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SHORT COMMUNICATION

Subtilisin-like serine proteases involved in N remobilization during grain filling in wheat

Irma N. Roberts · Carla Caputo · Mariana Kade · M. Victoria Criado · Atilio J. Barneix

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Abstract The induction of two subtilisin-like proteases (P1 and P2) associated with stress-induced senescence in young plants was investigated in adult wheat plants during the grain-filling period. Western blot analysis of flag leaf extracts showed that P1 was induced very late in the life cycle of the plants (9 days post-anthesis) and that 7 days later it reached a 2.5-fold increase with respect to the initial value at anthesis. On the other hand, the P2 signal was already detected previous to anthesis and increased soon after anthesis, reaching a fourfold increase by the end of the grain-filling period. The induction of P1 and P2 temporally correlates with the degradation of the Rubisco small and large subunits in the flag leaf, as well as with nitrogen (N) accumulation in the ears. At the same time, a decrease in the endogenous concentration of the cytokinins isopentenyladenine and isopentenyladenosine (iP + iPA) in the leaves was observed. In detached leaves senescing in the dark, the levels of both proteases were affected by 6-benzylaminopurine application: the induction of P1 was completely prevented, whereas the induction of P2 was reduced. Our findings demonstrate that both P1 and P2 are expressed in leaves of adult plants and are induced during natural senescence. These results enable us to postulate their participation in N remobilization to developing grains during monocarpic senescence and their regulation by a cytokinin-mediated mechanism.

Keywords *Triticum aestivum* · Senescence · Subtilisin · Proteases · Cytokinins

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I. N. Roberts (✉) · C. Caputo · M. Kade · M. V. Criado
Facultad de Agronomía, Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA), Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina
e-mail: iroberts@agro.uba.ar

A. J. Barneix
Instituto de Suelos, CIRN INTA,
De los Reseros y Nicolás Repetto 1686, Hurlingham,
Provincia de Buenos Aires, Argentina

Introduction

Grain protein concentration is one of the main determinants of wheat baking and nutritional quality. Wheat plants usually accumulate 80–90% of their final nitrogen (N) content before anthesis (Cregan and van Berkum 1984). During grain filling, N is remobilized to the ears, and the final N content in grains is mainly a consequence of the efficiency of the remobilization process (Reed et al. 1980).

The main reserves of N in the leaves are plastidial proteins, which can represent up to 75% of the total N in mesophyll cells (Peoples and Dalling 1988). During leaf senescence, degradation of these plastidial proteins, mainly Rubisco (EC: 4.1.1.39), provides the N available for remobilization. Although the induction of several senescence-associated proteases has been reported (Ueda et al. 2000; Parrott et al. 2005; Martínez et al. 2007), their role in protein breakdown during senescence is still unclear and so is the mechanism of Rubisco degradation (Feller et al. 2008; Gregersen et al. 2008).

Previously, we have described two subtilisin-like serine proteases induced in senescent wheat leaves, named P1 and P2. These enzymes were observed in detached leaves incubated in the dark (Roberts et al. 2003), and their induction in intact young plants subjected to N starvation or darkness was later demonstrated (Roberts et al. 2006).

However, there are no data on the expression or activity of these enzymes in wheat leaves of adult plants senescing in an age-dependent manner.

To evaluate the participation of these proteases in natural senescence, we studied the protein levels of P1 and P2 in the flag leaf of wheat plants during the grain-filling period. In this regard, we have analyzed the evolution of several parameters that are indicators of the senescence progress, including the decrease in the concentrations of the cytokinins isopentenyladenine and isopentenyladenosine (iP + iPA), the earliest signal triggering protein degradation in senescent wheat plants induced by N starvation (Criado et al. 2007). To study the effect of cytokinins in P1 and P2 expression, 6-benzylaminopurine (BAP) was applied to detached leaves senescing in the dark, the experimental system that elicits the strongest induction of both proteases.

