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Identification and expression analysis of 11 subtilase genes during natural and induced senescence of barley plants

Irma N. Roberts*, Cintia G. Veliz, María Victoria Criado, Ana Signorini, Ester Simonetti, Carla Caputo

Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA), CONICET, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE, Buenos Aires, Argentina

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ABSTRACT

Subtilases are one of the largest groups of the serine protease family and are involved in many aspects of plant development including senescence. In wheat, previous reports demonstrate an active participation of two senescence-induced subtilases, denominated P1 and P2, in nitrogen remobilization during whole plant senescence. The aim of the present study was to examine the participation of subtilases in senescence-associated proteolysis of barley leaves while comparing different senescence types. With this purpose, subtilase enzymatic activity, immunodetection with a heterologous antiserum and gene expression of 11 subtilase sequences identified in barley databases by homology to P1 were analyzed in barley leaves undergoing dark-induced or natural senescence at the vegetative or reproductive growth phase. Results showed that subtilase specific activity as well as two inmunoreactive bands representing putative subtilases increased in barley leaves submitted to natural and dark-induced senescence. Gene expression analysis showed that two of the eleven subtilase genes analyzed, *HvSBT3* and *HvSBT6*, were up-regulated in all the senescence conditions tested while *HvSBT2* was expressed and up-regulated only during dark-induced senescence. On the other hand, *HvSBT1*, *HvSBT4* and *HvSBT7* were down-regulated during senescence and two other subtilase genes (*HvSBT10* and *HvSBT11*) showed no significant changes. The remaining subtilase genes were not detected. Results demonstrate an active participation of subtilases in protein degradation during dark-induced and natural leaf senescence of barley plants both at the vegetative and reproductive stage, and, based on their expression profile, postulate *HvSBT3* and *HvSBT6* as key components of senescence-associated proteolysis.

1. Introduction

Proteases (also referred as peptidases or proteinases) are enzymes able to hydrolyze peptide bonds contained in proteins or peptides of various sizes. They are key components of a wide range of biological processes of great relevance to biology, medicine and biotechnology (Rawlings et al., 2016). In plants, their known biological roles are strikingly diverse, being demonstrated to participate in almost all stages of plant life including meiosis, morphogenesis, embryo development, defense responses, cell death and senescence (Van der Hoorn, 2008; Pesquet, 2012).

Senescence in plants refers to the last phase of development preceding death, either of the whole organism or of at least part of it. Age-related leaf senescence takes place sequentially during the vegetative growth phase allowing successive organ replacement and maintenance of high photosynthesis rates. In addition to single leaf senescence, monocarpic crops also undergo terminal senescence that refers to the whole plant death at the end of the life cycle (Distelfeld et al., 2014). Monocarpic senescence in annual crops is normally induced early in the reproductive phase (Davies and Gan, 2012). Senescence-associated proteolysis plays a crucial role by enabling the remobilization of nutrients, mainly nitrogen (N), from senescent tissues to new developing organs, both during vegetative and reproductive growth phases.

Abbreviations: CBB, Coomassie Brilliant Blue; CPs, cysteine proteases; DAS, days after sowing; N, nitrogen; NSL, non-senescent leaves; RLS, Rubisco large subunit; RSS, Rubisco small subunit; Rubisco, Ribulose-1,5-bisphosphate carboxylase oxygenase (EC 4.1.1.39); SL, senescent leaves; SPs, serine proteases; TCA, trichloroacetic acid; VSL, very senescent leaves.

* Corresponding author.
E-mail address: iroberts@agro.uba.ar (I.N. Roberts).

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Additionally to this nutrient recycling function, senescence-associated proteases are also involved in the regulation of the senescence process. The involvement of proteases in plant senescence is as intricate as reflected by the structural and functional diversity of the proteolytic enzymes that have been associated to this process (Roberts et al., 2012; Díaz-Mendoza et al., 2014). Although our knowledge on senescence-associated plant proteases keeps constantly expanding, for most of them many central questions such as the identity of their natural substrates, biological roles and regulation mechanisms still remain to be answered.

Two catalytic groups have been mainly related to plant senescence, cysteine proteases (CPs) and serine proteases (SPs). CPs, by far the most represented in senescent plant tissues as determined by transcriptomic studies (Bhalerao et al., 2003; Guo et al., 2004; Parrott et al., 2007), have been suggested to participate in leaf protein degradation during senescence in tobacco (Prins et al., 2008; Carrión et al., 2013), wheat (Martínez et al., 2007; Thoenen et al., 2007), barley (Parrott et al., 2010) and oilseed rape (Poret et al., 2016). Specifically, CPs belonging to the papain-like peptidase family C1A, as classified by the MEROPS database (Rawlings et al., 2016), seem to be strongly related to plant senescence development (Díaz-Mendoza et al., 2014). Moreover, the widely used senescence marker *SAG12* which expression strongly correlates with senescence progress in many plant species including barley (see references in Gregersen et al., 2013), encodes an L-like cathepsin belonging to the C1A family.

