneau and Newcomb, 1985). Key proteases (cysteine proteases and 26S proteasome) that are intrinsic to senescence are activated by ABA (Puppo et al., 2005). In contrast to ethylene and ABA, bioactive forms of gibberellins (GAs) may suppress nodule senescence, because the transcript levels of the gene encoding GA 2-oxidase (GA 2β-hydroxylase), which converts the active phytohormone into its inactive form, were found to increase during aging of *M. truncatula* nodules (Thomas et al., 1999; Van de Velde et al., 2006).

There are numerous mutants in different legumes with an early senescence phenotype; i.e., premature degradation of the symbiotic compartments. Certain genes, the mutations of which lead to the initiation of senescence, have been cloned from the model legumes *Lotus japonicus* (Regel) K. Larsen and *M. truncatula*. These genes encode various proteins involved in nodule functioning (Kumagai et al., 2007; Hakoyama et al., 2012; Bourcy et al., 2013; Xi et al., 2013). Both biotic and abiotic stresses cause early nodule senescence, leading to decreased agricultural yields (Puppo et al., 2005). Interestingly, mutants with a delayed senescence phenotype have not been obtained yet. However, *M. truncatula* transgenic plants with suppressed expression of *Cyp15a,* which encodes cysteine protease 15a, showed delayed nodule senescence (Sheokand et al., 2005). Similarly, *Astragalus sinicus* L. plants with decreased expression of the cysteine protease gene *Asnodf32* by RNA interference formed root nodules with delayed senescence and a prolonged nitrogen fixation period (Li et al., 2008).

In pea, mutations in the genes *sym13*, *sym25*, *sym26*, *sym27,* and *sym42* lead to early senescence in nodules (Borisov et al., 2007). This relatively high number of mutations suggests that there is precise plant genetic control over this important process. However, no mutant genes involved in nodule senescence have been cloned and sequenced yet. Ultrastructural analyses revealed a similar early senescence phenotype for the mutants of *sym13*, *sym25*, *sym26*, and *sym27* (Borisov et al., 1994, 1997; Morzhina et al., 2000). It is necessary to use molecular markers to discriminate among these mutants. Therefore, the first aim of the study was to develop a series of markers based on genes that are known to be involved in nodule senescence in pea and barrel medic (Granell et al., 1992; Kardailsky and Brewin, 1996; Peck and Kende, 1998; Martin et al., 1999; Thomas et al., 1999; Pariasca et al., 2001; Van de Velde et al., 2006; Zdunek-Zastocka, 2008; Guerra et al., 2010; D'haeseleer et al., 2010). The second aim was to analyze these molecular markers in the genetic system based on the symbiotic mutants E135F (*sym13*), with an early senescence phenotype (Kneen et al., 1990; Borisov et al., 1997), and Sprint-2Fix$^-$ (*sym31*), which lacks bacteroid differentiation and shows no morphological signs of nodule senescence (Borisov et al., 1997), and their corresponding wild-types (cv. Sparkle and line Sprint-2). We anticipated that all of the marker genes would be activated in the mutant E135F (*sym13*) but not in the mutant Sprint-2Fix$^-$ (*sym31*). Surprisingly, we observed the activation of markers in both mutants, although to a lesser extent in Sprint-2Fix$^-$ (*sym31*) than in E135F (*sym13*).

2. Materials and methods

2.1. Plant material, bacterial strain, and plant growth conditions

The mutant lines E135F (*sym13*), Sprint-2Fix$^-$ (*sym31*), and their corresponding wild-types cv. Sparkle and laboratory line Sprint-2 from pea (*Pisum sativum* L.) were used in this study (Table S1). Pea seeds were sterilized with concentrated sulfuric acid at room temperature for 7 min for the laboratory line Sprint-2 and the mutant Sprint-2Fix$^-$ (*sym31*), and for 15 min for cv. Sparkle and its mutant E135F (*sym13*). The seeds were then rinsed ten times with sterile water. Seeds were planted into sterile pots with 100 g of vermiculite and 200 mL of nitrogen-free medium (Fähraeus, 1957). Seeds were inoculated with the strain *Rhizobium leguminosarum* bv. *viciae* CIAM