Methyl jasmonate elicitation of common bean seedlings induces nucleotidase activity and the expression of several nucleotidase genes in radicles

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Abstract

Nucleotides are the basic elements of the genetic material, participate in bio-energetic processes, are cofactors and components of secondary metabolites, etc. Nucleotide hydrolases (nucleotidases) are phosphatases that remove the 5′-phosphate group from the nucleotides and play a crucial role in nucleotide metabolism. In this study, genes encoding putative nucleotidases in *Phaseolus vulgaris* have been identified, and the effect of methyl jasmonate (MeJA) on both nucleotidase activity and gene expression has been addressed. The predicted nucleotidase peptides include the conserved domains characteristic of the haloacid dehalogenase-like hydrolase superfamily. The analysis of the expression of the 11 identified genes in radicles of common bean seedlings elicited with MeJA showed that 3 of them are highly induced by this phytohormone in a dose-dependent manner. Nucleotidase activity in radicles from MeJA treated plants was higher than in not elicited seedlings, and this induction was observed with all the nucleotides assayed (mono-, di- or triphosphate) and with purine or pyrimidine nucleotides. MeJA is involved in biotic and abiotic stress, and the induction of nucleotide metabolism in response to this treatment suggests a relevant role for nucleotides in the seedlings response to unfavourable conditions.

Keywords: haloacid dehalogenase-like hydrolases superfamily, phosphatases, stresses.

Introduction

Nucleotides are essential molecules with implications for vital processes such as plant germination, as well as development and growth. Nucleotides are essential in the storage and recovery of genetic information acting as structural elements for the formation of DNA and RNA, act as a source of energy at the cellular level, and form parts of different cellular components such as vitamins (vitamin B12), hormones (such as cytokinin), or enzyme cofactors (such as NAD+) (Zrenner et al. 2006, Haferkamp et al. 2011). In addition, nucleotides have phosphate in their molecule, which can be transferable to other molecules (Zrenner et al. 2006).

Purine nucleotides have an additional key role in ureidic legumes, such as common bean or soybean. Ureidic legumes mobilize most of the nitrogen fixed in root nodules to the aerial organs of the plants in the form of ureides when they grow under nitrogen-fixing conditions (Todd et al. 2006). Ureides (allantoate and allantoin) are organic substances formed by the oxidation of purines with an elevated N:C ratio (1:1). In addition to their role as transporters of fixed nitrogen in ureidic legumes, ureides may be involved in other processes related to the storage and mobilization of nitrogen, and a protective role for ureides against reactive oxygen species has been suggested in stress situations (Watanabe et al. 2014), although this protective role could be related with...
its ability as nitrogen reservoir rather than stress protectant (Soltabayeva et al. 2018). In this way, legume seedlings show differences in antioxidant enzymatic activities in relation to their behaviour as ureidic or amide legumes (Quiles et al. 2019). Induction of ureide metabolism has been reported during germination and post-germinative development in common bean (Quiles et al. 2009), and ureide accumulation has been described during dark-induced bean leaf senescence (Lambert et al. 2017) and nitrogen starvation in Arabidopsis (Melino et al. 2018) associated, in both cases, with nucleic acid catabolism. The nucleic acid metabolism could be used to mobilize nitrogen and phosphorus during senescence (Lambert et al. 2016, 2017) and the importance of their mobilization during senescence has been reported (Soltabayeva et al. 2018).

The metabolic pathways of nucleotides have been studied fundamentally in animals, due to the importance of nucleotide metabolism in the investigation of genetic diseases and in cancer research (Camici et al. 2019). In plants, the knowledge about the enzymes involved in nucleotide metabolism is more limited. This could be due to the numerous reactions that participate in the de novo synthesis, salvage, and degradation (Zrenner et al. 2006). Both, the salvage and de novo synthesis pathways merge in the formation of nucleosides monophosphate. The catabolic pathway begins with the cleavage of the 5’-phosphate group, a reaction catalysed by a phosphatase that hydrolyses the nucleotides into nucleosides. However, it has not been elucidated if this reaction is carried out by different enzymes or if there are specific nucleotidases for each. A phosphatase with elevated affinity for nucleoside monophosphate was purified from embryonic axes of common bean (Cabello-Díaz et al. 2012), and the gene encoding the protein was identified (Cabello-Díaz et al. 2015), as well as another gene whose expression is higher in the radical nodules where ureides are synthesized (Galvez-Valdivieso et al. 2020).

