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Vacuole cysteine proteases and ribulose-1,5-bisphosphate carboxylase/oxygenase degradation during monocarpic senescence in cowpea leaves

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Abstract

Characterisation of proteases degrading ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO, EC: 4.1.1.39) was studied in the cowpea leaf during monocarpic senescence 3 and 9 d after flowering (DAF), representing early and mid pod fill. The stage at 3 DAF coincided with decrease in the metabolic parameters characterising senescence, *i.e.*, contents of total soluble proteins, RuBPCO, and leaf nitrogen. At 9 DAF, there was a decline in total soluble proteins and an appearance of a 48 kDa cysteine protease. Characterisation of the proteases was done using specific inhibitors. Subcellular localisation at 3 DAF was studied by following the degradation of RuBPCO large subunit (LSU) in the vacuole lysates using immunoblot analyses. Cysteine proteases played a predominant role in the degradation of RuBPCO LSU at the crude extract level. At 9 DAF, expression of cysteine protease isoforms was monitored using polyclonal antibodies against papain and two polypeptides of molecular masses 48 and 35 kDa were observed in the vacuole lysates. We confirmed thus the predominance of cysteine proteases in the vacuoles during different stages of pod development in cowpea leaf.

Additional key words: endopeptidase; iodoacetamide; net photosynthetic rate; D,L-norleucine; phenyl methyl sulphonyl fluoride; pod; proteins; *Vigna unguiculata*.

Introduction

Monocarpic senescence is the final phase of the development of the plant, initiated by the appearance of the pod/grain, and ultimately leads to the death of the plant. It is an internally programmed degeneration during which leaf cells experience sequential disorganisation of cellular organelles coupled with dramatic changes in the cellular metabolism (Nam 1997, Nooden *et al.* 1997). The metabolic changes occurring during leaf senescence include loss in photosynthetic activity and hydrolysis of macromolecules such as proteins and lipids. This hydrolytic activity is concomitant with the massive mobilisation of the hydrolysed compounds to the developing pod/grain (Hayati *et al.* 1995, Pennell and Lamb 1997). Thus, leaf senescence is not just a deteriorating process, but is also a critical process needed for the fitness of the plant and is regarded as an evolutionarily acquired genetic process (Roach 1993, Park *et al.* 1998).

Proteases are hydrolytic enzymes involved in the breakdown of proteins making them available for the developing reproductive sink during monocarpy. The process of proteolysis has potential biotechnological implications since crop productivity may be improved by regulating protein degradation (Vierstra 1996). Identification of new proteases, their localisation and characterisation is an on-going process for a better understanding of the mechanism behind proteolysis (Callis 1995). Senescence is often studied in detached systems and induced by environmental factors such as irradiance, temperature, *etc.* Molecular analysis of senescence associated genes

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Abbreviations: DAF, days after flowering; LSU, large subunit; β-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PCD, programmed cell death; PMSF, phenyl methyl sulphonyl fluoride; PVPP, polyvinylpolypyrrolidone; RuBPCO, ribulose-1,5-bisphosphate carboxylase; SDS, sodium dodecyl sulphate; SSU, small subunit; TCA, trichloracetic acid.
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(SAGs) has revealed significant differences in their expression in natural senescence *versus* artificially induced senescence in barley and *Arabidopsis* (Becker and Apel 1993, Weaver *et al.* 1998). Hence, the results obtained from detached systems should be extrapolated with caution to whole plant senescence occurring under natural environments.

Ribulose-1,5-bisphosphate carboxylase (RuBPCO; EC 4.1.1.39) is an important storage protein as it is a major nitrogen source providing amino acids for developing organs. It comprises about half of the total soluble proteins in the leaf (Peoples and Dalling 1988). RuBPCO is rapidly and selectively degraded during natural and stress induced senescence (Moreno *et al.* 1995). An understanding of the mechanism of breakdown of RuBPCO in naturally senescing leaves is required to establish an optimal use of leaf nitrogen as the major amino acid source of seed storage proteins in cereals and legumes (Mae *et al.* 1984, Srivalli and Khanna-Chopra 1998).

