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Research article

Role of the ubiquitin-proteasome pathway and some peptidases during seed germination and copper stress in bean cotyledons

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ABSTRACT

The role of the ubiquitin (Ub)-proteasome pathway and some endo- and aminopeptidases (EPs and APs, respectively) was studied in cotyledons of germinating bean seeds (*Phaseolus vulgaris* L.). The Ub system appeared to be important both in the early (3 days) and late (9 days) phases of germination. In the presence of copper, an increase in protein carbonylation and a decrease in reduced −SH pool occurred, indicating protein damage. This was associated with an enhancement in accumulation of malondialdehyde, a major product of lipid peroxidation, and an increase in content of hydrogen peroxide (H_2O_2), showing oxidative stress generation. Moreover, copper induced inactivation of the Ub-proteasome (EC 3.4.25) pathway and inhibition of leucine and proline aminopeptidase activities (EC 3.4.11.1 and EC 3.4.11.5, respectively), thus limiting their role in modulating essential metabolic processes, such as the removal of regulatory and oxidatively-damaged proteins. By contrast, total trypsin and chymotrypsin-like activities (EC 3.4.21.4 and EC 3.4.21.1, respectively) increased after copper exposure, in parallel with a decrease in their inhibitor capacities (i.e. trypsin inhibitor and chymotrypsin inhibitor activity), suggesting that these endoproteases are part of the protective mechanisms against copper stress.

1. Introduction

Copper (Cu) is one of the most toxic heavy metals that induces serious alterations in plant cells (Janas et al., 2010). One of the underlying causes of tissue injury following exposure of plants to heavy metals is generation of oxidative stress (Sharma and Dietz, 2008; Monferrán et al., 2009), which can damage almost all cellular components, including proteins. In particular, oxidation of side chains of amino acid residues and formation of protein–protein covalent cross-linkages can inactivate or denature proteins (Davie, 2001). If they are not rapidly eliminated, oxidatively-modified proteins can undergo direct fragmentation or form large aggregates, due to covalent cross-linkage and increased surface hydrophobicity, which affects cell division and can lead to cell death (Davie, 2001). In addition, an excessive production of reactive oxygen species (ROS) can induce structural and chemical alterations of cellular membranes, as well as peroxidation of lipids (Valavanidis et al., 2006). The process of lipid peroxidation involves a chain of reactions leading to the breakdown of polyunsaturated fatty acids that are relatively sensitive to oxidation. This process gives rise to a number of secondary products, such as malondialdehyde (MDA).

To cope with oxidative stress, organisms have developed multiple defense mechanisms against oxidative damage. In addition to antioxidants and antioxidant enzymes which metabolize ROS and molecular chaperones which repair oxidatively damaged proteins,

Abbreviations: CIA, chymotrypsin inhibitor activity; CLA, chymotrypsin-like activity; E-64, L-trans-epoxysuccinyl-leucylamide-4-guanidino-butane; LAP, leucine aminopeptidases; Leu-βNA, L-leucine-β-naphthylamide; MDA, malondialdehyde; MG-132, *N*-(Benzyloxycarbonyl)-leucinyl-leucinyl-leucinal; NEM, *N*-ethylmaleimide; PAP, proline aminopeptidases; PMSF, Phenylmethylsulfonylfluoride; Pro-βNA, L-proline-β-naphthylamide; s-LLVY-NH-Mec, Succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide; TIA, trypsin inhibitor activity; TLA, trypsin-like activity; TLCK, Tosyl-L-lysine chloromethyl ketone; TPCK, Tosyl-L-phenylalanine chloromethyl ketone; Ub, ubiquitin.

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proteolytic systems selectively degrade them (Grune et al., 2005). In plant cells, stress conditions trigger an increase in protein turnover and degradation (Brouquisse et al., 2001; Smalle and Vierstra, 2004). An important nonlysosomal proteolytic pathway, involved in the degradation of intracellular proteins under biotic and abiotic stress, is the ubiquitin(Ub)-proteasome system.

The 20S proteasome is a large multicatalytic proteinase complex involved in the ATP-dependent degradation of Ub-conjugated proteins (Smalle and Vierstra, 2004). Three proteolytic activities can be assigned to the proteasome, namely trypsin-like, chymotrypsin-like and peptidyl–glutamyl peptide hydrolase (Smalle and Vierstra, 2004). The presence of the proteasome is well established, it has been studied in many species and its role has been demonstrated in a diverse set of processes including cell cycle, antigen presentation, and selective intracellular proteolysis (Hershko and Ciechanover, 1992). In plants, the proteasome fulfills key functions in both Ub-dependent and Ub-independent non-lysosomal pathways for breakdown of oxidized proteins generated by stress (Smalle and Vierstra, 2004; Grune et al., 2003), thus minimizing their cytotoxicity. Ub-dependent proteolysis by the 26S proteasome could be summarized by the signaling of the protein by covalent attachment of multiple Ub molecules and degradation of the targeted protein with the release of free and reusable Ub (Hershko, and Ciechanover, 1992).

