15

Proteinases and Their Inhibitors in Plants: Role in Normal Growth and in Response to Various Stress Conditions

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Introduction

For several decades proteinases have been isolated from numerous plant latices and juices, just as proteinase inhibitors (PI) were obtained from seeds and other plant storage organs. In the beginning they were recognised not so much out of interest in plant physiology but because they were easily available, of commercial value, and last but not least, of scientific interest as model substances for research. Thus papain is doubtless the most studied cysteine proteinase with its primary (Mitchell, Chaiken and Smith, 1970) and crystal (Drenth *et al.*, 1971; Kamphius *et al.*, 1984) structure established. Later it was used as a tool for studying the mechanism of catalysis (Polgar and Halasz, 1982) and its mechanism of interaction has been studied in the crystallised complex with its PIs, the cystatins (Stubbs *et al.*,1990). The results of these investigations have been of general value in both plant and animal kingdoms.

Plant PIs were first reported over 60 years ago. Soybean trypsin inhibitor was crystallised by Kunitz in 1947 and, in complex with trypsin, in 1974 (Sweet *et al.*, 1974). This inhibitor was the main tool for studies that led to the elucidation of the standard mechanism of proteinase-inhibitor interaction for the majority of serine proteinases (Laskowski and Kato, 1980).

With their possible application in biotechnology, plant proteinases have been increasingly recognised as key regulatory agents in many physiological processes. Their role, however, has been relatively well established only during germination and senescence, both examples of nutrient mobilisation. As for plant PIs, which would be

Abbreviations: ABA, abscisic acid; AP, aspartic proteinase; API, aspartic proteinase inhibitor; CP, cysteine proteinase; CPI, cysteine proteinase inhibitor; E64, (L-*trans*-epoxysuccinylleucylamido-[4-guanidino]-butane; GA, gibberellic acid; JA, jasmonic acid; MC, multicystatin; MJ, methyl jasmonate; MP, metallo-proteinase; MPI, metallo-proteinase inhibitor; PI, proteinase inhibitor; PR, pathogenesis-related; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SAG, senescence associated gene; SBTI, soybean trypsin inhibitor; SP, serine proteinase; SPI, serine proteinase inhibitor.

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expected to control the activity of these enzymes, they appear to play a role in plant protection, whereas their endogenous function is still a matter of speculation. Nevertheless the present knowledge of the levels, compartmentation and activity of both proteinases and their inhibitors indicates that many physiological processes depend on their delicate balance. Our intention is to try to distinguish between those proteinases and inhibitors which are involved in endogenous protein degradation, and those which protect against pest digestive enzymes or enzymes essential to fungal growth and plant cell virus replication.

The main aim of this chapter is to deal with plant proteinases (or endopeptidases), which are responsible for the initiation and early stages of protein breakdown. Carboxypeptidases and aminopeptidases (exopeptidases), although their roles in regulation of peptide hormones like systemin and the complementation of action of proteinases should not be forgotten, will deliberately not be considered. Proteinases and their inhibitors, singly or through their interaction, will be considered wherever they are important in unstressed plant tissues or in pathological or stress conditions.

The task is complicated by the facts that plants are much more diversified than animals in the taxonomic sense, and that a single plant takes very different forms during its ontogenetical development from reproduction and growth stages to senescence, in both morphological and biochemical senses. In addition, plants react to environmental changes both biochemically and physiologically, which further complicates the picture. It will be seen that few physiologically important proteinases and inhibitors are sufficiently well defined, most often their primary structures and localisation in an organ or within the cell being lacking, so it is difficult to draw parallels between different plants or even between different life cycles of a single plant. Since the events during the developmental stages seem to be the most unifying between plants, we propose to use them for division into sections (seed, growth and senescence).

The experimental problems inherent in handling plant systems will be dealt with in a separate section. Some of these result simply from the fact that plants, in comparison to animals, have been much less studied, so that the general approach has tended to be 'old fashioned' and the methods 'primitive'. Only in recent papers have levels, and more importantly ratios, amino acid sequences, and compartmentation of both proteinases and inhibitors, and activities of proteinases towards *in situ* substrates been included. Particularly in studies of the interactions of proteinases and inhibitors, kinetic measurements have lacked the rigour and sophistication developed for use in animal systems, and are often handled improperly. Other difficulties in handling plant systems derive from their diversity, which makes comparisons difficult, and from their structural peculiarities, including rigid cell walls and the huge amounts of storage proteins which can mask minute amounts of sensitive active components.

The proteinases are listed as cysteine (CP), aspartic (AP), serine (SP) and metalloproteinases (MP) as defined by Barrett (1986) (see *Table 1*). Within a single catalytic type, proteinases will be classified into families following the suggestions by Rawlings and Barrett (1993). The classification of inhibitors will follow the proteinases according to principles set out by Laskowski and Kato (1980), reviewed by Barrett (1987), Ryan (1989) and Richardson (1991) and extended with later developments (see *Table 2*).

The chapter will focus on more recent developments. Earlier work has been

Table 1. Plant proteinases and their possible involvement in stress

Proteinase type	Cereals	Leguminoseae	Solanaceae	Others
Cysteine	barley aleurone (11), (12) rice seed (9), (8) barley seed (10) wheat seed (14) buckwheat seed (15) maize (13)	soybean seed (2), (3), (4), (6) castor bean seed (3) jack bean seed (5) pea shoot (48)* pea chloroplasts (34)* soybean leaf (45) *Phaseolus vulgaris* pod (1)* wetch seed (7)	tomato leaf (39), (41)* tobacco leaf (42)* potato sprouting tuber (28), (29)	*Brassica napus* (20) *Thaumatococcus* (22) milkweed latex (21) *Arabidopsis* root – Ca^{2+} dependant (30)
Aspartic	barley seed (25),(26) wheat seed (27) barley leaf, root (35)	soybean cotyledon (44)		*Cynara* flower (38)* *Nepenthes* (43)
Serine	wheat chloroplast (47) wheat leaf IWF (32) maize root (36), (37) rice coleoptile (40) rice chloroplast (49)	*Alnus glutinosa* nodules (46)	tomato leaf (31)	*Maclura pomifera* fruit (16) sunflower leaf (17) *Cucurbita* seed (19), (23), (24) *Arabidopsis* all organs (46)
Metallo	maize (13) buckwheat (15)	soybean cotyledon (44) soybean leaf (18)		
Unclassified				spinach leaf (33) kale seedlings (50)

*, senescence or stress related
IWF, intercellular washing fluid
References: (1) Tanaka *et al.*, 1993; (2) Kalinski *et al.*, 1992; (3) Hara-Nishimura *et al.*, 1995; (4) Scott *et al.*, 1992; (5) Abe *et al.*, 1993; (6) Muramatsu and Fukazawa, 1993; (7) Becker *et al.*, 1995; (8) Shintani, Yamauchi and Minamikawa, 1995; (9) Watanabe *et al.*, 1991; (10) Marttila, Jones and Mikkonen, 1995; (11) Koehler and Ho, 1990; (12) Rogers, Dean, Heck, 1985; (13) Mitsuhashi and Oats, 1994; (14) Cejudo *et al.*, 1992; (15) Dunaevsky, Belozersky and Voskoboynikova, 1993; (16) Rudenskaya *et al.*, 1995; (17) Rudenskaya *et al.*, 1987; (18) McGeehan *et al.*, 1992; (19) Kaneda *et al.*, 1986; (20) Dietrich *et al.*, 1989; (21) Tablero *et al.*, 1991; (22) Cusack *et al.*, 1991; (23) Dryanski *et al.*, 1990; (24) Hamato *et al.*, 1995; (25) Sarkkinen *et al.*, 1992; (26) Tormakangas *et al.*, 1994; (27) Belozersky, Sarbakanova and Dunaevsky, 1989; (28) Michaud *et al.*, 1994; (29) Isola and Franzoni, 1993; (30) Reddy *et al.*, 1994; (31) Schaller and Ryan, 1994; (32) Pinedo, Segarra and Conde, 1993; (33) Ozaki *et al.*, 1992; (34) Liu and Jagendorf, 1986; (35) Runeberg-Roos *et al.*, 1994; (36) Batt and Walace, 1989; (37) Goodfellow, Solomonson and Oaks, 1993; (38) Cordeiro *et al.*, 1994; (39) Linthorst *et al.*, 1993a; (40) Lee and Lin, 1995; (41) Vera and Conejero, 1988; (42) Linthorst, Woon and Chambers, 1993b; (43) Tokes *et al.*, 1974; (44) Bond and Bowles, 1983; (45) Huangpu and Graham, 1995; (46) Ribeiro *et al.*, 1995; (47) Gray, Hird and Dyer, 1990; (48) Guerrero, Johns and Mullet, 1990; (49) Hiratsuka *et al.*, 1989; (50) Wilimowska-Pelc *et al.*, 1991.

Table 2. Plant proteinase inhibitors and their possible involvement in stress[†]

PROTEINASE TYPE inhibitor (super)-family	Cereals	Leguminoseae	Solanaceae	Others
SERINE PROTEINASES				
Bowman Birk	rice seed (7)	R. (4), pea seed (41)	potato tubers (42)	
Proteinase inhibitor I	barley seed (21)	broad bean seed (22)	tomato (23)*, tobacco (52)*	
Proteinase inhibitor II			potato (51), tomato, (49)* (50)* (23)*, tobacco (49)* (46)*	
Squash				squash, cucumber seeds (3)
Cereal 'probable bifunctional'	barley (10), ragi (8), rice (12) seeds			
Ragi trypsin/α-amylase	ragi (11), barley (9) seeds			
Thaumatin-like bifunctional	maize (15), barley (16) seeds		tobacco (47)*, tomato (28)* leaves	
Kunitz SBTI, subtilisin/α-amylase	rice (13), wheat, rye, triticale (14) seeds	R. (4), (5), winged bean nodules (27)*	potato tuber (33), (34), (37)	aroid roots (6), radish (29)*, rapeseed (26)*, spinach (43), poplar (53)* leaves
unclassified				arrowhead root (1), white mustard seed (2)
CYSTEINE PROTEINASES				
cystatin	corn (18), rice (19)	soybean (20), chick pea (45)	potato (24)*, tomato (38)*	avocado fruit (48)*, short ragweed (17)
Kunitz SBTI			potato tuber (33), (35), (39), (40)	
unclassified		*Enterolobium* (44)		pineapple stem (25)
ASPARTIC PROTEINASES				
Kunitz SBTI			potato tuber (30), (31), (32), (36), (37)	

[†], for some proteins inhibitory activity is putative, based on their sequence homology to known inhibitors.

*, senescence or stress related.

R., reviewed.