Materials and methods

Adult plants

Wheat seedlings (*Triticum aestivum* cv. Pro INTA Isla Verde) were sown in 5-L pots containing homogenized soil (0.2% total N content), fertilized twice a week with 50 mL of a nutrient solution (Hoagland and Arnon 1950) and watered daily. Pots were kept in a greenhouse, and a randomized block design with five blocks was applied. At different times from anthesis to maturity, flag leaves were excised, frozen in liquid N and stored at −80°C, and ears were dried at 60°C for 48 h. At maturity, biochemical analysis of the flag leaf could not be performed, due to its advanced state of senescence.

Young plants

Wheat seedlings were sown in vermiculite and watered daily with nutrient solution (Hoagland and Arnon 1950) containing 10 mM KNO_3. Plants were kept in a growth chamber under a photoperiod of 16-h light/8-h darkness at 23°C and with an irradiance of 350 μmol photons m^{-2} s^{-1}. Fifteen days after sowing, the third leaf of each plant was detached, placed in plastic boxes with distilled water with or without 5 μM BAP and incubated in continuous darkness for 3 days. Three samples of each treatment were taken every day.

Biochemical determinations

Total N concentration in the flag leaf and grains was determined through micro-Kjeldahl distillation after wet digestion in concentrated H_2SO_4 and H_2O_2.

Frozen leaves (0.25 mg FW) were ground with liquid N in a mortar and extracted with 1 mL of 50 mM Tris–HCl buffer, pH 7.5, containing 1% (w/v) polyvinylpolypyrrolidone and 2 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000*g* for 30 min, and the supernatant was used for protein (Bradford 1976) and chlorophyll (Arnon 1949) measurements. Protease activity in the extracts was determined using azocasein as substrate after desalting on Sephadex G-25 columns as previously described (Roberts et al. 2003). Proteins (1 mg FW) were separated on SDS-PAGE 15% (Laemmli 1970) and stained with CBB R-250. Immunodetection of P1 and P2 was achieved after separation of proteins (3.5 mg FW) on SDS-PAGE 10% and transfer to a PVDF membrane (Immobilon, Millipore) (Towbin et al. 1979), with an antiserum raised against a sample of purified P1, which is able to recognize both P1 and P2 as previously reported (Roberts et al. 2006). Control lanes corresponding to purified samples (0.2 μg) of P1 and P2 were included in the blots. Bound antibodies were detected with alkaline phosphatase-conjugated anti-mouse IgG and NBT/BCIP solution (Pierce Biotechnology, Inc., Rockford, IL, USA). The integrated density of the bands was measured using Image J software, freely available at http://rsb.info.nih.gov/ij/index.html. The cytokinins iP + iPA were extracted from leaf tissue, as previously described (Criado et al. 2007), and measured by ELISA using monoclonal antibodies (Phytodetek, Agdia, Elkhart, IN, USA) following the protocol provided by the manufacturer.

Both experiments were conducted twice with similar results; therefore, only one of the two assays is presented here.

Results

Plants reached 50% anthesis 80 days after sowing (DAS) and grain filling ceased 16 days after that, as demonstrated by the timing of ear weight gain (Fig. 1a) and N accumulation in the developing grains (Fig. 1b). Flag leaf N content (data not shown), chlorophyll (Fig. 1c), soluble proteins (Fig. 1d), Rubisco large subunit (RL) (Fig. 2) and endogenous iP + iPA content (Fig. 1e) started to decrease soon after anthesis. In contrast, the decrease in the Rubisco small subunit (RS) (Fig. 2) was observed much later, concomitantly with the higher increase in protease activity (Fig. 1f).

Western blot analysis of flag leaf extracts showed that P2, but not P1, was present in non-senescent leaves (Fig. 2). The P2 signal started to increase before anthesis and showed a higher increase at 96 DAS, whereas P1 started to increase at 89 DAS and reached a 2.5-fold increase a week later (Fig. 2).