Jasmonates are phytohormones that regulate various physiological processes of plant development including root growth, flowering, and leaf senescence (Huang et al. 2017), and participate in the activation of the defence responses of plants to abiotic stress and pathogenic attacks (Dar et al. 2015, Raza et al. 2020, Wang et al. 2020). Jasmonates derive from lipids and include jasmonic acid (JA) and its derivatives, jasmonate iso-leucine conjugate and methyl jasmonate (MeJA) (Wasternack et al. 2018). The tissue damaged during wounding results in the release of cellular components to the extracellular space, including nucleic acids and nucleotides. Furthermore, extracellular ATP (eATP) is an emerging signalling molecule in plant metabolism that exerts its function acting synergistically with jasmonate (Tripathi et al. 2018, Pietrowska-Borek et al. 2020). To maintain its function, eATP homeostasis must be controlled and phosphatases should play a crucial role in this process (Clark et al. 2011).

To better understand the nucleotide metabolism in plants and its possible relationship with the response to stress, we have identified genes that code for nucleotidase members of the haloacid dehalogenase-like hydrolases (HAD) superfamily in Phaseolus vulgaris. Furthermore, we analysed their expression pattern in common bean radicles in response to MeJA treatment.

**Materials and methods**

**Plants and growing conditions:** Phaseolus vulgaris L. cv. Great Northern seeds were sterilized and germinated on Petri dishes as previously described (Lambert et al. 2014). After 5 d from the start of the imbibition, the seedlings were placed in new plates with filter paper moistened with distilled water (control treatment), or with the indicated concentration of MeJA and they were maintained in the growth chamber for 24 h. After that time, the seedlings were separated into radicles, the aerial part of embryonic axes and cotyledons and immediately frozen in liquid nitrogen.

**Preparation of crude extracts:** The plant material (approximately 100 mg) was pulverized with mortar in liquid nitrogen. The pulverized material was mixed with 4 volumes of extraction buffer consisting of 50 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES; pH 7.0) and 0.15% w/v sodium deoxycholate (DOC). Once homogenized, it was centrifuged at 24 000 g and 4 °C for 10 min. Finally, the supernatant was collected and considered as a crude extract.

**Enzymatic activities determination:** Phosphatase activities were assayed by monitoring the phosphate concentration in reaction mixtures as previously described (Cabello-Díaz et al. 2012). Total phosphatase activity was assayed in a standard reaction mixture containing 50 mM 2-(N-morpholino)ethanesulfonic acid (MES-HCl) buffer (pH 5.5), 2 mM para-nitrophenyl phosphate (pNPP) as substrate and an adequate amount of crude extract. Nucleotidase activity was determined in a mixture containing 50 mM MES-HCl buffer (pH 5.5) or 50 mM TES-NaOH (pH 7), 1 mM MgCl2, 2 mM nucleotide indicated as substrate, 5 mM molybdate and an adequate amount of enzyme crude extract. The reactions were initiated by the addition of the enzyme crude extract and were performed at 37 °C. Aliquots of 0.2 cm3 were extracted at various time points and phosphate concentration was determined (Cabello-Díaz et al. 2012).

**RNA isolation, cDNA synthesis and real-time PCR:** Total RNA isolation, cDNA synthesis, and real-time quantitative PCR (qPCR) were performed as previously described (Galvez-Valdivieso et al. 2020) using the specific primers indicated in Table 1 Suppl. Results were normalised using the geometric mean of ubiquitin and actin-2 using the 2ΔACT or 2-ΔΔACT method (Livak and Schmittgen 2001). The specificity of the pair of primers was verified by real-time PCR and sequencing of the products and following the amplicon dissociation curves.

**Analytical Determination:** Protein content in the crude extracts was determined by the protein-dye binding method.
method (Bradford 1976) using the Bio-Rad (Hercules, USA) reagent and with bovine serum albumin as standard. Total ureides were determined by a colorimetric method as described by Quiles et al. (2019).

**Sequences analysis:** The sequences were obtained from the Phytozone v. 12.1 (https://phytozone.jgi.doe.gov/pz/portal.html#) and NCBI (https://blast.ncbi.nlm.nih.gov/) databases. The alignment of sequences was performed with MegAlign from DNASTAR Lasergene 7.0.0. Specific primers were designed using Primer 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser et al. 2007). The phylogenetic tree was obtained after the alignment of the predicted proteins with the algorithms MUSCLE (Edgar 2004) from the program MEGA X 10.0.5 (Kumar et al. 2018). The phylogenetic tree was elaborated with the method neighbour-joining (NJ) (Saitou and Nei 1987) with a bootstrap value of 1 000 replicates. Subcellular localization of the predicted proteins was performed using DeepLoc-1.0 eukaryotic protein subcellular localization predictor (http://www.cbs.dtu.dk/services/DeepLoc/cite.php) (Armenteros et al. 2017). The platform New Place (https://www.dna.affrc.go.jp/LPLACE/?action=newplace) (Higo 1998) was used to analyse the promoter regions of the genes.