References: (1) Yang *et al.*, 1992; (2) Menegatti *et al.*, 1992; (3) Wieczorek *et al.*, 1985; (4) Laskowski and Kato, 1980; (5) Richardson, 1991; (6) Argall, Bradbury and Shaw, 1994; (7) Tashiro *et al.*, 1987; (8) Campos and Richardson, 1984; (9) Moralejo *et al.*, 1993; (10) Mundy and Rogers, 1986; (11) Campos and Richardson, 1983; (12) Yu *et al.*, 1988; (13) Ohtsubo and Richardson, 1992; (14) Mosolov and Shul'gin, 1986; (15) Richardson, Valdes-Rodrigues and Blanco-Labra, 1987; (16) Hejgaard, Jacobsen and Svendsen, 1991; (17) Rogers *et al.*, 1993; (18) Abe *et al.*, 1994; (19) Kondo *et al.*, 1990; (20) Brzin *et al.*, 1990; (21) Svendsen, Boisen and Hejgaard, 1982; (22) Svendsen, Hejgaard and Chavan, 1984; (23) Wingate, Franceschi and Ryan, 1991; (24) Walsh and Strickland, 1993; (25) Reddy *et al.*, 1974; (26) Reviron *et al.*, 1992; (27) Manen *et al.*, 1991; (28) King *et al.*, 1988; (29) Lopez *et al.*, 1994; (30) Mareš *et al.*, 1989; (31) Ritonja *et al.*, 1990; (32) Štrukelj *et al.*, 1990; (33) Brzin, Meško, Kregar, 1995; 34) Walsh and Twitchell, 1991; (35) Brzin *et al.*, 1988; (36) Meško *et al.*, 1989; (37) Ishikawa *et al.*, 1994; (38) Bolter, 1993; (39) Križaj *et al.*, 1993; (40) Rowan *et al.*, 1990; (41) Domoney *et al.*, 1995; (42) Hendriks, Vreugdenhill and Stiekema, 1991; (43) Trümper, Follmann and Haverlein, 1994; (44) Oliva, Sampaio and Sampaio, 1988; (45) Brzin (unpublished results); (46) Pearce, Johnson and Ryan, 1987; (47) Cornelissen, Hooft van Huijduijnen and Bol, 1986; (48) Dopico *et al.*, 1993; (49) Jongsma *et al.*, 1994; (50) Narvaez-Vasques, Franceschi and Ryan, 1993; (51) Hass *et al.*, 1982; (52) Linthorst *et al.*, 1993b; (53) Hollick and Gordon, 1993.

reviewed by Storey (1986) on general plant endopeptidases, Hatfield and Vierstra (1992) on protein turnover and storage protein formation, Bewley and Greenwood (1992) on protein utilisation in seeds, Feller (1986) and Huffaker (1990) on proteolytic activities during senescence, Dalling and Nettleton (1986) on proteolytic activities during chloroplast senescence and Vance, Reibach and Ellis (1986) on proteolytic activities in legume nodules and during nodule senescence.

Since multiple protein degradation systems operate within plant cells, several mechanisms and modes of control of proteolytic activity in plants are envisaged. They include synthesis (particularly the regulation of synthesis versus degradation), activation from proenzymes, their inherent high selectivity, their compartmentation (with possible influence of pH), redox potentials and metal ion concentration, and the presence of suitable endogenous inhibitors. Possible control of proteinase activity (as well as of their inhibitors, if involved) mediated by specific phytohormones will be emphasised particularly in relation to stress. The type and localisation of proteinase inhibitors will be considered in parallel, in an attempt to distinguish inhibitors which are intended for endogenous roles from those that are there to deter predators.

With the growing understanding of plant cells it is becoming increasingly apparent that the general organisation and activities within plant cells and animal cells are similar. Not everyone has been aware of this in the past, but it is advantageous to the plant biochemist, in terms of the ideas, approaches and methods available in the field of animal proteinase activity. Moreover, not only are some plant inhibitors recognised as potential anticancer drugs and as very specific inhibitors in the blood clotting cascade, but many animal inhibitors, when properly introduced into the plant genome, could help plants combat pests and other forms of stress, in a battle in which they now seem only partially successful.

Distribution, localisation and function of proteinases and their inhibitors

The majority of studies concern plants belonging to three families: Poaceae (later Graminaeae), Fabaceae and Solanaceae. The main work has been done on cereals (belonging to Poaceae), legume plants (Fabaceae) and tomato, potato and tobacco (belonging to Solanaceae). In the following part we shall consider plants according to these groups. All other examples are listed under 'Other Plants'.

The proteinases and PIs that are best characterised in terms of their properties are listed in *Table 1* and *Table 2* respectively.

SEED, TUBER AND FRUIT

Proteinases and their inhibitors have mostly been studied in seeds. Among them, CPs and their inhibitors are the best characterised.

Cysteine proteinases and inhibitors

Cereals. Among cereals, cysteine proteinases and inhibitors in barley have been studied in most detail. Three types of CP belonging to the papain family have been described. Aleurain was the first to be characterised (Rogers, Dean and Heck, 1985). This 32 kDa vacuolar CP which also shows aminopeptidase specificity appears to play

a specific role limited to intracellular protein catabolism, similar to that of mammalian cathepsin H (Holwerda *et al.*, 1990). The enzyme is stimulated by embryo-secreted plant hormones gibberellic acid (GA) and abscisic acid (ABA) and has not been observed outside the aleurone layer (Holwerda *et al.*, 1990), being in this respect similar to AP of barley (Törmäkangas *et al.*, 1994). It has been detected also in leaf and root tissues (Rogers, Dean and Heck, 1985) but is not involved in storage tissue mobilisation, resembling also in this respect AP (Törmäkangas *et al.*, 1994). Two other structurally closely related CPs of 30 kDa and 37 kDa (Koehler and Ho, 1990) appear during the first day and after three days of germination, respectively. They are synthesised *de novo,* in response to stimulation by GA, in the scutellar epithelium and aleurone cells, from where they are secreted to the starchy endosperm (Marttila, Jones and Mikkonen, 1995). It was shown that the 30 kDa CP degrades hordeins, the main storage proteins of barley endosperm (Poulle and Jones, 1988).

In other cereals, similar hydrolytic activities have been described in endosperm, mainly attributed to CPs, which degrade zeins in corn (Mitsuhashi and Oaks, 1994; de Barros and Larkins, 1990) and the 13S globular protein in buckwheat seeds (Dunaevsky, Belozersky and Voskoboynikova, 1993). They too, are synthesised *de novo*, increasing at different times after imbibition, or are present already in dry seed endosperm.

In wheat scutellum a cathepsin B-like CP has been described (Cejudo *et al.*, 1992), and a very specific CP, associated with the smaller ribosome unit, cleaving only methionyl-tRNA synthetase (de Vencay, Cenatiempo and Julien, 1991).

In rice seed, cDNA-deduced sequences of three CPs were found. Oryzains α and β are papain-like enzymes, while oryzain γ is aleurain or cathepsin H-like (Watanabe *et al.*, 1991). Oryzain β is present in dry seeds and the other two are synthesised *de novo* during germination. They are localised to aleurone or germ and their signal and prosequences suggest that they are secretory enzymes. Oryzain α isolated from rice seeds (Abe, Kondo and Arai, 1987) efficiently degrades glutelin, the major rice storage protein.

Rice was the first cereal plant in which inhibitors of CPs involved in germination were detected (Abe and Arai, 1985) and their gene and protein structures have been elucidated (Kondo *et al.*, 1990). They have all the features of cystatins and appear in the seed during the ripening stage. However, they disappear during germination, just after oryzains begin to appear (Kondo *et al.*, 1990).

Abe *et al.* in 1994 localised the corresponding corn cystatins by immunolocalisation, mostly to the aleurone layer and embryo, and only slightly in endosperm. This indicates that they are present at the site where all the *de novo* synthesised CPs engaged in storage protein degradation are synthesised at the onset of germination. Whether the physiological role of cereal cystatins is to protect against inappropriate endogenous proteolysis, against attacking insects, or both remains to be seen. Unanswered also are the questions as to whether cystatins are ubiquitous in cereals or limited only to rice and corn, and as to the way they are degraded during the early stages of germination.

Legumes. A rather complete picture has emerged of the content of CP in different leguminous seeds. CPs demonstrating the broad specificity of the papain superfamily have been isolated, among others, from vetch, *Vicia sativa,* (Shutov, Bulmaga and Vaintraub, 1984; Becker *et al.*, 1994) and moth bean, *Vigna aconitifolia*, (Kembhavi

et al., 1993). The latter was named vignain and shares the molecular mass 25–27 kDa of the other CPs from this group. They take part in general degradation of storage proteins for the growth of young seedlings, similarly to the CP described in cereal seeds. Although detectable in dry seeds their amount and specific activity increases significantly during the first days of germination, possibly due to *de novo* synthesis (Mitsuhashi and Minamikawa 1989; Kembhavi *et al.*, 1993), to some sort of activation from latent enzymes (Mitsuhashi and Minamikawa 1989; Becker *et al.*, 1994), or to inactivation of PIs, as proposed for mung beans by Baumgartner and Chrispeels (1976).

Proteins inhibiting papain as the test CP were in fact detected in cowpea seeds, *Vigna unguiculata*, (Rele, Vartak and Jagannathan, 1980) and subsequently more closely characterised (Fernandes *et al.*, 1991). They appear early after pollination during the seed filling phase. Their very low level in seed compared to trypsin inhibitors led the authors to the conclusion that they participate in endogenous metabolic control of CP rather than in seed protection. In soybean, inhibitors were identified as belonging to the cystatin superfamily (Brzin *et al.*, 1990) and are mostly concentrated in the hypocotyls of the seed (Hines, Osuala and Nielsen, 1991). The intracellular localisation of cystatins is unknown, as is their possible regulation of the CPs described earlier.

The second type of proteinase in leguminous seeds was originally designated as proteinase B in vetch (Becker *et al.*, 1995), legumain in moth bean, *Vigna aconitifolia*, (Kembhavi *et al.*, 1993) and vacuolar processing enzyme in castor bean and soybean (Hara-Nishimura, Inoue and Nishimura, 1991). This CP of molecular mass 33–39 kDa, active at acidic vacuolar pH, has rather atypical properties. It is selective for asparaginyl bonds and is not inhibited by E64 and cystatins, typical inhibitors of the papain superfamily of CPs. Some of its properties relate it to clostripain (Kembhavi *et al.*, 1993).

In legumes this CP contributes to the final stages of maturation of major storage proteins. The specific cleavage of proalbumins is essential for the final assembly of 11S albumins and proper deposition in protein bodies of storage tissue cells of maturing seeds (Scott *et al.*, 1992; Muramatsu and Fukazawa, 1993; Shimada *et al.*, 1994; Abe *et al.*, 1993).

The role of *de novo* synthesis of asparagine-specific proteinases in cotyledons of germinating seeds has, however, not been explained (Becker *et al.*, 1995). They may play a role in degradation of storage proteins after prior modification by papain-like CP, since they cannot cleave intact 11S proteins.

Asparagine-specific CPs are not limited to leguminous plants since similar activities and several cDNA homologues of the enzyme have been isolated in other plant genera (Hara-Nishimura *et al.*, 1995). Their major role seems to be vacuolar activation of many proteins from their inactive precursor forms, as exemplified by α-amylase inhibitor in common bean (*Phaseolus vulgaris*), (Pueyo, Hunt and Chrispeels, 1993), serine proteinase inhibitor (SPI) of PI-I family from tomato (Graham *et al.*, 1985) and tobacco leaves (Atkinson *et al.*, 1993) and acacia (*Acacia confusa*) Kunitz family trypsin inhibitor (Wu and Lin, 1993).

Regulation of asparagine-specific CPs is accomplished by strict developmental regulation of their expression and is limited to embryogenesis and germination. They are stored and activated in vacuoles and may not be active outside vacuoles since they

show acidic pH optima (Becker *et al.*, 1995). Their endogenous inhibitors are not known, and they are not inhibited by cystatins which are present in leguminous seeds (Fernandes *et al.*, 1991; Brzin *et al.*, 1990).