Among the various proteases, cysteine proteases have been implicated in the degradation of RuBPCO (Bhalla and Dalling 1986). The enhancement of their activity during leaf senescence was documented at the molecular level as seen by the increase in the transcript levels of the genes (Drake *et al.* 1996). They also emerge as the predominant enzymes involved in the self-destructive processes during senescence and other programmed cell death (PCD) processes in plants such as apoptosis, necrosis, *etc.* (Solomon *et al.* 1999). The expression of the cysteine proteinases during senescence varies in different plants and is subject to several regulatory factors (Xu and Chye 1999).

A question often raised is whether there are specific proteases involved in RuBPCO degradation during natural senescence and which proteases are more predominant? With this perspective in mind, we studied previously in a determinate cowpea (*Vigna unguiculata* cv. Komal) a tight nodal linkage between the source leaf and the developing pod (sink) at its axil (Khanna-Chopra and Reddy 1988). Our earlier studies during monocarpic senescence in the above system showed a bimodal pattern of endopeptidase activity, both at pH 4.8 and 7.0. We also observed that a 48-kDa cysteine protease, antigenically similar to papain appeared during pod development, the content of which increased up to 9 DAF (Khanna-Chopra *et al.* 1999).

In the present study we tried to characterise the subcellular localisation of proteases during monocarpic senescence at 3 and 9 DAF. The characterisation of proteases was done by measuring total proteolytic activity in crude leaf extracts using RuBPCO as a substrate. At 3 DAF we followed the degradation of RuBPCO LSU at the subcellular level using immunoblot analyses.

Materials and methods

Plants: A determinate cowpea (*Vigna unguiculata* L. cv. Komal) was grown in the field of Water Technology Centre, Indian Agricultural Research Institute, New Delhi following standard agronomic practices. The soil in the experimental area was of sandy loam type. Seeds were inoculated with *Rhizobium* sp. cowpea miscellany strain No. 6054 obtained from the Division of Microbiology, I.A.R.I., New Delhi. Commercial fertilisers were applied at the rate of 2 : 4 : 4 g m^{-2} of N, P, and K, respectively, at the time of sowing.

Sampling was done at three-day intervals from the day of flowering up to pod maturity. The leaf at the sixth node having flowers/fruits in the axil was used for biochemical analyses. The midrib was removed and the leaves were cut into small pieces after determining their fresh mass. All steps were carried out at 4 °C, unless otherwise stated. Three replicates were created for all measurements. The plant materials were dried in an oven at 80 °C to obtain dry matter. Characterisation of the proteases was studied in leaf samples collected 3 and 9 DAF.

Net photosynthetic rate (P_N) of cowpea leaves was measured in the morning from 10:00-11:00 using *Licor-6200* portable photosynthesis instrument (*Licor*, USA).

RuBPCO activity was estimated by RuBP-dependent incorporation of $^{14}CO_2$ into acid-stable products using the modified method of Jiang *et al.* (1993). 0.5 g of frozen leaf material was ground to a fine powder in liquid nitrogen and suspended in 3 cm^3 of 50 mM Bicine-KOH buffer, pH 7.8, containing 20 mM KCl, 5 mM DTT, 0.1 mM Na_2-EDTA, and 0.1 mM PMSF. 2 % (m/v) PVP was added exogenously. Aliquots were assayed for RuBPCO activity and total soluble protein using bovine serum albumin as a standard (Lowry *et al.* 1951). For determining RuBPCO activity, 10 mm^3 aliquots were incubated with 480 mm^3 of assay buffer containing 100 mM Bicine-KOH buffer, pH 8.2, 20 mM $MgCl_2$, 5 mM DTT, and 250 mM $NaH^{14}CO_3$ (specific activity 1 839 GBq mol^{-1}) at 25 °C for 9 min, after which 10 mm^3 of 20 mM RuBP was added. The reaction was terminated after 1 min by the addition of 200 mm^3 of 3 M HCl, and the sample was dried at 80 °C. The acid-stable compounds labelled with ^{14}C were estimated with liquid scintillation counter (*Wallac 1409, Pharmacia*).

Total nitrogen content in the leaves was determined in dried samples by *Kjeltec 1030 Autoanalyzer* (*Tecator*, USA) after digestion in sulphuric acid.