**Statistical analyses:** All results are means of three biological replicates. The analyses performed are indicated in the legend to figures. Statistical analyses were performed with SPSS Statistics, version 25.

**Results**

The effect of methyl jasmonate on nucleotidase and phosphatase activities was analysed in cotyledons, axes, and radicles of 6-d-old seedlings after 24 h of treatment with 50 µM of MeJA. Nucleotidase activity was lower in cotyledons with respect to axes and radicles (Fig. 1A). MeJA treatment did not produce any relevant effect on the nucleotidase activity in cotyledons and axes (Fig. 1A) while it caused an increase of nearly 100 % in radicle nucleotidase activity. The total phosphatase activity was much higher than nucleotidase activity in the three tissues, and no significant differences in activity were detected after the MeJA treatment (Fig. 1B). Ureide content in common bean seedlings parts was not affected by MeJA treatment (Fig. 1 Suppl.).

To identify the putative gene or genes responsible for the increase in nucleotidase activity in radicles after MeJA treatment a search in the database was performed. The sequences coding for putative nucleotidases PvNTD1 (Cabello-Diaz et al. 2015) and PvNTD2 (Galvez-Valdivieso et al. 2020) were used to search in the Phytozone and NCBI databases. In this way, 9 additional sequences were identified, which were named from PvNTD3 to PvNTD11 and their respective accession numbers in the Phytozone database are indicated in the legend to Fig. 2. All the sequences belong to the subfamily IIIB of HAD superfamily of phosphatases and are grouped in the family PF03767 in the Pfam database (https://pfam.xfam.org/). The predicted proteins encoded by these genes would range between 251 and 312 amino acids. The alignment of these sequences as well as the domains shared by the acid phosphatases of the HAD family (Burroughs et al. 2006) is shown in Fig. 2. A crucial nucleophilic asparagine and the DxDx(T/V)(L/V) consensus sequence form part of the HAD signature motif I, and this motif together with motif IV are involved in the coordination of Mg^2+ in the active site. Motif II consists in the consensus sequence hhh(S/T), where h represents a hydrophobic residue and S/T amino acids serin and threonin. The degree of conservation of Motif III is reduced when compared to the other motifs, with a lysin residue mostly conserved. Motif IV generally contains the consensus sequence hhS(D/E), but, alternatively, a DD signature instead of a Dx↓↓D sequence has been also observed (Seifried et al. 2013). A phylogenetic tree was elaborated, and 2 differentiated groups were observed (Fig. 3). PvNTD4 and PvNTD6 are in a separate branch from all the other sequences. The other branch is divided into two groups, with PvNTD1, PvNTD2, PvNTD9, PvNTD10, and PvNTD11 in the same group. The two previously characterized genes (PvNTD1 and PvNTD2) are in the same branch, and the other three are in a separate one.

To elucidate the possible location of the proteins that encode these genes, analysis of the amino acid sequences was carried out using DeepLoc-1.0 (Table 1). The results suggest that most of the proteins could be extracytosolic, located either in the extracellular space (PvNTD1, PvNTD2, PvNTD3, PvNTD9, PvNTD10, and PvNTD11), or in organelles such as the lysosome or the vacuole,
endoplasmic reticulum, or in the membrane (PvNTD4, PvNTD5, PvNTD6, PvNTD7, and PvNTD8). Of all the sequences analysed, PvNTD4 and PvNTD6 appear to be membrane proteins, while all the others appear to be soluble.

Since the nucleotidase activity changed in response to MeJA in radicles (Fig. 1), the expression of the 11 identified genes from common bean was analysed in radicles from both control and 50 µM MeJA treated seedlings (Fig. 4). All the genes were detected in non-treated radicles and the expressions were very variable, being PvNTD5 (relative gene expression of 0.0002) and PvNTD10 (relative gene expression of 0.78) the genes with the lowest and highest transcriptions, respectively (Fig. 4). Treatment with MeJA for 24 h induced the expression of PvNTD9, PvNTD10, and PvNTD11, whereas did not significantly affect the expressions of the other genes (Fig. 4). The MeJA treatment resulted in a 4.4-, 3.2- and 14.1-fold increase in the relative expression of PvNTD9, PvNTD10, and PvNTD11, respectively.