Solanacous plants. In solanacous protein storage organs only potato (*Solanum tuberosum*) tubers have been investigated for their content of CPs. A 25 kDa protease, sensitive to CP inhibitors, has been observed in ungerminated tubers (Isola and Franzoni, 1993) and a similar enzyme detected in early sprouting tubers (Kitamura and Maruyama, 1986). In the absence of SP, AP and MP an increased number of CPs were observed in the close vicinity of older sprouts during prolonged sprouting (Michaud *et al.*, 1994). Interestingly, the latter activities were not inhibited by oryzacystatins but their activity was reduced by E64 and iodoacetamide. The significance of these proteinases in the storage protein degradation during sprouting, despite their demonstrated ability to degrade the major soluble tuber proteins (Isola and Franzoni, 1993), is unclear, especially in view of the significant amounts of inhibitors of CP (CPI) shown to be present in potato tubers (Brzin *et al.*, 1988; Brzin, Meško and Kregar, 1995; Walsh and Strickland, 1993). It may be that these tuber CPs are not inhibited by the latter CPIs (no such experiment has been reported) or that the enzyme-inhibitor complexes can be dissociated under the rather harsh conditions used during the isolation of CP (Isola and Franzoni, 1993) or during their electrophoretic separation in gels (Michaud *et al.*, 1994), both of which could release protease activities that are not initially detected in the potato juice as starting material.

Potato tuber juice has proved to be a rich source of inhibitors of proteinases of several mechanistic classes, among which two different types of CPIs have been purified and characterised. The first group (Brzin *et al.*, 1988) proved to be one of the very few non-cystatin type of inhibitors, as its sequence classified it as belonging to the Kunitz type superfamily (Križaj *et al.*, 1993). The differences in inhibitory specificity also reflect those of distinct superfamilies (Rowan *et al.*, 1990). The very strong inhibition of human cathepsin L compared to papain may indicate that these potato CPIs are designed to encounter a specific potato or pest CP, similar to this important mammalian protease. These CPIs have in their preregion the characteristic motif that encodes common vacuolar sorting information (Chrispeels and Raikhel, 1992), in agreement with its recent immunolocalisation in vacuolar dense protein bodies. The potato CPIs are detectable in other plant organs only in minute quantities, but can be elevated upon induction with the plant hormone jasmonic acid (Gruden-Gričar, unpublished results).

Recently another type of CPI was isolated from potato tubers (Walsh and Strickland, 1993) and described as protein cubic crystals in the cytoplasm of peridermal cells of tuber (Rodis and Hoff, 1984). Its cDNA-derived sequence classifies it as a 85 kDa polypeptide (Waldron *et al.*, 1993) named multicystatin (MC), composed of 8 tandem cystatin domains which can be easily cleaved quite non-specifically by trypsin and some other proteinases into single cystatin inhibitory domains. Whether this process occurs naturally in tubers and is physiologically significant is uncertain, since the complete MC also has the capacity to bind 8 papain molecules simultaneously. MC accumulates as an intact 85 kDa polypeptide protein in tubers and in leaves under stress. MC is present in small amounts in potato leaves but is enhanced after mechanical wounding and reaches a maximal level of up to 2 % of total protein in

leaves (Akers and Hoff, 1980). Its expression can thus be influenced both developmentally and environmentally (Walsh and Strickland, 1993). No secretory peptide was found at the N-terminal, which confirms its cytoplasmic localisation.

Other plants. Cysteine proteinases from other plant families are poorly described where they have been isolated, or their properties are assumed from their cDNA sequences only. Dietrich *et al.*, (1989) described special expression of a gene in post-germinative growth in *Brassica napus*. It encoded an aleurain-like CP, supposedly not engaged in storage protein degradation. Thaumatopain, a papain-like CP degrading thaumatin, a plant protein known by its intense sweetness, was extracted from the arils of *Thaumatococcus danielli* (Cusack *et al.*, 1991).

CPIs are not abundant but are present in almost all plant species. Total papain inhibitory levels were determined in seed extracts from more than 30 plants of different botanical species. Low but comparable amounts were measured for all of them and the authors suggested their metabolic involvement, as opposed to the protective role of much higher amounts of trypsin inhibitors measured in parallel in the same seeds (Fernandes *et al.*, 1991). Two of these CPIs were partly characterised, a high molecular mass protein from *Enterolobium contortisiliquum* (Oliva, Sampaio and Sampaio, 1988) and a low molecular mass inhibitor from a cucurbit (Zimacheva, Ievleva and Mosolov, 1988).

Apart from CPIs from pineapple stem (Reddy *et al.*, 1975) all other CPIs whose primary structures have been elucidated, belong to the cystatin superfamily. Such is the cystatin-like major allergen protein from the pollen of short ragweed, *Ambrosia artemisiifolia* (Rogers *et al.*, 1993).

Aspartic proteinases and inhibitors

Cereals. Compared to animal, microbial and retroviral aspartic proteinases, those from plants are less well documented. APs have been purified from monocotyledonous rice (Doi *et al.*, 1980), wheat (Belozersky, Sarbakanova and Dunaevsky, 1989), barley (Sarkkinen *et al.*, 1992) and buckwheat (Elpidina, Dunaevsky and Belozersky, 1990). The primary structures of the barley (Runeberg-Roos *et al.*, 1994) and rice (Hashimoto *et al.*, 1992) APs relate them to yeast proteinase A and, even more closely, to human cathepsin D (Guruprasad *et al.*, 1994). APs in barley are present in vacuole-like protein bodies present in resting seeds in all tissues except endosperm, and their amount increases slightly during germination. They have been detected also in all living tissue of barley, roots, flowers and leaves. Their role is obscure, but must be that of one of the typical intracellular enzymes, that is vacuolar turnover, processing and activation of enzymes and other proteins, for example the processing of prolectin in roots (Runeberg-Roos *et al.*, 1994). AP shares its localisation with barley aleurain (Holwerda *et al.*, 1990) and is apparently not secreted to barley endosperm. This is in contrast to the reports that in wheat the majority of seed AP is localised in endosperm, and that the *in vitro* rate of hydrolysis of gliadin by *de novo* synthesised GA-dependent CP was dramatically enhanced when gliadin was first incubated with AP isolated from seeds (Dunaevsky, Sarbakanova and Belozersky, 1989).

No inhibition of AP has been observed in either cereal seeds or in any other part of the plant. In general, very few proteinaceous inhibitors of APs are known, which may

suggest that the fact that they are active only in the rather acidic media of vacuoles provides sufficient cellular protection. However, cereal seeds contain proteins of the Kunitz superfamily which in cereals play the role of α-amylase-subtilisin inhibitor. In potato several members of this superfamily are potent inhibitors of cathepsin D, therefore it is possible that similar proteins may be discovered in cereals as well.

Other plants. An AP was purified and characterised from seeds of cucumber and squash (Polanowski *et al.*, 1985), from two genera of pine seeds (Bourgeois and Malek, 1991; Salmia, 1981), and from *Arabidopsis* seed (D'Hondt *et al.*, 1993) processing its seed 2S albumin precursor *in vitro*.

Serine proteinases and inhibitors

Cereals. Serine proteinases in Poaceae seed are seldom mentioned in the literature. In germinating rice coleoptiles a trypsin-like activity was measured (Lee and Lin, 1995), but this is a rather special case of anaerobic germination. No other SP activity has been described in cereal grains, resting or germinating. Either they are not involved in proteolytic events in the seed, or they were possibly simply overlooked because of their narrow specificity or because of cohabitation with significant amount of SPIs.

Besides the rice bran trypsin and subtilisin inhibitor, with the structure of the duplicated Bowman Birk inhibitor (Tashiro *et al.*, 1987), and the barley grain CI-1 inhibitor of chymotrypsin and subtilisin (Svendsen, Boisen and Hejgaard, 1982), the many other cereal inhibitors had to await elucidation of primary structures to be classified into one of several evolutionarily distinct classes of bifunctional α-amylase/SP inhibitors (reviewed by Richardson, 1991).

The first group of inhibitors initially characterised from Indian finger millet (ragi) (Campos and Richardson, 1983) and later found in the endosperm of many cereals, inhibits bovine-type trypsin and exogenous α-amylase, some of the latter enzymes originating from the gut of pests. Molecular masses of these inhibitors are 13.3 kDa and they have high cysteine contents (10 cysteines per molecule).

The second group of inhibitors have molecular masses around 10 kDa, contain 7 cystatins, and have been isolated from the aleurone layer of mature cereal seeds (Campos and Richardson, 1984). They were called probable α-amylase protease inhibitors (Mundy and Rogers, 1986) because, in spite of their homology to other α-amylase inhibitors and to segments of Bowman Birk SPI, no target enzymes have been found for them. Their localisation in seed suggests their role as a protection against exogenous α-amylases and SP of pests.

Tryptic inhibitory activity and inhibition of beetle α-amylases is exhibited by the third type of bifunctional inhibitor from maize (*Zea mays*) seed (Richardson, Valdez-Rodriguez and Blanco-Labra, 1987). No defined target enzymes for the barley counterpart have been identified. The amino acid sequences of these 23 kDa proteins with high cysteine content (16 cysteines per molecule) relate them to solanacous PR5 (pathogenesis related) proteins (Cornelissen, Hooft van Huijsduijnen and Bol, 1986). Both proteins are structurally related to thaumatin and are therefore named thaumatin-like proteins, and are able to inhibit fungal growth by an as yet unknown mechanism (Hejgaard, Jacobsen and Svendsen, 1991).

Finally, the fourth type of bifunctional inhibitor, originally isolated from barley

endosperm and later also from other cereals, is specific for microbial protease, subtilisin and some fungal SPs. Its function is probably in seed protection and in inhibition of endogenous germination-specific α-amylases, possibly playing a role in the control of germination. Its amino acid sequence (Mundy, Hejgaard and Svendsen, 1984) and crystal structure (Zemke *et al.*, 1991) clearly place these proteins among the Kunitz superfamily of proteins. They exhibit quite diverse functions also in potato (Brzin, Meško and Kregar, 1995; Ishikawa *et al.*, 1994) and other plants: sweet potato (Hattori *et al.*, 1991), spinach, *Spinacia oleracea*, (Trümper, Follmann and Häberlein, 1994) and the shrub of the miracle fruit, *Richadella dulcifica* (Theerasilp *et al.*, 1989).

Legumes. Mammalian trypsin was used in the early days to identify a great number of leguminous SPIs. It was assumed that their role in seed ought to be one of controlling the action of endogenous SPs. Although some papers brought reports about SP activity in seeds from vetch (Shutov and Vaintraub, 1987), and soybean (Nishikata, 1984), it now seems clear that trypsin-like SP generally do not take part in seed protein formation and its general degradation during mobilisation. One could hardly imagine them to be active, surrounded with SP inhibitors constituting up to 30% of the total storage protein to be degraded, and in the acidic environment of vacuoles. Furthermore, it was shown that a powerful synthetic inhibitor of SP failed to prevent the degradation of the major storage protein in common bean (Nielsen and Liener, 1984), while on the other hand complete lack of Kunitz inhibitor activity in soybean (Orf and Hymowitz, 1979) has no effect on seed formation and germination.