RuBPCO content: Frozen leaf samples were ground in a mortar with liquid nitrogen and extracted (3 cm^3 per 0.25 g fresh mass) in 30 mM Tris buffer, pH 7.8, containing 1 mM ascorbic acid, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF (Zivy *et al.* 1983). PVPP was added at the time of grinding [0.05 g per 1 g(FM)]. The homogenate was centrifuged at 10 000×*g* for 20 min. The extracted samples were passed through cheesecloth and centrifuged at 10 000×*g* for 20 min. To the supernatant, eight volumes of acetone were added and the protein precipitated overnight. The samples were again centrifuged at 10 000×*g* for 20 min. To the pellet, electrophoresis sample solution (125 mM Tris-Cl buffer, pH 6.8, 10 % glycerol, 5 % β-ME, and 2 % SDS) was added and boiled for 4 min. Total soluble proteins was measured according to Lowry *et al.* (1951). Aliquots of 20 mm^3 of the protein samples were subjected to electrophoresis on a 10 % SDS-PAGE (Laemmli 1970). In all cases 30 µg of total protein was loaded. After electrophoresis, the gel was stained with Coomassie Brilliant Blue *G-250* (*Sigma Chemical Co.*, St. Louis, USA) and destained with a methanol (40 %) and glacial acetic acid (10 %) solution. RuBPCO LSU and SSU were quantified by scanning the gels using a laser densitometer (*Pharmacia LKB, Ultroscan XL*).

Preparation of antibodies: Papain (*Sigma Chemical Co.*, St. Louis, USA) was emulsified with Freunds incomplete adjuvant (2 mg of Tris-Cl per 1 cm^3, pH 7.5). 0.75 cm^3 was inoculated subcutaneously into a rabbit. This procedure was followed every week for three weeks. Blood was collected from the ear of the rabbit. Serum was prepared by incubating the blood at 37 °C for two hours and overnight at 4 °C. The serum was centrifuged at 2 000×*g* for 15 min. The clear supernatant was distributed into aliquots and stored at –80 °C. One such aliquot was tested for the titre level by ELISA using anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate antiserum with *p*-nitrophenyl phosphate as substrate. The titre obtained was 1 : 2 000. For localisation studies, purified antibodies of RuBPCO were obtained from the University of Agricultural Sciences, Bangalore. The titre was 1 : 3 000.

Proteases in the crude leaf extracts were characterised at 3 and 9 DAF using specific inhibitors (Cercos and Carbonell 1993). Endopeptidase activity was measured following the modified version of Peoples *et al.* (1983). Frozen samples were ground in a mortar using liquid nitrogen and suspended in 250 mM Tris-Cl buffer, pH 7.0 containing 10 mM β-ME [4 cm^3 per 1 g(FW)]. During homogenisation 2 % PVP (m/v) was added and the extract centrifuged at 10 000×*g* for 20 min at 4 °C. The supernatant was collected and passed through three layers of cheesecloth. The supernatant was dialysed overnight against 25 mM Tris-Cl buffer, pH 7.0, containing 10 mM β-ME. The reaction mixture contained 100 mm^3 of crude extract, 250 mm^3 of either 250 mM Tris-Cl buffer, pH 7.0, containing 10 mM β-ME or 250 mM sodium acetate buffer, pH 4.8 containing 10 mM β-ME and 150 mm^3 of RuBPCO. For inhibitor study, the reaction mixture was supplemented separately with 7.5 mm^3 of 2 mM PMSF, 37.5 mm^3 of 10 mM EDTA, 18.8 mm^3 of 25 µM DL-norleucine, and 75 mm^3 of 1 mM iodoacetamide solution for serine-, cysteine-, metallo-, and aspartate-proteases, respectively. All the inhibitors were dissolved in water except PMSF, which was dissolved in isopropanol. After incubation at 50 °C for 1 h, the reaction was stopped by adding 1 mm^3 10 % TCA solution, and incubated at 4 °C for 1 h. After centrifugation at 25 000×*g* for 10 min, TCA-soluble peptides generated during the reaction were estimated at A_{340} of the supernatants. Blanks were determined both for zero time and without substrate. In the latter case, the substrate was added after the termination of the reaction. One unit of proteolytic activity was defined as an increment of 0.01 in A_{340} in 1 h.