Despite the considerable progress that has been made in elucidating the proteasome mode of action, little is known about the involvement of the Ub system in the degradation of specific cellular proteins. Recent evidence implicates the Ub system in the degradation and post-translational processing of several important regulatory proteins, regulation of gene expression, DNA repair, import of proteins into mitochondria, biogenesis of peroxisomes, programmed cell death and involvement in the cellular stress response via lysosomal proteolysis (Chen and Chen, 2013; Girzalsky et al., 2010; Hammond-Martel et al., 2012; Jackson and Durocher, 2013; Li and Vierstra, 2012). In all these processes ubiquitination signals degradation, but it can also serve nonproteolytic functions.

In plant tissues, in addition to the 20S proteasome, some proteases, namely endopeptidases (EPs) and aminopeptidases (APs) have been proposed to play a role in the degradation of moderately oxidized protein during oxidative stress (Davie, 2001; Grune et al., 2003). Hence, degradation of oxidized proteins starts by the action of the 20S proteasome and continues through a set of proteases - leucine aminopeptidases (LAP; EC 3.4.11.1), proline aminopeptidases (PAP; EC 3.4.11.5) and tripeptidyl peptidase II (EC 3.4.14.10) – which act downstream of the proteasome to finish the degradation in the cytosol (Book et al., 2005; Polge et al., 2009). APs belong to a group of exopeptidases that require the presence of a free amino group and hydrolyze the amide bonds of amino acids, dipeptides and oligopeptides consecutively from the N-terminal end. The APs' classification parameters include substrate specificity, cellular location, catalytic function, requirement for cofactors, and optimal pH (Sanz, 2007).

Under stress conditions, other enzymes could be involved in the cellular protective mechanisms, in particular trypsin- and chymotrypsin-like proteases. In fact, many investigations have demonstrated that the up-regulation of trypsin-like activity (TLA) and chymotrypsin-like activity (CLA) argues in favor of the potential involvement of these enzymes in the elimination of damaged proteins, thus promoting plant survival (Ahsan et al., 2007; Boojar and Goodarzi, 2007; Domash et al., 2008).

In a previous paper, copper treatment has been shown to delay bean seed germination and embryo growth by affecting nitrogen release from cotyledonary proteins; notably cysteine-, aspartic- and metallo-EPs activities were inhibited. In contrast, activity of serine-EPs was enhanced (Karmous et al., 2012). In light of these findings, TLA, CLA and their inhibitor capacities (trypsin inhibitor activity, TIA, and chymotrypsin inhibitor activity, CIA) were investigated under copper stress. In addition, the effect of this metal on the Ub-proteasome system and LAP and PAP activities were studied as well. The aim of this investigation was to shed more light on the mechanism of copper toxicity and cell defense response.

2. Materials and methods

2.1. Germination and copper treatment conditions

Dry seeds of bean (*Phaseolus vulgaris* L. var. soisson nain hatif) were washed under running tap water, surface-sterilized with 2% of sodium hypochlorite for 10 min, rinsed twice and soaked in distilled water. They were then plated in Petri dishes with two filter-paper discs imbibed with distilled water or a 200 μM $CuCl_2$ solution and germinated at 25 ± 1.5 °C in the darkness. Cotyledons from seedlings were collected after 3, 6 and 9 days. Day 0 corresponds to 4-h imbibed seeds in distilled water. The 200 μM $CuCl_2$ concentration was chosen on the basis of preliminary experiments in which this concentration was demonstrated to cause 50% of inhibition of growth parameters.

2.2. Carbonyl content determination

Quantification of protein carbonyl groups was carried out according to the classical approach of Reznick and Packer (1994) using a spectrophotometric DNPH method. The mixture of protein extract and dinitrophenylhydrazine (10 mM, prepared in 2 N HCl) was allowed to stand in the dark at room temperature for 1 h with continuous vortexing, and then precipitated with cold trichloroacetic acid (20% final concentration) and centrifuged at 20,000 × g for 15 min. The protein pellet was washed with 20% trichloroacetic acid, and then three times with ethanol/acetic acid (v/v). Samples were then resuspended in 6 M guanidine hydrochloride (dissolved in 2 N HCl) and incubated at 40 °C for 30 min with vortex mixing. Carbonyl content was determined from the absorbance at 480 nm (ε = 22,000 M^{-1} cm^{-1}) as described by Levine et al. (1994). The protein-carbonyl content was expressed as nmol mg^{-1} protein.

2.3. SH determination

Total protein thiols were assayed according to Ellman (1959). The reduction of 5.5′-dithiobis (2-nitrobenzoic acid), (ε = 13,600 M^{-1} cm^{-1}), was followed by measuring the increase in the absorbance at 412 nm.

2.4. Lipid peroxidation

Lipid peroxidation was measured by determining MDA, a decomposition product of polyunsaturated fatty acids. Samples (about 3 g) were homogenized in 20 mM Tris–HCl (pH 7.4; 1:3, w/v), centrifuged at 3000 × g for 20 min and then derivatized in a 1 mL reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol, 3/1, v/v), HCl 32%, 100 μL water and an equal volume of sample or 0–6 μM 1,1,3,3-tetramethoxypropane in 20 mM Tris–HCl (pH 7.4). After 40 min of incubation at 45 °C, samples were cooled on ice, centrifuged at 15,000 × g for 10 min and the absorbance of the supernatant was recorded at 586 nm. Levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol mg^{-1} protein.