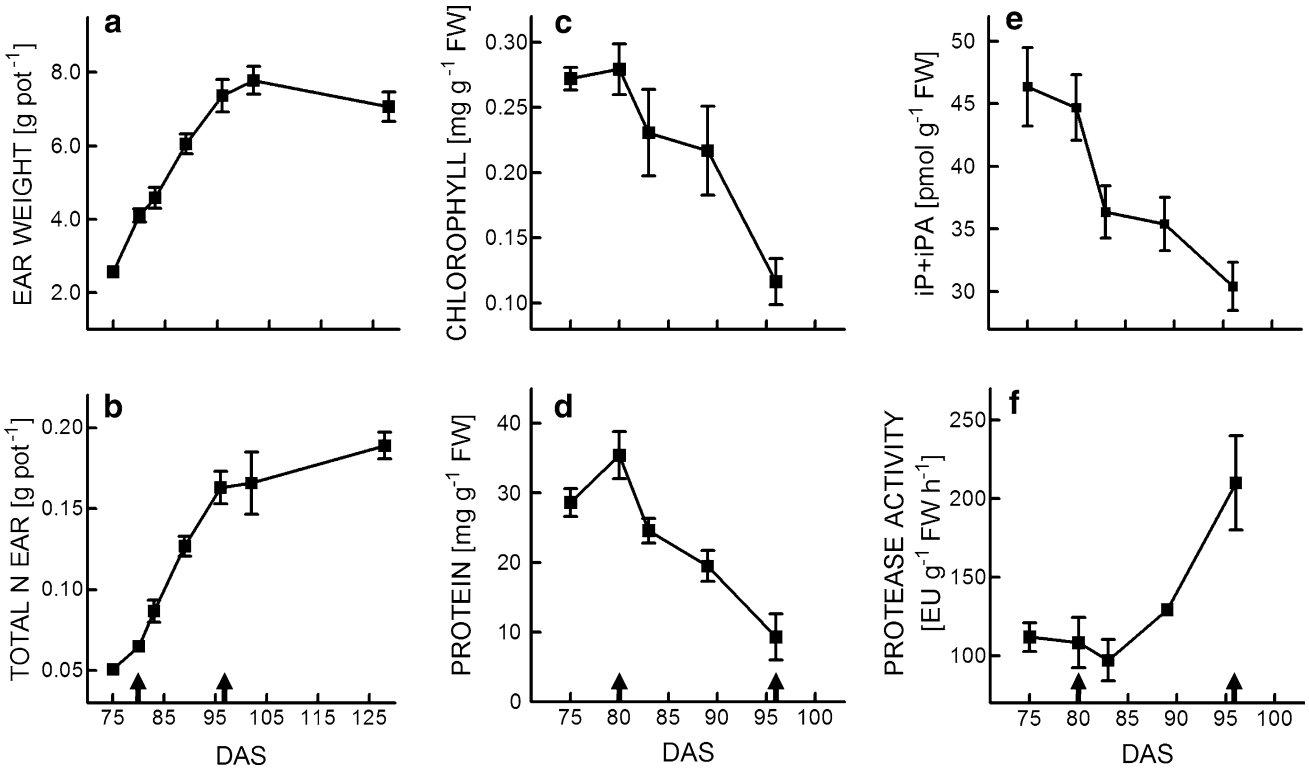

Fig. 1 Changes in ear weight (**a**), total N content in ears (**b**) and in the concentration of chlorophyll (**c**), protein (**d**), iP + iPA (**e**) and protease activity (**f**) in the flag leaf of wheat plants during grain filling. Data are the mean ± SE ($n = 5$). *Arrows* indicate the moment of 50% anthesis (80 DAS) and ending of grain-filling period (96 DAS)