Vacuoles were isolated following the method of Yoshida and Minamikawa (1996). 20 g of fresh leaf material were cut into small pieces, put into 100 cm^3 of maceration medium containing 5 mM MES-KOH, pH 5.5, 0.2 % (m/v) cellulase, 0.05 % (m/v) pectinase, 0.4 M mannitol, and 1 mM $CaCl_2$ and incubated for 2 h at 25 °C with gentle oscillation. During the incubation, the maceration medium was changed twice. The medium containing protoplasts was passed through a single layer of cheesecloth and the filtrate was centrifuged at 100×*g* for 5 min. The pellet was washed three times with 5 mM MES-KOH, pH 5.5, containing 0.4 M mannitol and 1 mM $CaCl_2$. To the pellet 12 cm^3 of 5 mM Hepes-NaOH, pH 7.8, containing 0.2 M mannitol, 20 mM EDTA, and 10 % (m/v) *Percoll* (*Sigma Chemical Co.*, USA) was added. The suspension was allowed to stand for 30 min at 4 °C and centrifuged at 100 000×*g* for 20 min (*Beckman* ultracentrifuge using *SW 40 Ti* rotor). The top layer (1 cm^3) containing an abundance of vacuoles was collected. The vacuole preparations were dialysed overnight against 50 mM sodium acetate, pH 5.4, containing 10 mM β-ME. After centrifugation at 20 000×*g* for 5 min, the supernatant was used as vacuole lysate.

Protease activity determination using immunoblot analysis: 10 µg protein of the vacuole lysate was incubated with 120 µg of RuBPCO for 0, 15, 30, 60, and 120 min at 50 °C. The assay buffer used in the reaction mixture was 250 mM sodium acetate, pH 4.8, containing 10 mM β-ME. The total volume was 200 mm^3. The reaction was stopped using electrophoresis sample buffer. This served as the control. The content of total soluble proteins was estimated according to Lowry *et al.* (1951). 10 µg was loaded on a 10 % SDS-PAGE gel (Laemmli 1970) and proceeded for Western blotting using purified polyclonal antibodies raised against RuBPCO. This was

done for vacuole samples at 3 DAF. For inhibitor studies, PMSF (2 mM), iodoacetamide (1 mM), EDTA (10 mM), and D,L-norleucine (25 μM) were used as inhibitors for serine proteases, cysteine proteases, metallo proteases, and aspartate proteases, respectively.

Immunoblot analysis of cysteine proteases belonging to the papain family: 10 μg of the vacuole lysate was loaded onto a 10 % SDS-PAGE and proceeded for Western blotting. For Western blot analysis, proteins were electro-transferred at 4 °C onto nitrocellulose membranes (*Bio-Rad*, Richmond, CA, USA) at 50 V for 1 h in a *Transblot* unit (*Bio-Rad*, Richmond, CA, USA). Immunodetection was carried out with polyclonal antibodies directed against papain. The blot was incubated with the primary antibody and immunoreactive protein bands were visualised using anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate antiserum with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

Results

The pods of cowpea plants cv. Komal developed for a period of 18 d. The period was divided into three stages: early pod fill (0-6 DAF), mid pod fill (6-12 DAF), and late pod fill (12-18 DAF).

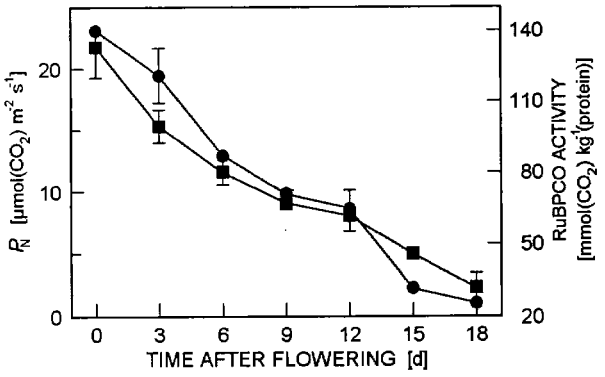

Fig. 1. Effect of pod development on net photosynthetic rate (P_N; •) and RuBPCO activity (■) in cowpea leaf. Vertical bars indicate SE ($n = 4$ for P_N; $n = 3$ for RuBPCO). In some cases error bars are smaller than the symbols.

