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Occurrence of polyamines in root nodules of *Phaseolus vulgaris* in symbiosis with *Rhizobium tropici* in response to salt stress

Miguel López-Gómez *, Libertad Cobos-Porras, Javier Hidalgo-Castellanos, Carmen Lluch

Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, Campus de Fuentenueva s/n, 18071 Granada, Spain

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ABSTRACT

Polyamines (PAs) are low molecular weight aliphatic compounds that have been shown to be an important part of plant responses to salt stress. For that reason in this work we have investigated the involvement of PAs in the response to salt stress in root nodules of *Phaseolus vulgaris* in symbiosis with *Rhizobium tropici*. The level and variety of PAs was higher in nodules, compared to leaves and roots, and in addition to the common PAs (putrescine, spermidine and spermine) we found homospermidine (Homspd) as the most abundant polyamine in nodules. UPLC-mass spectrometry analysis revealed the presence of 4-aminobutylcadaverine (4-ABcad), only described in nodules of *Vigna angularis* before. Indeed, the analysis of different nodular fractions revealed higher level of 4-ABcad, as well as Homspd, in bacteroids which indicate the production of these PAs by the bacteria in symbiosis. The genes involved in PAs biosynthesis in nodules displayed an induction under salt stress conditions which was not consistent with the decline of free PAs levels, probably due to the nitrogen limitations provoked by the nitrogenase activity depletion and/or the conversion of free PAs to theirs soluble conjugated forms, that seems to be one of the mechanisms involved in the regulation of PAs levels. On the contrary, cadaverine (Cad) and 4-ABcad concentrations augmented by the salinity, which might be due to their involvement in the response of bacteroids to hyper-osmotic conditions. In conclusion, the results shown in this work suggest the alteration of the bacteroidal metabolism towards the production of uncommon PAs such as 4-ABcad in the response to salt stress in legume root nodules.

1. Introduction

Polyamines (PAs) are low molecular weight aliphatic nitrogen compounds that are found in a wide range of organism from bacteria to plants and animals (Alcázar et al., 2006). Common PAs putrescine (Put), spermidine (Spd) and spermine (Spm) are the most abundant in the plant cells and have been proposed as a new category of plant growth regulators involved in a variety of physiological processes such as cell division and elongation, root growth, flower and fruit development and membrane stabilization among others (Bais and Ravishankar, 2002; Liu et al., 2007; Kusano et al., 2008; Zhao and Yang, 2008). PAs are also implicated in the protection against of a wide variety of environmental stresses including salinity (Shevyakova et al., 2013) due to their capacity to stabilize macromolecules such as RNA, DNA, proteins and phospholipids, and to their free radical scavenging activity (Groppa and Benavides, 2008; Hussain et al., 2011). In plant cells, PAs occur as free bases but may also be covalently liked to small molecules such as hydroxycinnamic acids to form soluble conjugated PAs. In plants and some bacteria, the diamine Put is synthesized by decarboxylation of arginine or ornithine by arginine and ornithine decarboxylases (ADC, ODC), respectively. Spd and Spm are synthesized from Put through the successive activities of spermidine synthase (SPDS) and spermine synthase (SPMS) by the addition of aminopropyl groups derived from methionine, which is first converted to S-adenosylmethionine (Sam) and then decarboxylated via S-adenosylmethionine decarboxylase (SAMDC). It is interesting to note that some of these enzymes are synthesized as inactive precursors which are subjected to post-translational processing to be activated (Perez-Amador et al., 1995). In addition to the common polyamines Put, Spd and Spm, many structural analogs including long-chain and branched PAs have been found in different organisms, as a result of a broad survey (Niitsu et al., 1993). Legumes root nodules, which can fix atmospheric nitrogen as result of the symbiotic interaction with soil bacteria known as rhizobia, contains a variety of PAs some of which are nodule specific and synthesized by the rhizobia (Fujihara, 2009). One of this uncommon polyamine is homospermidine (Homspd), found in nodules of *Medicago sativa* as one of the most abundant

* Corresponding author. Tel.: +34 958241310; fax: +34 958248995.
E-mail address: mlgomez@ugr.es (M. López-Gómez).

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(López-Gómez et al., 2014) and the major cellular polyamine in different rhizobia species (Hamana et al., 1990). In addition to Homspd, the occurrence of 4-aminobutylcadaverine (4-ABcad) which was not yet recognized in any other living system before, was found to be specifically present in root nodules of adzuki bean (*Vigna angularis*) (Fujihara et al., 1995). Linear correlations between the total concentration of PAs in nodules and the nitrogenase activity, responsible for the nitrogen fixation, have been reported in field grown mung bean (Lahiri et al., 2004), however, little is known about the physiological role of PAs in the response to salt stress of root nodules.

In this work we have investigated the involvement of PAs in the response to salt stress in the symbiotic interaction between *Phaseolus vulgaris* and *Rhizobium tropici* since soil salinity is one of the main factor limiting production of this important crop for human consumption (Khadri et al., 2006). The results reported here reveal the presence in the nodules of the uncommon polyamine 4-ABcad which showed a concentration increase in response to salt stress. Data concerning nitrogen fixation as well as gene expression and activity of the enzymes involved in the biosynthesis of PAs were also examined.