It is generally accepted that leguminous SPIs of the Bowman Birk and Kunitz families (Richardson, 1991) are intended for protection from attack by pest and pathogens, and, during germination, utilised as a source for plantlet growth. A set of proteolytic activities degrading Kunitz trypsin inhibitors in germinating soybeans has been described (Wilson *et al.*, 1988).

Other plants. Several other SPs have been isolated from plants of various taxonomic classes, all confirming the absence of SP of the trypsin and chymotrypsin class in plants. Thus macluralisin from *Maclura pomifera* fruit (Rudenskaya *et al.*, 1995), cucumisin from melon seed and fruit (Kaneda *et al.*, 1986) and a proteinase from cucurbit seeds (Dryjanski *et al.*, 1990) are subtilisin-like proteinases. The seed enzyme is not inhibited by protein inhibitors of SPs like Kunitz, Bowman Birk and squash trypsin inhibitor from the same seeds. The latter is degraded by the proteinase; the significance of this fact is unknown (Dryjanski *et al.*, 1990).

Most other inhibitors of SPs belong to one of the already well established inhibitor families, as based on their amino acid sequences, position of reactive site and disulphide bridges, and common standard mechanism (Laskowski and Kato, 1980). In recent years these families have been expanded with new entries from additional plant sources, exhibiting interesting new inhibitory and other functions. Thus the novel inhibitor of the squash family (Wieczorek *et al.*, 1985) inhibits human cathepsin G and elastase (McWherter *et al.*, 1989) and β factor XIIa (Wynn and Laskowski, 1990), but no endogenous protease. New members of the Kunitz family have been found in Solanaceae monocotyledons and Araceae, tropical root crops (Hammer, Shaw and Bradbury, 1989; Argall, Bradbury and Shaw, 1994).

Two inhibitors characterised from white mustard (Menegatti *et al.*, 1992) and

arrowhead (Yang *et al.*, 1992) cannot be classified in any existing inhibitor families, on the basis of detailed knowledge of their primary structures and activities. Whether they constitute distinct families remains to be seen.

Other proteinases

Among other proteolytic activities, two metalloenzymes have been described in maize and buckwheat extracts (Mitsuhashi and Oaks, 1994; Dunaevsky, Belozersky and Voskoboynikova, 1993). The structure and function of these enzymes are not known, but they are supposed to accompany other proteinases in certain steps of storage protein cleavage.

LEAVES

The status of proteinases and their inhibitors in mature leaves growing under steady state turnover is the least well-defined. There have been many studies but proteinases at least have often been only generally described without defining their catalytic class, localisation and *in situ* substrate (Storey, 1986). Degradation processes in leaves, which are a part of normal turnover, take place in the neutral or slightly alkaline environment of the cytosol, in the acidic environment of lysosomes and in chloroplasts with an alkaline milieu. The mitochondrial, cell wall and nuclear compartments are not well documented. The pH of these cell compartments is an important factor in the regulation of proteinase activity, with the additional possibility of regulation by the environmental redox potential. Finally, intracellular localisation of proteinases limits the zone of their activity, forming part of the overall control of proteolysis. Bearing in mind these factors, proteinases in this section are grouped under their respective locations, to the extent that this is known. The role of PI in leaves has only recently become a subject of study.

Vacuoles

A cDNA clone encoding a CP was obtained from tomato leaves (Linthorst *et al.*, 1993a). Its signal sequence suggests putative vacuolar localisation. Its sequence shows that it is related to tobacco leaf CP (Linthorst *et al.*, 1993b) and clearly assigns it to the papain superfamily. It is also clearly distinct from the cold-induced CP of tomato (Schaffer and Fischer, 1988).

In cereals, acidic proteinase activity at pH 4.5 was reported in vacuoles from oats (*Avena sativa*) leaves (Van der Valk and Van Loon, 1988) and shown to belong to AP in barley (Runeberg-Roos *et al.*, 1994). The vacuoles obtained from isolated barley protoplasts were shown to contain the entire AP activity of leaf protoplasts. The same AP was immunolocalised also in vacuoles of barley root cells. Degradation of endogenous proteins seems to be regulated by their selective transfer into vacuoles, as suggested by Canut *et al.* (1986) in sycamore (*Acer pseudoplatanus*) cells.

Vacuoles in leaves, being highly proteolytically active, would not be expected to contain significant amounts of inhibitors, particularly of APs and CPs which are the two major components. Indeed, only SPI of the PI-I and II type have so far been shown to be compartmentalised in vacuoles in tomato leaf cells (Walker-Simmons and Ryan, 1977). In tuber and seed vacuolar protein bodies the presence of these and other PIs can be explained by their regulatory function during the resting period.

Chloroplasts

Proteolytic activities in chloroplasts are difficult to characterise as they seem to be of high specificity and low activity, involving danger of contamination from other organelles. The total activity was found to increase during leaf growth and decrease during leaf senescence (Ragster and Chrispeels, 1981). A 93 kDa protease sensitive to sulphydryl reagents has been described in stroma of pea chloroplasts (Liu and Jagendorf, 1986). It has a maximum activity at pH 7.7, is Ca^{2+} or Mg^{2+} dependent and ATP-independent. Earlier, an ATP-dependent activity was detected in thylakoid membranes (Liu and Jagendorf, 1984). The role of these proteases seems to be the removal of abnormal and mature, but not assembled, proteins (Malek *et al.*, 1984) or the degradation of other chloroplast-specific proteins (Robinson and Ellis, 1984). Wheat (Gray, Hird and Dyer, 1990) and rice (Hiratsuka *et al.*, 1989) chloroplasts contain an ATP-dependent proteinase belonging to the S14 SP family (Rawlings and Barrett, 1993).

No PIs have been found in chloroplasts, apart from a remarkable protein from spinach (Trümper, Follmann and Häberlein, 1994). The first 14 amino acids of this protein are identical to soybean Kunitz inhibitor. It functions as an inhibitor of SPs in its oxidised form and as dehydroascorbate reductase when reduced. Its target enzyme is not known. Whether this uncommon regulation is reserved for this protein, or will be found for other members of the Kunitz superfamily (Križaj *et al.*, 1993; Ishikawa *et al.*, 1994) remains to be seen.

Cytoplasm

Very few proteinases have been assigned unambiguously to the cytoplasm, probably because of the difficulties of contamination from other organellar structures. Localisation within the cytoplasmic compartment was assumed for an Azocoll degrading neutral proteinase in oats (Van der Valk and Van Loon, 1988). Better defined are the multicatalytic protease complexes or proteosomes, which are responsible for the proteolysis sometimes linked with ubiquitin in the cytosol and nuclei of all eucaryotes (Schliephacke *et al.*, 1991; Rivett, 1993). Recently they have been found in plants as well, including spinach leaves (Ozaki *et al.*, 1992) and potato cells (Vallon and Kull, 1994). Trypsin- like and chymotrypsin-like activities were measured *in vitro* (Ozaki *et al.*, 1992) at neutral and slightly alkaline pH, but the catalytic site of proteosomes is still unknown (Rawlings and Barrett, 1993).

The role of plant proteosomes is not understood. It is assumed that they are involved in the non-lysosomal ATP-dependent pathway of protein breakdown, possibly of the cytoskeleton network and of short-lived regulatory proteins. Since the structure of proteosomes and the sequence of oats ubiquitin (Vierstra, Langan and Schaller, 1986) – only three among the 76 amino acids of human ubiquitin are substituted – are remarkably conserved, highly effective control of proteosome activity through co-operative subunit regulation can be expected, similar to that in mammalian proteosomes (Orlowski, 1990).

CELL WALL AND EXTRACELLULAR SPACE

Cell walls and intercellular space also contain proteolytic activities which mostly belong

to the serine class. In intercellular washing fluid of wheat leaves a proteolytic activity assigned to the serine type was reported (Pinedo, Segarra and Conde, 1993). The suggested role of this high molecular mass protease was the turnover of some constitutive proteins of intercellular space. In tomato leaf plasma membrane a 50 kDa SP was identified (Schaller and Ryan, 1994). This subtilisin-like enzyme has been be classified among prohormone convertases of the SP family (Rawlings and Barrett, 1993).

In the extracellular fluid of common bean (*Phaseolus vulgaris*) a 37 kDa proteinase with alkaline pH optimum was found, probably localised in the cell wall (Van der Wilden, Segers and Chrispeels, 1983). A similar 60 kDa proteinase was described in young leaves of soybean (Huangpu and Graham, 1995). Their activities, tested by inhibitors, resemble that of CP but their physiological role is a matter of speculation. A 69 kDa proteinase with some characteristics of a CP and corresponding to major PR protein band was described in tomato leaves (Vera and Conejero, 1988). From tobacco leaves, however, a 36 to 40 kDa extracellular AP was isolated, possibly involved in PR protein degradation (Rodrigo *et al.*, 1991).

ROOT AND FLOWER

In a soluble protein extract of *Arabidopsis* root culture, a calcium-dependent CP was observed (Reddy *et al.*, 1994). This calpain-like activity, rarely reported in plants, is common in vertebrates, invertebrates and fungi. The possibility of its regulation by Ca^{2+} concentration has been considered. Cyprosin, an AP, possibly involved in the senescence process, was isolated from epidermal cell layers of styles of mature flowers of *Cynara cardunculus* (Cordeiro *et al.*, 1994). It is not present in other parts of the plant. Its sequence is slightly more similar to human cathepsin D than to bovine chymosin. The milk clotting property of the plant is used in cheese-making in Portugal.

An SP, in several respects atypical and exhibiting elastase specificity, has been obtained from maize roots (Batt and Wallace, 1989; Goodfellow, Solomonson and Oaks, 1993). It is active at close to neutral pH and its physiological role could be in the turnover of nitrate reductase involved in nitrate assimilation.

LATICES

Latices and juices of many different plants contain highly active proteinases, mainly of the cysteine type (Lyn and Clevette-Radford, 1988; Brocklehurst, Willenbrock and Salih, 1987). In contrast to their biochemical properties, we still have much to learn about their physiological role, the purpose of their abundances, and the control of these potentially hazardous proteinases. Preproregions, which are removed at the appropriate time and place, may be one of the answers (Revell *et al.*, 1993), the other being possibly the requirement of a reducing environment for activity.

SENESCENCE

Genetically programmed senescence of leaves is characterised by, among other metabolic changes, enhanced proteolysis (Feller, 1986). This is switched on to effect ordered degradation of previously assimilated proteins and translocation of the

released amino acids, which are used during formation of young growing tissue and eventually, of seeds and fruits. Senescence involves well-organised and controlled changes in metabolic regulation so that, despite degradative processes that affect a number of molecules, plant cells are enabled to remain viable (Smart, 1994). This process is beneficial to plants.

Changes in proteolytic activities have also been observed during senescence of other organs such as the pods, ovaries and root nodules of leguminous plants (Storey and Beevers, 1977; Carrasco and Carbonell, 1988; Vercher, Carrasco and Carbonell, 1989; Cercós *et al.*, 1992; Pladys and Rigaud, 1985; Pladys and Vance, 1993).