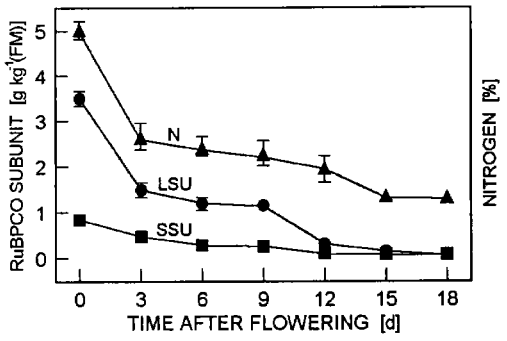

Fig. 2. Effect of pod development on degradation of RuBPCO large (LSU) and small (SSU) subunits and total nitrogen content (N) in cowpea leaf. Vertical bars indicate SE ($n = 3$). In some cases error bars are smaller than the symbols.

There was a gradual loss in P_N and RuBPCO activity during the early pod fill stage (Fig. 1). This was followed by a rapid rate of decline during the late pod fill stage. RuBPCO LSU declined faster than its small subunit (SSU) throughout pod development (Fig. 2). This decline in RuBPCO LSU was 59 % as compared to the 41 % decline of RuBPCO SSU in the first 3 DAF. There was a steady pattern of decline in leaf nitrogen during pod development with a sharp dip at 3 DAF (Fig. 2).

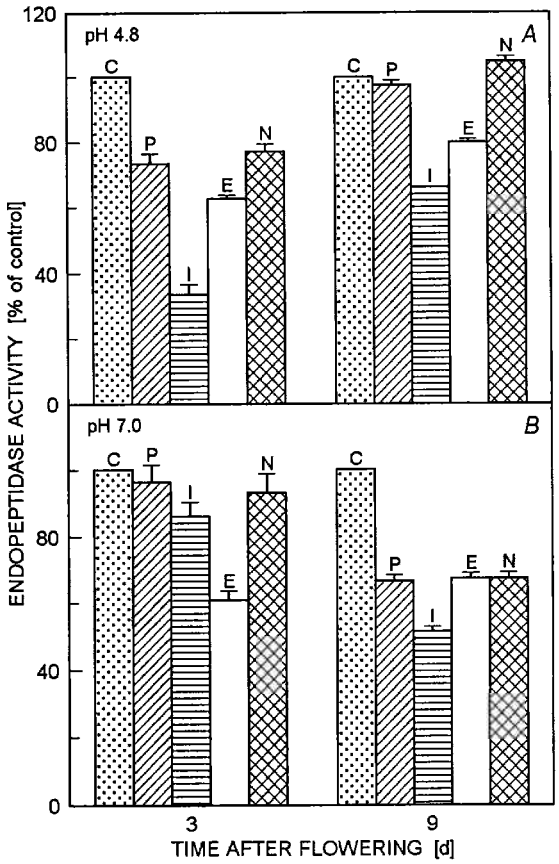

Fig. 3. Effect of inhibitors on endopeptidase activity at pH 4.8 (*A*) and at pH 7.0 (*B*) during pod development in cowpea leaf. Vertical bars indicate SE ($n = 3$). In some cases error bars are smaller than the symbols. C, control. P (PMSF), I (iodoacetamide), E (EDTA), N (DL-norleucine) are the inhibitors for serine-, cysteine-, metallo- and aspartate proteases, respectively.

Characterization of the endopeptidases was studied in the crude extracts at 3 and 9 DAF at pH 4.8 and 7.0. At pH 4.8, cysteine proteases were predominant in RuBPCO degradation at both 3 and 9 DAF because iodoacetamide inhibited the endopeptidase activity by 68 and 34 %, respectively (Fig. 3*A*). At 9 DAF, there was requirement