2. Results and discussion

2.1. Occurrence of polyamines in nodules of P. vulgaris

Polyamines (PAs) levels in leaves, roots and nodules of *P. vulgaris* (inoculated with *Rizobium tropici*) were determined by HPLC as described in the material and methods section. In Fig. 1 is shown a typical elution chromatogram corresponding to the three organs

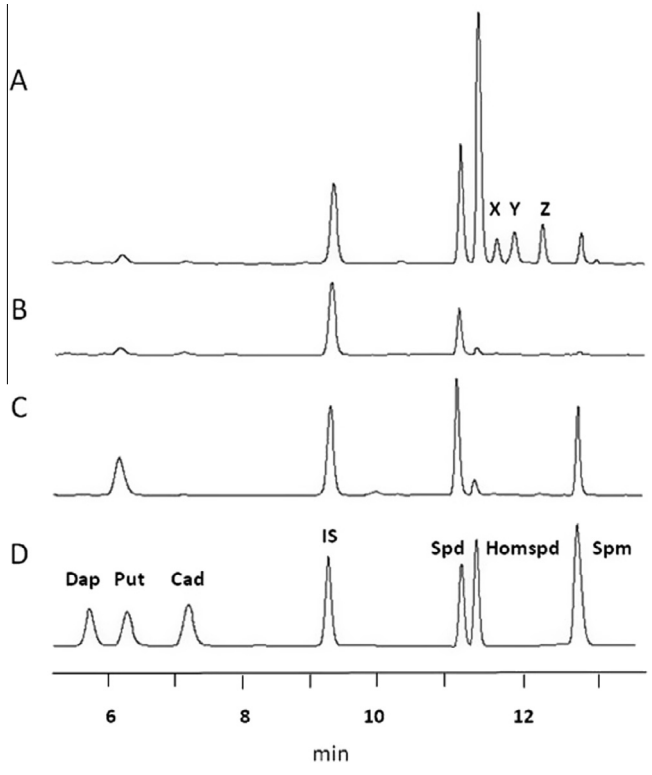

Fig. 1. Elution profile of polyamines in *P. vulgaris* nodules (A), roots (B) and leaves (C) standard polyamines (D). Polyamines were extracted and derivatized with dansyl chloride before subjecting to HPLC analysis. 1,7-diaminoheptane was used as a internal standard (IS). Peaks X, Y and Z in panel A denotes unknown compounds. Peaks in panel D: Dap; diaminopropane, Put; putrescine, Cad; cadaverine, Spd; spermidine, Homspd; homospermidine, Spm; spermine.

mentioned above and as can be observed, the number of PAs detected was higher in nodules compared to leaves and roots. In the chromatogram corresponding to nodules, four peaks between Spd and Spm were detected and three of them could not be assigned to any of the standard PAs used. These peaks were only observed in the nodular tissue while in leaves and roots, only common PAs such as Put, Spd and Spm were found. The peak posterior to Spd corresponded to the Homspd standard and seems to be the most abundant polyamine in nodules, as confirmed by the quantitative assay shown in Table 1, which has been previously observed in nodules of *P. vulgaris* (Smith, 1977) as well as in others legumes such as *M. sativa* (López-Gómez et al., 2014) or *V. angularis* (Fujihara et al., 1995). On the contrary, common PAs such as Put and Spm were majority in leaves, compared to roots and nodules, while cadaverine (Cad) was minority in all plant organs, with no detectable levels in leaves (Table 1). The major presence of Homspd in the nodular tissue has been attributed to its bacteroidal origin (Fujihara, 2009). In this regard, the distribution of PAs in different nodular fractions were analyzed and compared with the free-living bacteria in order to determine their plant or bacterial origin in the nodular tissue (Table 2). Homospd was the most abundant polyamine in the free-living bacteria and in bacteroids, confirming the bacteroidal origin of this polyamine in nodules. On the contrary, Cad was only detected at low concentrations in the plant cytosolic fraction (Table 2) which suggest its plant origin.

To identify the standard unavailable compounds (X, Y, Z), the dansylated nodule extracts were analyzed by UPLC-MS and the molecular formula was established by high accuracy quasi-molecular ion such as $[M+H]^+$ within a mass error of 5 ppm. Then the most rational molecular formula was searched in chemical databases (www.chemspider.com). The mass fragmentation pattern of the compound X showed ion at *m*/*z* 873 (Fig. 1S) with a mass error of 0.3 ppm which matched with dansylated 4-ABcad. However, no coincidences with PAs were found for the peaks Y and Z. The occurrence of 4-ABcad has only been described in root nodules of *V. angularis* (Fujihara et al., 1995) and has been considered to be an unusual polyamine occurring in rhizobial cells under specific environmental conditions. Indeed, the analysis of different nodular fractions (Table 2) reveals a higher relative level of 4-ABcad in bacteroids than in the cytosol, while this polyamine was not detected in the free living bacteria, which suggest that this compound is synthesized by the bacteroids to counteract the hyper-osmotic conditions found into the nodule. It is also possible that within the nodule bacteroids are supplied with Cad, precursor for the synthesis of 4-ABcad, as described by Fujihara et al. (1995) in *Bradyrhizobium japonicum* bacteroids isolated from *V. angularis* plants. This possibility is supported by the low levels of Cad detected in the nodular tissue (Table 1) that would indicate its consumption by the bacteroids to produce 4-ABcad.

2.2. Response of nodular polyamines levels to salt stress conditions

In order to study the implication of PAs in the nodular response to salt stress, *P. vulgaris* plants were subjected to 100 mM NaCl treatments for 3 and 7 days before harvest. The quantification of free PAs shown in Fig. 2 reveals a reduction in response to salinity of about 20% for all of them, except for Cad which augmented 20% under salt stress. This may be due to the fact that Cad, differing from PAs of the Put family (Spd and Spm), is the product of another metabolic pathway starting from aspartate and formed by lysine decarboxylation (Kuznetsov et al., 2007), and would not be in competition with Put derived from glutamate. Therefore, Cad accumulation under salt stress conditions compensates the decrease in the content of Put-family PAs, as previously observed by Kuznetsov et al. (2007). The Homspd reduction observed in nodules is consistent with previous studies where rhizobial strains subjected to salt