During leaf senescence mainly enzymes and membrane proteins are degraded (Huffaker and Peterson, 1974), a large portion of them in chloroplasts (Peterson and Huffaker, 1975). It is particularly true for the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), one of the most important in the chain of photosynthesis and one of the rare identified natural substrates of proteolytic enzymes (Ragster and Chrispeels, 1981). Obviously this process greatly affects photosynthesis, just as the senescence of root nodules affects nitrogen fixation (Pladys and Rigaud, 1985). Hence, the elucidation of proteolytic events involved in senescence is of great importance both for a better understanding of the role of proteases in plant cells and for understanding how to delay senescence for practical purposes. The possibility of constructing transgenic plants expressing the ability to maintain photosynthesis and/or nitrogen fixation during seed-fill, in order to obtain greater crop yields and/or nitrogen nutrition value would be of interest in agriculture (Huffaker, 1990; Pladys and Rigaud, 1985).

Leaves

Although the expected simple correlation between increase of protease activity and the hydrolysis of proteins is not always realised (Vierskov and Thimann, 1988), it has been generally accepted that specific alterations, often an increase, in proteolytic activities occur during senescence of different plant organs, (Pladys and Rigaud, 1985; Feller, 1986; Huffaker, 1990; Peeters and Van Leere, 1992; Cercós *et al.*, 1992).

Several extensive reviews have appeared considering proteolysis in relation to leaf senescence in particular (Feller, 1986; Dalling and Nettleton, 1986; Huffaker, 1990). Until recently the only more detailed characterisation of proteases supposed to be involved in senescence has related to family identification by the use of inhibitors, partial purification and determination of pH optimum, pI and molecular mass (Feller, 1986). A brief summary is given here as well as some more recent results.

One of the conclusions of the above reviews that attracts attention is that there is no unequivocal evidence for the existence of any proteinase specific for senescence. The same seems to be true for leaf as for the whole organ and for chloroplasts (Dalling and Nettleton, 1986). It appears that protein degradation is carried out by proteinases constitutively expressed prior to senescence (Huffaker, 1990). The same conclusions could be reached from recent results concerning gene expression associated with senescence (Hensel *et al.*, 1993; Smart *et al.*, 1995). It should be noticed, however, that the properties of the proteolytic activities (e.g. pH optimum) observed in senescing leaves differ from those detected in germinating seeds of the same species (Feller, 1986), where proteinases are also involved in protein mobili-

sation. Changes in the pattern of endopeptidases occur during senescence (Feller, 1986; Huffaker, 1990) and this has led to the conclusion that senescence is characterised by alteration of the proportions of different enzyme activities present in all stages of leaf development, rather than by the induction of specific protease(s).

Different groups of proteolytic enzymes do not react in the same manner. It is difficult to generalise since an increase of some endoproteolytic activities has been observed and a decrease of others. Considerable differences exist between the levels of proteinase activities and their increase or decrease during senescence in various groups of annual and perennial plants and this requires separate discussion in every case (Feller, 1986). For example, significant increases of proteolytic activities were observed in the leaves of Poaceae during senescence, but corresponding observations for Fabaceae have been reported only in a few cases (Feller, 1986). Also, although several proteolytic activities were separated by means of chromatography and the change in their profile observed, the isolation and biochemical characterisation of protease(s) whose contribution to cellular proteolysis alters during senescence has not been reported.

It has been suggested that chloroplast senescence is not achieved through pinocytotic relation with vacuoles and the evidence would lead to the conclusion that degradative enzymes operate from within the chloroplasts (Dalling and Nettleton, 1986). Most of the suggested involvement of proteolytic enzymes in chloroplast senescence has been largely speculative although, despite the danger of chloroplast contamination by vacuolar enzymes, proteases are associated with this organelle and its different fractions (Huffaker, 1990; Roy-Macauley *et al.*, 1992).

According to available data, mainly dependent on the sensitivity of observed proteolytic activities to specific PIs, the conclusion is that CPs are most frequently involved in leaf senescence (Feller, 1986; Huffaker, 1990).

Leaf senescence has only recently been studied at the level of gene expression. Techniques of molecular biology and immunology now enable new approaches that promise further progress. mRNA that increases in abundance during leaf senescence has been detected and the corresponding cDNA clones have been isolated from a number of plant cDNA libraries (e.g. Smart *et al.*, 1995). Relatively few cDNAs have been assigned to genes with known translation products. Among them one from *Arabidopsis thaliana* has been identified as coding for a CP (Hensel *et al.*, 1993). This putative protease is likely to be related to the family of CPs that includes oryzain γ and aleurain which are specifically expressed in germinating grain seeds. Recently two cDNAs for senescence-enhanced mRNAs of maize leaf have been isolated and show sequence homology with cDNA for seed CP protease oryzain γ from rice and a protein-processing enzyme from castor bean seeds. The latter is also a CP but is not related to the former (Smart *et al.*, 1995). It has been pointed out that for oryzain γ, it is not surprising because seed germination and leaf senescence have features in common. Since processing enzyme is possibly involved in post-translational modification, this process is suggested to be a major feature of leaf senescence.

It is worth noting that in a number of cases CPs have been reported to be involved in leaf senescence. It has to be clarified whether this is the result of easier experimental access or specific connection of this family of plant proteases with senescence.

Ovaries

In the case of proteolysis in ovary as opposed to leaf senescence the involvement of a senescence-specific protease has been proposed (Cercós and Carbonell, 1993). The series of investigations that led to this suggestion has been one of the rare detailed studies of proteases related to senescence.

In unpollinated pea ovaries a neutral proteolytic activity is involved in senescence (Carrasco and Carbonell, 1988). Further examination of the same model system revealed the simultaneous appearance of endoproteolytic activities detected by electrophoresis of the ovary extracts at the beginning of senescence and histochemically in the endocarp (Vercher, Carrasco and Carbonell, 1989). It has been suggested that this is senescence-specific proteolysis and that it may be the operating mechanism controlling Rubisco levels (Cercós *et al.*, 1992). Beside the new proteolytic activity at neutral pH, an enhancement of acidic proteolytic activity was detected only in senescent ovaries. A protease proposed to be senescence-specific has been purified by affinity chromatography on Rubisco-Sepharose and suggested to be a CP (Cercós and Carbonell, 1993). Purified antibodies enabled its induction to be followed during senescence and during the suppression of its synthesis by GA. Immunolocalisation of this CP (Cercós, Harris and Carbonell, 1993) indicates that it is associated with the vacuolar membrane and also within the vacuole, the site of degradative processes in senescent ovaries.

Expression of a gene encoding a putative thiol-proteinase has also been reported to take place at the beginning of ovary senescence (Granell *et al.*, 1992).

As in the case of leaves, it appears that CPs are associated with senescence.

Root nodules

Proteinases appear to be involved also in nodule senescence (for review, see Vance, Reibach and Ellis, 1986). A decrease in the soluble proteins of root nodules of French bean (*Phaseolus vulgaris*) correlated with increase of two distinct proteolytic activities against leghaemoglobin with acidic and alkaline pH optima (Pladys and Rigaud, 1985). After partial purification, the two enzymes were shown to be CPs. They were reported to be characteristic of senescing nodules, although it has not been demonstrated that they are expressed by the plant itself. Increased cysteine protease activity with acidic pH optimum was also observed in alfalfa nodules (Pladys and Vance, 1993).

These enzymes showed significant affinity for leghaemoglobin (Pladys and Rigaud, 1985; Pladys and Vance, 1993) and were able *in vitro* to partially digest the peptidoglycans of bacteroid cell walls (Pladys and Rigaud, 1988).

The result of sequence analysis of cDNA corresponding to a protein that is specifically expressed in wing bean nodules on the onset of senescence is interesting (Manen *et al.*, 1991). This protein has proved to be a PI of the Kunitz type. Its localisation is exclusively in infected senescent cells of the nodule and it has been suggested that specific expression of this PI occurs during senescence. It has been speculated that this PI interacts specifically with a protease (plant or bacteroid) that is present in functional nodules but disappears at senescence. The importance for the maintenance of symbiosis has been suggested.

These results indicate that a delicate balance of different proteolytic enzymes and their inhibitors is important for the regulation of cellular processes.

Regulation of proteolysis

Although new approaches are already giving interesting data, old questions (Feller, 1986) still wait to be answered. Structural properties of the proteases involved, their cellular and subcellular localisation, the possibility of existence of different proteolytic systems responsible for the breakdown of proteins from various cell compartments and, particularly, control mechanisms of proteolysis, all have to be elucidated in order to understand how proteases function in senescence, and if and how to use them in plant improvement.

One of the most intriguing questions is the nature of the signals that trigger the proteolytic breakdown of proteins and the regulation of this process. Although it is a matter of speculation, some general ideas have been suggested that still act as a useful guide. Among them, the influence of lipid metabolism on changes in membrane proteins, interactions of proteins with low-molecular-weight compounds that specifically affect the susceptibility to proteolysis of some proteins, regulatory effects of pH, inorganic ions, other metabolites, susceptibility of substrate proteins and intracellular compartmentation have been mentioned (Feller, 1986). In view of recent findings, the possibility of enhancement of putative protease gene expression must be added (Hensel *et al.*, 1993; Smart *et al.*, 1995).

Targeting proteins for degradation must be very selective and this field attracts great attention, but in plants experimental evidence has only begun to appear. Some of the points likely to be important are selective movement of proteins into vacuoles that serve as lysosomes in plant cells, substrate selectivity, regulation by ATP, ubiquitin-pathway involvement and N-end rule (for reviews, see Huffaker, 1990; Vierstra, 1993).

A recent model has been presented (Hensel *et al.*, 1993) in which leaf senescence is postulated to be triggered by age-related decline in photosynthetic processes that is under control of maturation signals at full leaf expansion. Consequently, the concentration of metabolites of photosynthesis, which act to repress the expression of senescence-associated gene (SAG) in young leaf, declines and the derepressed SAG gene starts to produce substances that mobilise nutrients and promote the senescence of the leaf tissue. Future research will show if and how proteolysis associated to senescence fits in this model and if it can be applied in plant improvement. The availability of mutants and variants with altered senescence patterns will be useful for studying the molecular basis of senescence, as well as for sense or antisense transformation of plants (Smart *et al.*, 1995).

Proteinases and inhibitors in plants under stress

An adequate response at the cellular level to external stimuli is of great importance for plants. They are rooted in the soil and cannot move and hence cannot respond to environment in ways open to animals. This is particularly true in the case of the environmental conditions which deviate significantly from those optimal for the organism in question, whether originating from other organisms including predators

or from different stress conditions. During evolution plants have, therefore, developed both physiological and biochemical responses. Proteases and their inhibitors that are connected to the protein breakdown that is essential to adaptation to environmental conditions (Vierstra, 1993) constitute an important molecular mechanism involved in response to stress.

There is an increasing volume of research concerned with the involvement of proteolytic enzymes and their inhibitors in certain kinds of stress. Numerous studies have focused on their role in plant response to wounding and pathogenic attack by different organisms and the most notable results have been achieved in just that field. Recognition of the importance of these enzymes and their inhibitors in plant resistance to herbivores and plant pathogens, and hence their potential biotechnological value, justifies this orientation. Relatively smaller and more fragmentary investigations have been directed towards the elucidation of the molecular basis and mechanisms of modification of proteolytic activities in plants in response to stress such as drought, salinity and high or low temperatures. Despite this fact, as well as that different plant species, organs or cultured cells were investigated, rendering comparisons and generalisations more difficult, these studies have shown that the levels of some proteolytic enzymes and/or their inhibitors correlate with environmental stress and are probably involved in the plant response. Having in mind the importance of coping with states of stress in plants, this should be an encouragement to further investigation in the field.