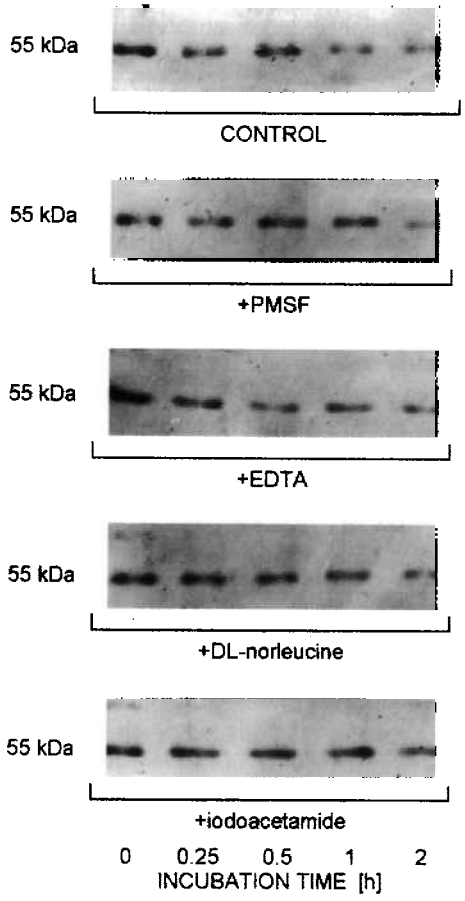

Fig. 4. Immunoblot showing the degradation of RuBPCO LSU which was incubated with the vacuole lysates with (*control*) and without the presence of the protease inhibitors at different time intervals. The experiment was done at 3 DAF. The reaction mixture was assayed at pH 4.8 at 50 °C. Purified anti-RuBPCO antibodies were obtained from Prof. M. Udaykumar, UAS, Bangalore, India.

of divalent cations because EDTA reduced the endopeptidase activity by 21 %. At pH 7.0, the predominant role in RuBPCO degradation was played by metalloproteases at 3 DAF, followed by cysteine proteases because EDTA and iodoacetamide inhibited the endopeptidase activity by 40 and 15 %, respectively (Fig. 3*B*). Cysteine proteases played a major role 9 DAF as iodoacetamide reduced the endopeptidase activity by 50 %.

When RuBPCO was incubated with the vacuole lysates at pH 4.8, there was a steady loss in the RuBPCO LSU at 3 DAF (Fig. 4). However, no degradation products were observed. The Western blot showed that within 15 min RuBPCO was degraded and continued to degrade with increase in time. Inhibitor studies revealed that at 3 DAF, degradation of RuBPCO LSU was inhibited in the presence of 10 mM iodoacetamide. Both PMSF, a serine protease inhibitor, and DL-norleucine, an aspartate protease inhibitor, also inhibited the degradation of RuBPCO LSU although to a lesser extent (Fig. 4). These results suggest that cysteine proteases play a predominant role in the degradation of RuBPCO in vacuoles at 3 DAF, although other proteases are also involved to a certain extent. These results were similar to those observed *via* inhibition of endopeptidase activity by protease specific inhibitors in crude extracts at 3 DAF.

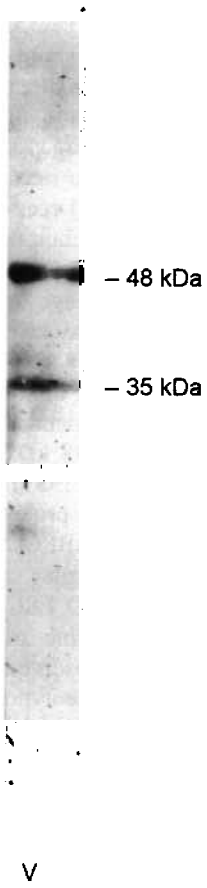

Fig. 5. Immunoblot showing the presence of polypeptides antigenically similar to papain in the vacuole lysates in cowpea leaf, 9 DAF. V, vacuole lysates. 10 μg protein was loaded in each lane. Immunoblotting using anti-papain antibodies was done according to Khanna-Chopra *et al.* (1999).

The expression of cysteine proteases was monitored in the vacuole lysates using polyclonal antibodies against papain in cowpea leaf at 9 DAF (Fig. 5). 48 and 35 kDa proteins antigenically related to the protease papain were present in the vacuole lysates.