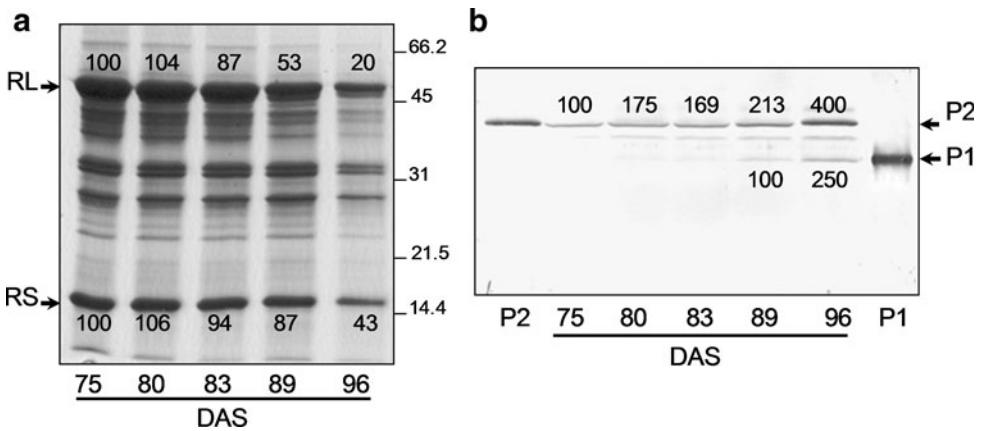

Fig. 2 **a** Changes in the levels of Rubisco large (*RL*) and small (*RS*) subunits in 15% SDS-PAGE stained with CBB R-250. The molecular masses of the protein markers are indicated on the *right*. **b** Immunodetection of *P1* and *P2* subtilisin-like serine proteases in the flag leaf of wheat plants during grain filling. Samples corresponding to 0.2 μg of purified *P2* and *P1* were included as controls. *Arrows* indicate the position of *RL*, *RS* and the immunoreactive bands. *Numbers* in *each lane* represent the average density ($n = 5$) of the bands as a percentage of the first sampling day (*RL*, *RS* and *P2*) or the first signal detected (*P1*)

The effect of cytokinins on P1 and P2 expression was investigated in dark-induced senescent leaves (Fig. 3). Totally expanded leaves were detached from young wheat plants and incubated in the dark for 3 days in the presence or absence of BAP. Both Rubisco subunits were degraded in the absence of BAP (Fig. 3b). RL started to decrease already after 1 day in the dark, whereas RS degradation was observed only after 3 days. Only P2 was present in non-senescent leaves (day 0) and increased with time, whereas the P1 signal was detected later, after 2 days in the dark. BAP addition reduced the dark-induced degradation of both Rubisco subunits and completely reversed the

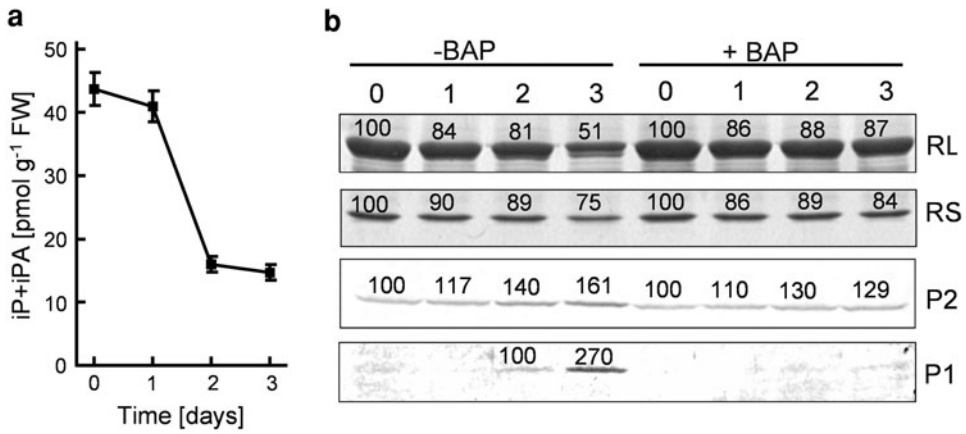

Fig. 3 **a** Concentration of iP + iPA in detached leaves senescing in darkness. Data are the mean ± SE ($n = 3$). **b** Changes in the levels of Rubisco large (*RL*) and small (*RS*) subunits in 15% SDS-PAGE stained with CBB R-250 and immunodetection of *P1* and *P2* subtilisin-like serine proteases in detached leaves senescing in darkness in the absence (−) or presence (+) of 5 μM BAP. *Numbers* in *each lane* represent the average density ($n = 3$) of the bands as a percentage of the first sampling day (*RL*, *RS* and *P2*) or the first signal detected (*P1*)

induction of P1. An attenuation effect on the increase in P2 was observed between the second and third day of treatment when BAP was applied, although the induction of this protease was not totally prevented. The endogenous concentration of iP + iPA decreased during incubation in darkness in control plants (Fig. 3a) and increased dramatically in the BAP-treated plants (data not shown).