In the natural environment, factors that induce a state of stress seldom act individually. On the other hand, different stress conditions may have the same effects at the cellular level and response to them may share a common molecular mechanism. Such a relationship between drought, salt stress and cold is well known (Bartels and Nelson, 1994; McKersie and Leshem, 1994). Also many kinds of stress in cultivated plants induce the same partially reduced forms of oxygen which are involved in different disorders and dysfunctions at the molecular level (McKersie and Leshem, 1994). Moreover, the senescence processes, especially degenerative changes in cellular membranes common to different kinds of environmental stress, can cause surface lesions, degradation of cellular membrane structures and the leakage of solutes (McKersie and Leshem, 1994). This not only changes the local environment in the plant cell and thus denatures some molecules, but also enables pathogens, another stress for plants, to grow on such tissue.

Because of the complexity of the different kinds of stress it is difficult to establish exact correlations between a particular stress and the corresponding response. Further, as noted above, the same molecules or molecular systems are in some cases involved in defence against different kinds of stress. This is particularly true for certain plant hormones, especially ABA (Hetherington and Quatrano, 1991), but can also be true for at least some proteases and/or their inhibitors, although no strong proof is yet available (e.g. Williams *et al.*, 1994). The significant homology of senescence-enhanced cDNA from maize leaves to the CP oryzain γ from rice seeds, involved in seed germination, shows that the same or similar proteases can be involved in different (although with some common features) processes in plants (Smart *et al.*, 1995). Nevertheless, with regard to the type of response and the present state of knowledge, this survey is divided into two parts. One considers the role of proteases and their inhibitors in defence against wounding and pathogenic attack, the other their role in

plant response to abiotic environmental stress such as drought, salinity and cold (referred to in the following text as environmental stress) whose common feature is that they all lead to cellular dehydration.

WOUNDING AND PATHOGEN ATTACK

In a valuable source of information about plant proteolytic enzymes which appeared about ten years ago, the chapter considering the roles of these enzymes in interaction of plants with other organisms had a comparatively small section about proteases in defence against pathogens (Boller, 1986). It was pointed out that the increased proteinase activity observed in several cases was often accounted for by the enzymes of the pathogen rather than of the plant host. On the basis of a few reports, however, it was concluded that the role of proteases in defence against pathogens should be considered. The involvement of wounded tissue, often a point of entry for pathogens or viruses, was also underlined. In such tissue, vacuoles are broken and it was suggested that release of vacuolar hydrolases may form a first line of defence. The wound-regulated synthesis and vacuolar compartmentation of PIs in plant leaves has also been cited (Ryan, 1980), mentioning that these inhibitors do not inhibit plant proteases but block animal and fungal digestive proteases. Because of this, plant tissue becomes indigestible and/or at least partially resistant to insects and other pathogens.

Since then a number of reports concerning studies in this field have appeared, especially concerning PIs. The interest arises mainly because these molecules could be potential factors for plant resistance and hence of biotechnological value for plant improvement. A number of studies focus on the activation of transduction of the signal for PI synthesis in response to wounding and pathogen attack, thus constituting a useful model system for studying the regulation of gene expression and signal-transduction pathways in plants.

Combating insects and pathogens

Insects and pathogens (fungi, bacteria and viruses) menace plants either by wounding and eating them, or by causing diseases. Insects, especially larvae, attack leaves and seeds. It has been observed that growth and development of insects from a variety of genera including *Heliothus, Spedoptera, Diabiotica* and *Tribolium* can be seriously menaced by endogenous plant PI (Broadway and Duffey, 1986). Attack by different pathogens provokes a hypersensitive reaction which involves a number of physiological and biochemical adaptations, among them *de novo* expression of a set of host-encoded PR proteins (Bowles, 1990). These proteins are induced by different forms of stress and some show proteolytic activity (Linthorst, 1991). It appears that both proteases and their inhibitors have an important role in plant defence against predators: the former can digest predators' proteins and the latter can inhibit their digestive proteinases.

The majority of reports consider members of Solanaceae (leaves of potato, tomato and tobacco, potato tubers), Fabaceae (seeds of leguminous plants, alfalfa leaves) and *Arabidopsis thaliana*, a model plant in molecular biology studies. Though this approach makes relevant comparisons possible – not often a case in the field of plant proteases and their inhibitors – one still has to be prudent. For example, in the case of

wounding, distinction must be made between leaves in the vicinity and those far from the primary site of attack by insects, since local and systemic responses differ. The age of leaves is also reflected in results, and even the time of day when samples for analysis are taken (Roberts, 1992; Linthorst *et al.*, 1993b).

Phytophagous insects and micro-organisms differ considerably in their digestive proteinases. Those most extensively studied are SPs (which predominate in Lepidoptera) and CPs (which predominate in Coleoptera including the well known Colorado potato beetle). This has been reflected in investigations and potential use of their respective inhibitors (Ryan, 1989).

Proteinase inhibitors in defence

The involvement of PIs in plant response to this kind of stress is now well established (Ryan, 1990). Some of them have been characterised, their localisation determined and their gene expression described (Farmer, Johnson and Ryan, 1992). Genes encoding some of the known PIs, as well as systemin, a probable signal that triggers PI synthesis (McGurl *et al.*, 1992, 1994), have been isolated and used in plant transformation (Sánchez-Serrano *et al.*, 1987; Jongsma *et al.*, 1995). Moreover, some features of the complex signalling and regulatory pathway have been elucidated (Farmer and Ryan, 1990, 1992; Farmer *et al.*, 1994). On this basis hypotheses about the overall pathways have been proposed but several disagreements exist and many molecular details are still missing (Farmer *et al.*, 1994). No doubt, this will be one of the central points in future research. The most recent attempts at the use of PIs as anti-pest agents in plants are summarised in *Table 3*.

Physical damage to leaves of plants from a number of families results in the synthesis of PIs at the wound site as well in distant unwounded leaves (Green and Ryan, 1972; Walker-Simmons and Ryan, 1977; Brown and Ryan, 1984). In response to wounding an increased accumulation of inhibitors of SPs, CPs and APs is observed (Hildmann *et al.*, 1992; Suh, Stiekema and Hannapel, 1991; Štrukelj *et al.*, 1992; Walsh and Strickland, 1993). Members of four of the SPI families (out of eight identified in plants, see *Table 2*) are known to be systemically induced in various plants, among them PI-I and PI-II from tomato, potato and tobacco leaves (Plunkett *et al.*, 1982; Pearce, Johnson and Ryan, 1993), Bowman-Birk inhibitors in alfalfa (Brown and Ryan, 1984) and a Kunitz inhibitor in poplar trees (Bradshaw *et al.*, 1989). An SPI, probably belonging to PI-I family, is strongly induced in tobacco leaves reacting hypersensitively to tobacco mosaic virus (Geoffroy, Legrand and Fritig, 1990). See also *Table 2* and *Table 3*.

Despite the focus on PI-I and II, it has become clear that the induction of SPIs may not be an effective defence mechanism against all insect species (Wolfson and Murdock, 1990). Benz (1978) demonstrated that, although wounding potato leaves led to a doubling of SPI activity, the growth and development of Colorado potato beetles provided with previously injured plants was not significantly different from those reared on intact plants. It was shown that Kunitz trypsin inhibitor had no effect on the proteinase activity in homogenates of larval bruchid beetles (Kitch and Murdock, 1986) or cowpea weevils (Gatehouse *et al.*, 1985). Eventually it has been demonstrated that CPs are responsible for most of the proteolytic activity in the digestive tract of many Coleoptera (Murdock *et al.*, 1987). Growth suppression in the

Table 3. Effects of plant inhibitors on plant pests and pathogens

Inhibitor type	Bacteria and fungi	Viruses	Insects
Serine PI			
PI I	tobacco PI I-several microbial proteinases: **inhibition,** (5)		transgenic tobacco-*Manduca sexta*: **enhanced resistance** (7)
PI II			transgenic tobacco-*Spodoptera exigua*: **no effect** (11)
Bowman Birk inhibitors			transgenic tobacco-*Heliothis virescens*: **enhanced resistance** (13)
Kunitz soybean trypsin inhibitor	*Botrytis cinerea*: **no effect** (2) *Fusarium solani*: **no effect (2)** *Alternaria brassicicola*: **no effect** (2)	poliovirus infected Vero cells: **no effect** (3)	transgenic tobacco-*Spodoptera littoralis*: **complete resistance to pests** (9)
Cabbage leaf SPIs	*Botrytis cinerea*: **antifungal activity** (2) *Fusarium solani*: **antifungal activity** (2) *Alternaria brassicicola*: **no effect** (2)		several herbivorous Lepidoptera: **varying biological effect due to adaptation** (1)
Cysteine PI			
Oryzacystatin		poliovirus infected Vero cells: **inhibition of replication** (3)	*Tribolium castaneum*: **suppression of growth** (4) *Leptinotarsa decemlineata* Say: **strong inhibition of gut proteinases** (8)
Potato tuber multicystatin			*Diabrotica* larvae: **growth inhibition (in diet) and inhibition of gut proteinases *in vitro*** (16)
Cowpea embryo extract		cowpea mosaic virus: **inhibition of *in vitro* proteolytic activity** (6)	
Cowpea cystatin			Bruchid beetles: **no effect on resistance** (10)
Soybean cystatin			*Callosobruchus maculatus*: **strong inhibition of gut extracts** (12) *Tribolium castaneum*: **strong inhibition of gut extracts** (12) *Acanthoscelides obtectus*: **weak inhibition of gut extracts** (12)
Microbial E-64		poliovirus infected Vero cells: **no effect** (3)	*Callosobruchus maculatus*: **strong suppression of development**(15) *Acanthoscelides obtectus*: **strong suppression of development** (14) *Diabrotica* larvae: **strong inhibition of gut proteinases *in vitro*** (16)

References: (1) Broadway, 1995; (2) Lorito *et al.*, 1994; (3) Kondo *et al.*, 1992; (4) Chen *et al.*, 1992; (5) Geoffroy, Legrand and Fritig, 1990; (6) Shih *et al.*, 1987; (7) Johnson *et al.*, 1989; (8) Michaud, Nguyen-Quoc and Yelle, 1993; (9) Marchetti *et al.*, 1994; (10) Fernandes *et al.*, 1993; (11) Jongsma *et al.*, 1995; (12) Hines *et al.*, 1991; (13) Hilder *et al.*, 1987; (14) Hines, Osuala and Nielsen, 1990; (15) Murdock *et al.*, 1988; (16) Orr, Strickland and Walsh, 1994.

Colorado potato beetle is not surprising, since it is known now that CPs cathepsin B and H are two of the most important proteinases of the gut of the insect (Thie and Houseman, 1990). It has been suggested that utilisation of CPs may be an evolutionary adaptation that enables insects to consume legume seeds and other plant materials that are naturally high in SPIs (Ryan, 1990).