Discussion

Monocarpic senescence was characterised by the simultaneous decline in P_N, RuBPCO activity and content (Figs. 1 and 2). In cowpea leaves, the degradation of RuBPCO LSU was faster compared to the SSU (Fig. 2). This is in contrast to studies in water stressed sugar beet, where SSU declined at a faster rate than the LSU (Dreesmann *et al.* 1994). In soybean seedlings both subunits degraded at a steady rate (Majumdar *et al.* 1990). This discrepancy was explained as a reflection of leaf age or differences in plant species. In cowpea leaf SSU may be a more stable protein during senescence.

Monocarpic senescence in cowpea observed in an earlier study showed a bimodal pattern of enhancement of the proteolytic activity with an appearance of a 48 kDa cysteine protease at 9 DAF (Khanna-Chopra *et al.* 1999). The results in crude extract showed that no single protease was responsible for the degradation of the RuBPCO protein although cysteine proteases appeared to play a predominant role (Fig. 3). Incubation of RuBPCO LSU with vacuole lysates resulted in a steady decline of RuBPCO LSU, but no degradation product was observed (Fig. 4). This may be because of multiple proteolytic activities involved, which makes the detection of partial breakdown products difficult (Vierstra 1996). Yoshida and Minamikawa (1996) observed in French bean leaves the degradation of RuBPCO LSU to a 41 kDa polypeptide through three intermediates of 50, 48, and 42 kDa involving a cysteine protease and a serine protease. Degradation products of RuBPCO were also observed in wheat when incubated with vacuole lysates (Bhalla and Dalling 1986). The mechanism of RuBPCO breakdown may differ in fully expanded green leaves and senescent leaves. Even in senescent leaves, differences may be between natural senescence and artificially induced senescence (Becker and Apel 1983, Park *et al.* 1998, Weaver *et al.* 1998).

Inhibitor studies revealed that cysteine proteases along with serine and aspartate proteases play a role in the degradation of RuBPCO in vacuoles at 3 DAF (Fig. 4). Recently, a cysteine proteinase, victorin, a host-selective toxin produced by *Cochliobolus victoriae*, the causal agent of victoria blight of oats was implicated in triggering senescence by inducing a specific proteolytic cleavage of the RuBPCO LSU (Navarre and Wolpert 1999). The importance of cysteine proteases in the degradation of RuBPCO LSU were shown in crops such as wheat (Bhalla and Dalling 1986, Nettleton *et al.* 1985) and barley (Miller and Huffaker 1982).

The 48 kDa cysteine protease observed during pod development was present in the vacuole lysates (Fig. 5) (Khanna-Chopra *et al.* 1999). Morris *et al.* (1996) showed in the detached leaves of *Lolium temulentum* papain-like antigens of lower molecular masses (<60 000) whereas a 58 kDa papain-like polypeptide was detected in the unpollinated ovaries of pea in the presenescent stage (Aguero *et al.* 1996). Cysteine proteases may be predominant in the vacuoles as compared to chloroplasts where serine proteases such as Clp proteases are more abundant (Clarke 1999, Sokolenko *et al.* 1997, 1998). This could be related to the autophagic nature of the vacuoles (Baba *et al.* 1994) and also by the fact that they have an abundance of active proteases.

Cysteine proteases have emerged as important enzymes in the regulation of phenomenon, such as necrosis, apoptosis, *etc.* (Kidd 1998, Kroemer *et al.* 1998). In soybean cells plant PCD can be regulated by activity poised between the cysteine proteases and the cysteine protease inhibitors (Solomon *et al.* 1999). Our studies showing the predominance of cysteine proteases during monocarpy in cowpea are in consonance with molecular studies in senescing leaves showing an increase in the transcript levels of cysteine proteases such as tomato SENU2 and SENU3 (Drake *et al.* 1996), *Arabidopsis* SAG2 (Hensel *et al.* 1993) and SAG12 (Lohman *et al.* 1994), and *Brassica* cysteine proteinase (Buchanan-Wollaston and Ainsworth 1997).

In conclusion, our study on proteases in cowpea leaf showed that cysteine proteases are important in degradation of RuBPCO LSU during monocarpic senescence. Two isoforms of cysteine proteases of 48 and 35 kDa were detected in the vacuoles. There was a preferential degradation of LSU as compared to SSU during pod development. Whether the degradation product(s) will be seen if RuBPCO is incubated at a much shorter interval remains to be further elucidated.

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