The role of SPs in senescence have received less attention although they are highly abundant in plants (Tripathi and Sowdhamini, 2006; Van der Hoorn, 2008). According to the MEROPS database plant SPs are divided into 14 families, of which the family S8, known as subtilisin-like proteases or subtilases, is one of the largest. As much as 56 subtilisin-like serine proteases have been identified in the proteome of *Arabidopsis thaliana*, while 63 have been found in rice (Tripathi and Sowdhamini, 2006), all of them belonging to the subfamily S8A. More recently, a total of 80 subtilase genes were identified in the genome of grape (Cao et al., 2014). Despite their abundance, information on the biological role of plant subtilases is still very limited (Schaller et al., 2012) and for many crop species even the number of subtilase genes is unknown. So far, only two wheat peptidases have been classified as subtilases in the MEROPS database, while in barley genome of the 346 counts of known and putative peptidases predicted, only one still unassigned peptidase sequence have been recognized as a member of S8A subfamily (Rawlings et al., 2016).

At present, there is evidence supporting both bulk protein degradation and highly specific regulatory and signaling functions for plant subtilases (Schaller, 2004; Rautengarten et al., 2005; Vartapetian et al., 2011). Subtilases mRNA expression has been shown to substantially increase during senescence in barley (Parrott et al., 2007; Hollmann et al., 2014). Also, increased enzymatic activity at senescence has been reported in different plant species such as wheat (Wang et al., 2013), *A. thaliana* (Martinez et al., 2015), and common bean (Budič et al., 2013). Very recently, the increase in SPs activity measured in leaves of *Brassica napus* senescing in response to N starvation has been attributed in part to four different subtilisins (Poret et al., 2016). In wheat, the purification and characterization of two highly active subtilisin-like proteases from senescent leaves has been previously reported (Roberts et al., 2003, 2006). These proteases were denominated P1 and P2, and it has been suggested that they play an important role in N remobilization during whole plant senescence (Roberts et al., 2011).

Taking into account our previous work in wheat, the aim of the present study was to examine the participation of subtilases in senescence-associated proteolysis in barley. For this purpose, subtilases enzymatic activity was analyzed in dark-induced and naturally senescent leaves of barley plants at the vegetative and reproductive stage. As a complementary approach, immunodetection of putative subtilases in barley leaf protein extracts was attempted by using a heterologous antiserum raised against subtilase P1 from wheat. In addition, barley databases were searched in order to identify sequences coding for putative subtilases related to P1 subtilase from wheat and its mRNA expression profile was examined by qRT-PCR in leaves senescing under dark-induced or natural senescence.

2. Materials and methods

2.1. Plant growth conditions

Studies were conducted in growth chamber or greenhouse. In both cases, barley (*Hordeum vulgare* L.) seeds of the cultivar Scarlett were germinated on filter paper soaked with distilled water for 48 h, before transferring to pots (five seedlings/pot).

2.2. Growth chamber experiments

Plants were grown on vermiculite, watered daily and fertilized every two days with nutrient solution (Hoagland and Arnon, 1950) containing 10 mM KNO_3 and maintained under a photoperiod of 16 h light/8 h dark, at 23 °C and an irradiance of 350 μmol m^{-2} s^{-1}. Fifteen days after sowing (DAS), the last expanded leaf (third leaf) of barley plants was excised (t = 0 d), placed in plastic boxes with distilled water and incubated in complete darkness to induce senescence or maintained under the light/dark cycle used for plant growth as control. Samples of the incubated leaves were taken at 0, 2, 4 and 6 days after detaching. For the analysis of natural senescence in young barley plants, samples of the second, third and fourth leaf were taken starting at 15 DAS and at 21, 25, 28, 32 and 34 DAS. Sampling period took place from full expansion to advanced senescence of the third leaf.

Leaf chlorophyll content was estimated by *in vivo* determination of the greenness index using a SPAD-502 (Konica Minolta Inc., Osaka, Japan). Growth chamber experiments were repeated at least three times. All samples were taken by triplicate.

2.3. Greenhouse experiment

Greenhouse studies were conducted at the School of Agriculture of the University of Buenos Aires in 2013 and repeated in 2014, obtaining similar results. Seedlings were sown in 6-L pots containing a mix of vermiculite, perlite and soil (1:1:2 v/v), fertilized every two weeks with nutrient solution (Hoagland and Arnon, 1950) containing 10 mM KNO_3 and watered periodically to keep substrate at field capacity. Leaf samples were harvested by quadruplicate at 125 DAS and dissected according to age. Plants at this stage, 5.5–5.7 of the Zadoks scale, (Zadoks et al., 1974) had both yellow and green leaves together with developing spikes, indicating a stage of active nutrient remobilization at monocarpic senescence. Three senescence ranks were defined for leaves: non-senescent leaves (NSL, including flag leaf and the last 2 developed leaves), senescent leaves (SL, showing about 40–60% of NSL chlorophyll content) and very senescent leaves (VSL, with less than 10% of NSL chlorophyll content).

Both in growth chamber and greenhouse studies, each replicate consisted of one pot containing five plants. Collected tissues were immediately frozen in liquid N_2 and stored at −80 °C.

2.4. Extracts preparation and biochemical determinations

Frozen leaves were ground with mortar and pestle in liquid N_2 and the powder obtained was extracted with 50 mM Tris-HCl