The rice CPI, oryzacystatin significantly inhibits the digestive proteinases of two species of stored grain beetles (Liang *et al.*, 1991). In addition, a specific inhibitor of CPs, E-64, supplied in the insect diet, suppresses the growth and development of larval cowpea weevils (Murdock, Shade and Pomeroy, 1988), common bean weevils (Hines *et al.*, 1990) and Colorado potato beetles (Wolfson and Murdock, 1987). In potato an increase in mRNA that translated into a peptide with homology to the oryzacystatin was observed (Hildmann *et al.*, 1992). Other examples of CPIs that inhibit insect digestive proteolytic activity include soybean cystatin (Hines, Osuala and Nielsen, 1991).

The high sulfhydryl content of many trypsin inhibitors makes them susceptible to alkylation by plant-derived quinones so that PIs with a low potential for alkylative inactivation may be most appropriate for host-plant resistance (Felton, Broadway and Duffey, 1989). With their low content of disulphide bonds, CPIs would seem to be good candidates.

Relatively few APIs have been found so far (see *Table 2*). Experiments were reported that showed an increase of API in potato leaves after wounding (Kregar *et al.*, 1994).

Proteinases in defence

The high abundance of proteases in the vegetative organs of certain plants has been noted for a long time. It has been difficult to explain this observation in terms of an intrinsic function. The possible role of these proteases as defence enzymes has been suggested. This proposal is supported by the finding that one of the major pathogenesis related proteins of tomato is an alkaline proteinase (Vera and Conejero, 1988).

In tubers, an increase of CP upon wounding has been established (Isola and Franzoni, 1993). One of the proteases possibly involved in response to wounding is a tobacco CP (Linthorst *et al.*, 1993b). Expression of genes encoding putative proteinases is considerably enhanced upon incision wounding, but this has not been observed in other kinds of stress such as infection with tobacco mosaic virus. It has been suggested that the products of these activated genes play a role in the degradation of intracellular proteins that are no longer functional.

Regulation of proteinase inhibitors

Most of the studies on regulation of plant proteinases and PIs have been in the context of insect attack or severe wounding. In this process a number of genes are expressed, among them those encoding for PIs. This induction happens in both local and distal leaves and obviously requires a mobile systemic signal(s) that will transmit the information to distal tissues. Its identity is still not clear, although a number of candidates have been proposed: oligogalacturonides from the damaged cell wall (Bishop *et al.*, 1981, 1984), plant growth regulators ABA and auxin (Peña-Cortés, *et*

al., 1989, Hildmann *et al.*, 1992), jasmonic acid (JA) and its ester methyl jasmonate (MJ) (Hildmann *et al.*, 1992), secondary products of lipoxygenase-catalysed oxidation of linolenic acid involved in regulating several physiological responses in plants and in specific gene regulation (Farmer *et al.*, 1994), octadecanoid precursors of JA, particularly linolenic acid (Farmer and Ryan, 1992), an 18-amino acid polypeptide derived from plants, called systemin (Pearce *et al.*, 1991; McGurl *et al.*, 1994) and electrochemical signals (Wildon *et al.*, 1992). PI synthesis is known to be induced by fungal oligosaccharides such as chitosan (Walker-Simmons and Ryan, 1984) and β-glucan-containing elicitors (Rickauer, Fournier and Esquerré-Tugayé, 1989).

The involvement of at least some of these substances is a matter of lively discussion and there are still arguments *pro et contra*, especially concerning their properties that are not fully consistent with observations and expectations of primary, long-range systemic signals (Roberts, 1992; Farmer *et al.*, 1994). The possibility remains, nevertheless, that they could be components in the pathway for pathogen-stimulated PI gene expression caused by wounding or pathogens, some of them being involved in long-distance signalling and some being intracellular signal intermediates.

Having in mind that any signal-transduction pathway for PI synthesis probably involves a complex array of substances, it is reasonable to expect new findings that will link together primary signal and gene expression. One such observation has led to the hypothesis that signal transduction for PI synthesis in tomato, activated in response to wounding and systemin, involves an octadecanoid hydroperoxide intermediate derived from linolenic acid by lipoxygenase activity (Farmer *et al.*, 1994).

Biotechnological application

In designing PI for improving plants, the mechanistic class and structural aspects of possible protease-PI complexes have to be considered. The interaction between them has to be sufficiently strong to provide effective inhibition of the enzyme. It has been suggested that insect CPs and SPs are the most likely targets for inhibition by engineered PI since it appears that APIs or MPIs may not have adequate potential for improving plant defence (Ryan, 1989).

Several PI genes have already been used to transform plants to assess their defensive capabilities. One of the first successful attempts was the enhancement of resistance of tobacco plants to tobacco bud worm. Cowpea trypsin inhibitor was constitutively expressed in the leaves of tobacco plants (Hilder *et al.*, 1987). Since then there have been a number of reports of transgenic plants expressing genes for PI and genes for inhibitors I and II from tomato and potato (Sánchez-Serrano *et al.*, 1987; see also *Table 3*) but it has to be recognised that adequate plant resistance has not yet been achieved.

It appears reasonable to assume that in the future new PIs involved in plant defence will be discovered or designed and used in plant improvement. One of the most interesting characteristics of PIs is the possibility of great variability of amino acid sequence in that part of polypeptide which is responsible for specific interaction with proteases (Ryan, 1989). This is important for the ability of plants to adapt to different insect or pathogen attacks and allows the production of new and effective PI mutants.

Many of the results of PI applications appear promising and, at the same time, intriguing. Some of them are summarised in *Table 3*.

As at least two groups of plant viruses, como- and potyviruses, require a proteolytic step in their replication, proteinase inhibitor, if internalised into infected cells, could be of potential value as an anti-viral agent. Analogous experiments in animal systems have been promising (Korant, Brzin and Turk, 1985).

For bacterial and fungal proteinases many inhibitors of the subtilisin family exist in cereals (q.v.) but some others also seem to be effective (Geoffroy, Legrand and Fritig, 1990; Lorito *et al.*, 1994).

Most of the experiments have dealt with inhibition of insect gut proteinases *in vitro*, or by feeding them on different amounts and types of inhibitors and observing their effects. Many of the initial expectations proved to be oversimplified, as insects seem to be able to adapt in a matter of hours to the presence of inhibitors in their diet, simply by mobilising a set of insensitive proteinases (Broadway, 1995; Jongsma *et al.*, 1995). The effects of extremes of pH in insect gut on the effectiveness of inhibition and the sensitivity of inhibitors to proteolytic inactivation need to be taken into account. The introduction of inhibitory cocktails should also be envisaged.

ENVIRONMENTAL STRESS

In contrast to the number of reports concerning the exogenous role of proteases and their inhibitors in defence against pathogens and damage caused by wounding, there is a lack of information concerning the involvement and endogenous function of plant proteases and/or their inhibitors in response to environmental stress. The conclusion that proteolytic enzymes could be specifically involved in plant response to environmental stress originated in observations, on the one hand, that stress conditions often bring about the senescence of plant tissue and, on the other hand, that senescence is closely connected with enhanced proteolysis in the same tissue.

Environmental stress and senescence

The enhancement of leaf senescence caused by a number of stress conditions such as drought or salinity is well known. Loss of proteins has usually been observed during drought stress (Dungey and Davies, 1982). More severe chilling stress also promotes cellular autolysis and senescence (Saltveit and Morris, 1990). Although the relationship between stress and senescence, at least at the level of gene expression, is not always straightforward (Becker and Apel, 1993), it is reasonable to conclude that proteolytic enzymes form part of plants' molecular mechanisms of defence against environmental stress. It should be noted that increased proteolytic activity may be due to change in substrate as well as in protease activity. For example, activated oxygen which is formed during different kinds of stress can alter protein structures and increase their susceptibility to proteolysis (Davies, 1987).

Changes in proteolytic activity have been observed in maize root tips during carbohydrate starvation, which is known to produce similar metabolic changes to those in senescence (James *et al.*, 1993). Acidic endopeptidase activities were increased in response to starvation and appear to be different from those present in non-starved root tips. It was suggested that this is accounted for by a new set of SPs and CPs.

Although the number of reports in this field has increased during the last few years,

adequate molecular details are still not known. Generally, two main approaches in these investigations have been adopted: (i) detection and characterisation of changes of proteolytic activities in plants under stress and (ii) determination of genes whose expression is induced by stress, and elucidation of the identity of the gene products as proteases or their inhibitors.

Changes of proteolytic activities

It is important to keep in mind that an observed increase of proteolytic activity can be due either to the activation of already synthesised enzymes (activation of proenzymes, alterations in inhibitor content or in the interaction with proteases, interaction with effectors, changes in cellular pH, alteration of possible isoforms), or to the activation of corresponding genes. A decrease of protease activity can be due either to increased inhibition, to alteration of environment or to increasing turnover rates and alteration of possible isoforms. It is also important to be aware that, since in the majority of studies proteolytic activities are measured *in vitro*, usually with non-natural substrates, the observed activity does not necessarily reflect the activity in the cell.

As regards the interpretation of enhanced expression of genes that correlate to environmental stress and that are assumed, on the basis of gene sequences, to encode proteases or their inhibitors, (a) the functions of polypeptides encoded by these genes are still putative and (b) it is not certain that the genetic potential would always be completely realised in complex events within the cells.

Investigations of changes of proteolytic activity under stress have shown that in several cases they were increased (Roy-Macauley *et al.*, 1992; Guérin *et al.*, 1991) although the opposite has been also shown for other systems (Pierre and Savouré, 1990). SPs are involved in several cases, although not in the same way in different tissues (Guérin *et al.*, 1991; Pierre and Savouré, 1990; Roy-Macauley, 1993).

Proteolytic activity in the leaves of water stressed leguminous plants *Phaseolus vulgaris* and *Vigna unguiculata* cultivars correlates with the sensitivity of cultivars to water stress. The leaf extracts exhibit three peaks of endoproteolytic activity with different pH optima. Water deficit hardly induced any change in this pattern of activity in the resistant cultivar, moderately sensitive cultivars showed some changes and in the sensitive cultivar a clear stimulation at all pH values tested was observed (Roy-Macauley *et al.*, 1992). In soluble, membrane and chloroplast cell fractions from stressed plants proteolytic activities increased and this effect was greatest in the sensitive cultivar. The increase was most marked in the soluble (mainly vacuolar) fraction, particularly in the acidic pH range, and correlated with the level of drought sensitivity of the plants. The increase was later identified as being in APs (Roy-Macauley, 1993). In the same study the drought resistant cultivars showed a decrease of activity of CPs and SPs, which was explained by the possible induction and/or activation of the corresponding inhibitors during water deficit.

An SP activity (pH optimum of 8) measured in cytosolic extracts of nodules of faba beans (*Vicia faba*) approximately doubled under water deficit. In the same tissue acidic proteolysis remained low (Guérin *et al.*, 1991). In contrast, in needles of *Picea abies* water stress led to a decrease in proteinase activities, especially those specific for SP (Pierre and Savouré, 1990). Since the levels of these activities start decreasing before any significant loss in protein, it could mean that in *P. abies*

needles, proteinase that are not responsible for degradation of total soluble proteins under water-stress are sensitive very early to changes in the water status of the tissue.