Fig. 2. Alignment of amino acid sequences of French bean genes belonging to pfam family PF03767. The corresponding accession numbers for each gene are PvNTD1 (Phvul.004G174200), PvNTD2 (Phvul.011G182400), PvNTD3 (Phvul.001G164000), PvNTD4 (Phvul.001G240100), PvNTD5 (Phvul.007G270800), PvNTD6 (Phvul.008G227000), PvNTD7 (Phvul.010G058800), PvNTD8 (Phvul.010G059000), PvNTD9 (Phvul.010G144200), PvNTD10 (Phvul.010G144300), PvNTD11 (Phvul.010G144600). The amino acids identical to the consensus sequence are shadowed in black. The different motifs of the haloacid dehalogenase (HAD) superfamily (Burroughs et al. 2006) are underlined. The sequences were aligned using the ClustalW method.
Since the transcriptions of *PvNTD9*, *PvNTD10*, and *PvNTD11* increased significantly in the presence of 50 µM MeJA, the effect of different concentrations of MeJA on their expression was analysed. As shown in Fig. 5, there was a dose-dependent relation between the concentration of MeJA and the transcriptions of these 3 genes, being *PvNTD11* the gene that shows the strongest response to the addition of MeJA (Fig. 5).

The region containing the 1000 nucleotides upstream from the translation start was identified in the Phytozome database, and the cis-regulatory elements of the three genes induced by MeJA were analysed. In the three promoter regions, various motifs of response to water stress, wounding, pathogens, and hormones were identified (Table 2 Suppl.). Motifs related to biotic and abiotic stresses were identified in promoter regions of these genes (Table 2 Suppl.). In two of them, *PvNTD9* and *PvNTD11*, there were motifs of a specific response to methyl jasomate.

Since 250 µM of MeJA provokes a higher increase in the expressions of *PvNTD9*, *PvNTD10*, and *PvNTD11*, nucleotidase activity was determined in crude extracts from radicles of seedling after 24 h of treatment with 250 µM of MeJA, using different nucleotides as substrates at both pH 5.5 and pH 7.0 (Fig. 6). The activity in radicles of untreated seedlings was very similar with most of the substrates assayed, being generally higher at pH 7.0 than at pH 5.5. Thus, at pH 5.5, the specific activity values ranged from 37 mU mg⁻¹(protein) min⁻¹ for AMP to 87 mU mg⁻¹(protein) min⁻¹ for UMP, whereas at pH 7.0 it ranged from 70 mU mg⁻¹(protein) min⁻¹ for AMP to 139 mU mg⁻¹(protein) min⁻¹ for ATP. Only with ADP, the activity was markedly higher, with values of 269 and 616 mU mg⁻¹(protein) min⁻¹ at pH 5.5 and 7.0, respectively (Fig. 6). With all nucleotides used at both pHs, the MeJA treatment resulted in a significant increase in nucleotidase activity. At both pH 5.5 and pH 7.0, the highest nucleotidase activity was recorded with ADP, being almost 3 times higher when the assay was performed at pH 7.0 and with a value of 3 U mg⁻¹(protein) min⁻¹ (Fig. 6).
Discussion

Jasmonates are phytohormones that are involved in the defence of the plant against attack by herbivores and pathogens, in tolerance to abiotic stress, and regulation of various aspects of plant physiology such as root growth, flowering, and leaf senescence (Huang et al. 2017). The application of methyl jasmonate resulted in an increase in phosphatase activity for nucleotides as substrates in the presence of molybdate in the radicles of common bean seedlings. Molybdate is an inhibitor of unspecific phosphatases (Duff et al. 1994) and therefore in these assays, the phosphatase activity should correspond to a nucleotidase (Cabello-Diaz et al. 2012). In comparison to total phosphatase activity, nucleotidase represents only a small fraction. In fact, when total phosphatase was determined in the absence of molybdate and with pNPP as substrate, no differences were obtained after MeJA application. Induction of nucleotidase activity was observed only in radicles. The radicle is also the part of the seedling that shows changes in ribonuclease in response to salt stress (Diaz-Baena et al. 2020). Plant roots are organs with a particularly high diversity of responses to challenging environments, rearranging their development to cope with adverse situations (Gruber et al. 2013).