Discussion

Senescence triggers the start of global changes in wheat leaf metabolism as shown by a coordinated and gradual decrease in total N, chlorophyll and soluble proteins (Figs. 1, 2). These changes characterize the remobilization of assimilates to the developing grains (Feller and Fischer 1994; Kamachi et al. 1991). However, there are still many questions to be answered. In particular, little is known about the proteases involved in senescence. The present work was conducted to study the involvement of two previously identified wheat proteases (P1 and P2) in protein degradation during natural senescence. P1 and P2 are the two main proteases involved in stress-induced senescence of leaves of young plants in wheat (Roberts et al. 2003) and are strongly induced in detached leaves senescing in the dark and intact plants subjected to N starvation (Roberts et al. 2006). Despite the widespread use of these and other methods to artificially induce the senescence of plant tissues, the results obtained should always be validated in naturally senescing tissues. We demonstrated that both P1 and P2 were indeed expressed and induced in the flag leaf of adult wheat plants during the grain-filling period. However, a clear difference was observed in the timing of expression of both proteases. The P2 signal was already present previous to anthesis, whereas P1 was detected much later in senescent leaves. The present results are in agreement with our previous reports on young plants subjected to senescence induced either by dark or N starvation. In those experiments, P2 increased soon after the induction of senescence, whereas P1 was induced much later, even after the characteristic oxidative burst preceding cell death (Roberts et al. 2006; Criado et al. 2007). The late induction of P1 points to a possible involvement in the execution of cell death. On the other hand, the early induction of P2 suggests that its activity is most likely directed at protein degradation along the first stages of senescence, when the integrity of the cells is still maintained. However, and since N concentration in ears keeps on increasing until very late, both P2 and P1 activities seem to contribute to N remobilization to the developing grains in advanced senescent tissues.

The decrease in soluble protein concentration observed after anthesis (Fig. 1d) was caused mainly by degradation of Rubisco, which contributes up to 50% of the leaf soluble protein (Lawlor et al. 1989). In this experiment, Rubisco content decreased from anthesis to the end of ear growth (Fig. 2), concomitantly with a decrease in protein content in the flag leaf (Fig. 1d) and N accumulation in the developing grains (Fig. 1b). RL degradation started earlier than RS degradation, in agreement with that reported by Bernard et al. (2008). The greatest decrease in RL and RS content was recorded between 89 and 96 DAS, correlating with the main increase in P1 and P2 (Fig. 2), suggesting that both proteases might be involved in Rubisco degradation.

Numerous physiological studies have demonstrated that the level of endogenous cytokinins decreases in most senescing tissues and that exogenous application of cytokinins causes a delay in senescence (Gan and Amasino 1997;

Buchanan-Wollaston et al. 2003; Criado et al. 2007). In addition, we have shown that a sharp decrease in endogenous iP + iPA content is the earliest event associated with protein degradation and senescence development in young wheat plants when N is depleted from the nutrient solution (Criado et al. 2007). In agreement, we observed that both under natural or dark-induced senescence, a decrease in iP + iPA concentration precedes the induction of proteolytic activity in wheat leaves. These findings, together with the fact that P1 induction was repressed and that P2 was attenuated by BAP application, suggest a cytokinin-mediated regulation of these proteases. However, only P1 was synthesized de novo after iP + iPA concentration decreased in advanced senescent leaves. P2, instead, was already present at basal levels in non-senescent leaves and showed a two-stage induction. A first increase at the beginning of the senescence phase before any variations in iP + iPA concentration was observed, and a second increase later, after the decrease in cytokinins. These findings suggest that either P2 is very sensitive to small variations in cytokinin concentration or that there are two pathways for P2 induction: one operating earlier and independently of cytokinin levels and another one working later when the repressor effect of this hormone has been abolished. The latter hypothesis also provides an explanation of the limited effect of BAP on this protease in detached leaves senescing in the dark.

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