To determine the gene or genes responsible for the induction of nucleotidase activity in response to MeJA, nine additional genes were identified based on similarity to previously characterized \(PvNTD1\) (Cabello-Diaz et al. 2015) and \(PvNTD2\) (Galvez-Valdivieso et al. 2020). All of them have the typical domains of the HAD superfamily, which groups, among other enzymes, hydrolases, phosphatases, nucleotidases and several phosphotransferases (Bogan and Brenner 2010). The catalytic action of HAD phosphatases...
differs from other well-known phosphatases. This group of phosphatases use an Asp residue as a nucleophile in an Mg-dependent phosphoaspartyl transferase reaction (Seifried et al. 2013). The effect of magnesium was demonstrated for the nucleotidase PvNTD1 purified from embryonic axes from common bean, being the only cation that activated the purified enzyme (Cabello-Diaz et al. 2012). This distinctive feature of HAD phosphatases in relation to the transferase reaction is also responsible for their lack of sensitivity against the most common inhibitors of phosphatases (Seifried et al. 2013). Apart from these motifs, the sequence similarity between HAD phosphatases is very low.

The three genes that are induced in response to MeJA are in the same branch in the phylogenetic tree and they are located very close in the same chromosome in the common bean. A wound-induced gene, PtdAP1, from poplar (Veljanovski et al. 2010) would be located within this branch (data not shown), showing the highest similarity to PvNTD9 and PvNTD11. Microarrays analysis in poplar demonstrates the important role of the acid phosphatase in the poplar defence mechanism against herbivores since this gene is one of the most strongly upregulated in response to wounding (Major and Constabel 2006).

The possible extracellular localization of the proteins encoded by the three genes induced by MeJA (Table 1) suggests that in response to methyl jasmonate, radicles would increase their ability to degrade nucleotides in the extracellular space. These nucleotides could come from the degradation of nucleic acids that are released into the environment when cells are damaged, or from the decomposition of organic matter in the soil. These enzymes could also participate in the maintenance of extracellular ATP homeostasis. This compound acts as a signalling molecule in plants, participating in functions as diverse as growth, development, and the response to abiotic and biotic stresses (Möhlmann et al. 2014). An eATP is released into the apoplastic space by exocytosis, damaged membrane or by specific transporter. In Arabidopsis, the eATP receptor has been identified (Choi et al. 2014) that gives rise to a signalling cascade in which eATP acts crosstalk with jasmonate, salicylic acid, and ethylene signalling pathways (Tripathi et al. 2018, Jewell et al. 2019). This cascade leads to downstream changes aimed to protect the plant against stresses and guaranteeing plant growth.

![Fig. 6. Nucleotidase activity with different nucleotides at pH 5.5 and pH 7.0. The activity was tested with the nucleotides indicated at both pHs in crude extracts from radicles treated with 0 or 250 µM MeJA. The asterisks refer to the significant difference in nucleotidase activity between the control (0 µM MeJA) and the treatment (250 µM MeJA) (Student's t-test; P < 0.001). Means ± SEs of three biological replicates.](image-url)
and development (Jewell et al., 2019, Pietrowska-Borek et al., 2020). The eATP content must be finely regulated to maintain cell physiology. The eATP is removed from the apoplast by the action of a set of enzymes, among which are e-nucleotidases, which are responsible for hydrolyzing nucleotides, tri- and diphosphates, the 5′-nucleotidases, which degrade AMP giving rise to phosphate adenosine, and apoplastic nucleosidases that hydrolyse adenosine to adenine. Both molecules, adenosine and adenine, can be re-incorporated into cells via nucleoside transporters or purine permeases and thus recycled (Möhlmann et al., 2014). A model for the plant eATP signal transduction pathway with the involvement of Ca2+, reactive oxygen species, and MAP kinases has been recently proposed (Pietrowska-Borek et al., 2020). Interestingly, the promoter of the MeJA inducible genes is enriched in motif related to response to pathogenesis and several hormones including jasmonates, ethylene, and salicylic acid.

The nucleotidase activity in radicles from seedlings elicited with MeJA cannot rule out any of the hypotheses indicated for the induction in nucleotidase gene expression. The sequences of the members of the HAD superfamily are highly divergent, and the catalysed reactions and substrate specificities of each enzyme can only be determined empirically (Kuznetsova et al., 2006). The nucleotidase activity determined in the radicles increased with the treatment with methyl jasmonate regardless of the nucleotide tested, purine or pyrimidine, and of the pH, acidic or neutral. The increase was not specific for nucleotides monophosphate, but it was also obtained for ADP and ATP. Therefore, the enzyme or enzymes induced by MeJA do not show great substrate specificity.

We describe here that MeJA induces nucleotide metabolism in common bean radicles. Among the identified gene candidates to code for nucleotidase, three of them induce its expression as far as after 24 h of MeJA elicitation in a dose-dependent manner. The induction of gene expression coincides with an increase in nucleotidase activity both at acidic and neutral pH, and with all the nucleotides assayed, either purine or pyrimidine. It will be interesting to demonstrate if these genes are specifically induced in other adverse situations and to clearly identify the subcellular space where they show catalytic activity.

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