Gene expression

The other, complementary, approach is to study gene expression in response to stress and to use sequence comparison for identification of the corresponding protein products. The alteration of the levels of a number of translatable mRNAs in different plant tissues in response to environmental stress is well documented (e.g. Hurkman, Fornari and Tanaka, 1989; Hurkman, Tao and Tanaka, 1991; Robinson, Tanaka and Hurkman, 1990; Curry and Walker-Simmons, 1993). Recently, some of these genes have been suggested to encode proteases or their inhibitors. All of them are CPs, but belonging to different families (Schaffer and Fischer, 1988; Guerrero, Jones and Mullet, 1990; Koizumi *et al.*, 1993; Williams *et al.*, 1994).

Analysis of mRNA that accumulates in tomato in response to low temperature has led to identification of a CP gene (Schaffer and Fischer, 1988). In wilted pea shoots the reduction of turgor also correlates with enhanced expression of a gene for CP but the latter seems not to be in the same subfamily as the tomato protease (Guerrero, Jones and Mullet, 1990). As a reaction to drought stress, two genes apparently encoding different types of CP belonging to the papain superfamily were activated in *Arabidopsis thaliana* (Koizumi *et al.*, 1993). One of these proteinases resembles the rice CPs oryzains α and β (Watanabe *et al.*, 1991) and the above mentioned tomato putative CP (Schaffer and Fischer, 1988), while the other is closely related to the stress induced proteinase of pea shoot (Guerrero, Jones and Mullet, 1990). These enzymes contain sequences homologous to signal peptides that function in protein secretion. mRNA transcription of both genes was not responsive to cold or heat stress but was strongly induced after exposure to high salt conditions. In the same plant a gene for another putative CP whose mRNA accumulated upon wilting has been demonstrated (Williams *et al.*, 1994). In contrast to the former genes, its mRNA accumulated during exposure to low temperature although heat shock had only a minor effect.

Reports concerning the involvement of plant PIs in response to environmental stress are even more rare. Based on sequence data for osmotin, one of the best characterised salt stress-induced proteins, possesses homology with the sweet tasting protein thaumatin and a pathogenesis-related protein from tobacco (Singh *et al.*, 1987) – its role as a CPI has been suggested. Osmotin has been identified in tobacco (Singh *et al.*, 1987) and tomato (King *et al.*, 1988), but its true biochemical function has yet to be unequivocally established. The mRNA for a protein, whose sequence is related to the Kunitz PI family, accumulated in leaves of *Brassica napus* during rapid or slow drought stress (Lee Downing *et al.*, 1992) and in *Raphanus sativus* after exposure to NaCl and rapid drought stress (Lopez *et al.*, 1994). It has been pointed out however that, although a soybean Kunitz pattern is present in the sequence, it is not known whether this protein exhibits a trypsin inhibitory activity.

Regulation of proteolysis

The way in which a particular environmental stress induces changes at the molecular

level, including mechanisms of signal reception by the cell and its transduction into altered gene expression or activity of already existing proteases and their inhibitors, is still not understood.

Existing evidence is fragmentary and does not allow any generalisation. For example, in the same plant the expression of different genes for putative CP did not show the same correlation with different kinds of stress (Koizumi *et al.*, 1993; Williams *et al.*, 1994). Since in some cases the genes for putative proteinases were activated by drought and salt stress, and not by heat or cold stress, it has been suggested that they may be induced by changes in osmotic potential of plant cells (Koizumi *et al.*, 1993). But the same conclusion could not be drawn for another putative proteinase (Williams *et al.*, 1994) since the expression characteristics were completely different.

Abscisic acid (ABA) has been proposed as a common mediator for plant responses to stress (Hetherington and Quatrano, 1991). It has been shown that a number of genes expressed as a result of stress such as dehydration can also be induced in unstressed tissue in response to ABA (Skriver and Mundy, 1990). The same has been shown for some genes encoding putative proteases and their inhibitors but here again different phenomena are seen. The expression of one gene encoding a putative CP from *Arabidopsis thaliana* was induced independently by wilting and by ABA. However, accumulation of the corresponding mRNA was stimulated exclusively by ABA synthesis. The complexity of the regulatory pathways is further shown by the fact that transcription of two genes encoding putative proteinases in the same plant was induced by drought and salt stress but was insensitive to ABA (Koizumi *et al.*, 1993); the same was shown to be true for one pea gene also encoding a putative CP (Guerrero, Jones and Mullet, 1990). The above mentioned gene encoding a putative PI similar to the Kunitz PI from *B. napus* was expressed after exposure to drought and also after treatment of plants with ABA (Lee Downing *et al.*, 1992). The involvement of ABA *in vivo* is therefore not clear-cut.

Another control mechanism for proteolytic degradation is marking of substrate. Ubiquitin is a well known and highly conserved marker for selective proteolysis of damaged proteins, present in all eukaryotic cells, including plants, where it is particularly abundant in chloroplasts (Lindquist, 1986; Wettern *et al.*, 1990; Vierstra, 1993). Involvement of this small polypeptide in response to stress is well documented. It has been shown, for example, that the synthesis of ubiquitin in *Arabidopsis* is induced at high temperature (Burke, Callis and Vierstra, 1988). A study of gene expression associated with water-stress adaptation of rice cells showed that, in adapted cells the ubiquitin gene was preferentially expressed relative to several other genes (Borkird *et al.*, 1991).

Role of proteinases and proteinase inhibitors

Although several possible roles of proteinases and/or their inhibitors in the response of plants to environmental stress can be envisaged, the real functions of particular enzymes induced by stress have not yet been elucidated. Nevertheless it is obvious that potentially the whole cellular biochemistry may be changed by the induction of proteolytic activities.

The enhanced activity of proteinases can have different kinds of impact on plants. An uncontrolled increase in non-specific proteolysis connected to environmentally

induced senescence would be harmful to plants once it abolished photosynthesis. In such a case inhibition of proteinase activity would be beneficial. The results of studies on water deficit show that particular systems react differently as regard the correlation of enhancement of proteolytic activity with decrease in protein content. In nodules of the legume plant *Vicia faba* a stimulation of proteolysis under water stress has been observed which corresponds to a diminution of leghaemoglobin content. A parallel can be made with degradation of this protein by proteolytic enzymes during nodule senescence (Guérin *et al.*, 1991). This haemoprotein is essential for an adequate oxygen supply and nitrogen fixation in nodules and hence its degradation is unfavourable. The same correlation between protein degradation and increase in proteolytic activity was observed in glucose-starved maize root tips (James *et al.*, 1993). In chloroplasts, a particular target for stress-associated senescence, the same correlation has been observed in legume plants during drought (Roy-Macauley *et al.*, 1992) and was significantly stronger in more sensitive cultivars. In contrast, water deficit led to a decrease in the proteinase activity in needles of *Picea abies* several days before the breakdown of total soluble proteins (Pierre and Savouré, 1990). In the light of the harmful effects of proteolysis caused by stress, the accumulation of PIs during stress can be beneficial, as has been suggested by the accumulation of mRNA for a putative PI in *B. napus* during water stress (Lee Downing *et al.*, 1992).

On the other hand, the role of proteinases in the response to stress can have several beneficial aspects. The induction of proteolytic activities may alter the pattern of metabolic pathways by increasing turnover rates of specific enzymes. Enzymes, inhibitors or other regulatory proteins can be activated by limited proteolysis. It has been suggested that induction of CPs, which are known to be responsible for processing precursor proteins to their mature forms in plant vacuoles, may act as a switch to activate specific proteins (Koizumi *et al.*, 1993). The involvement of proteolytic enzymes in the degradation of vegetative storage proteins located in vacuoles is also a possibility. This could provide amino acids for *de novo* synthesis of proteins important for adaptation to new environmental conditions (Guerrero, Jones and Mullet, 1990).

Another positive effect can be the degradation of aberrant proteins which result from cell stress (Ryan and Walker-Simmons, 1981; Guerrero, Jones and Mullet, 1990). There are many reasons for the formation of aberrant proteins as a consequence of different kinds of stress and one has been already pointed out: that oxygen free radicals can modify protein structure and thus mark it for proteolysis (Stadtman, 1986). It has been suggested that such proteins could be degraded by specific proteases by analogy to *E. coli* proteases that degrade oxidised proteins (Farr and Kogoma, 1991). Moreover, the breakdown of cellular structures, characteristic for senescence associated to stress conditions, with consequent leakage of solutes and electrolytes, may also lead to conformational changes in proteins.

It is known that mechanisms for the proteolytic degradation of proteins whose conformation was changed as a consequence of environmental stress, are activated in eukaryotic cells (Ananthan, Goldberg and Voellmy, 1986) including plants. For example, it has been reported that the degradation of an important part of abnormal proteins in *Acer pseudoplatanus* cells occurred inside the vacuoles, apparently not by a specific proteolytic system. The investigation showed that the degradation of aberrant proteins was dependent on their preferential transfer from the cytoplasm to

the vacuole. This has led to the conclusion that, in contrast to animal cells where the abnormal protein breakdown is due to cytosolic processes, in plant cells it is essentially a vacuolar process (Canut *et al.*, 1986)

The observed changes in proteolytic activities and gene expression correlated with stress are significant indications which now need to be confirmed by more precise and detailed information. To obtain clues to the identity and functions of the proteinases and their inhibitors involved, they have to be isolated, purified, localised within the cell, and related to physiologically relevant substrates, proteolytic pathways and regulatory substances. Their amounts in cells have to be determined more precisely. Recombinant DNA technology, including antisense or sense construction of target RNAs and their introduction into the corresponding plants, should also provide further insight.

Problems in handling plant systems

In the isolation of proteinases and their inhibitors, the nature of plant material itself presents some specific problems such as tough cell walls, heavy pigmentation and in some organs presence of huge amounts of proteinaceous and carbohydrate materials in the form of inactive storage materials. Ways of overcoming these problems have been described (Rhodes, 1977). The proper function of the many proteolytic systems in eucaryotic cells is based on compartmentation and organised and controlled trafficking of the many components, which, amongst other things, prevents proteins from encountering their potential substrates and inhibitors at inappropriate times and places. For example, the attempt to isolate soybean Azocoll-digesting enzyme was unsuccessful from whole leaf homogenate, but relatively easy from intercellular wash fluid alone (Huangpu and Graham, 1995). The requirement that such compartments should be isolated as intact as possible cannot easily be met in dealing with plant materials which, due to rigid cell walls, often require homogenisation by freezing, thawing and grinding thus reducing the yield of intact organelles. As far as proteinases and their inhibitors are concerned a rapid, virtually irreversible formation of complexes may be expected to occur, and the remaining measurable activity will only indicate the component that had been in excess, often leading to quite misleading conclusions. Such problems would be further aggravated in organs or tissues with high proteolytic or inhibitory potential, such as plant latices and juices, and plant storage organs.

In principle it is often possible to design the following isolation steps in such a way that they will favour isolation of either proteinases or inhibitors. Acidification of homogenates, a method that had been previously used in isolation of cysteine cathepsins from animal and human tissues (Mason, Green and Barrett, 1985) has proved to be beneficial for isolation of two CPs from moth bean. Inactivation or proteolysis of inhibitors, release of proteinases from complexes, or activation of latent enzymes were claimed to contribute to this process (Kembhavi *et al.*, 1993). The presence of chaotropic ions (Jarvinen, 1978), alkaline pH (Brzin, Meško and Kregar, 1982) and heating (Turk *et al.*, 1983) as used in the isolation of human cystatins, proved to be advantageous in isolation of cystatin from chick peas (Brzin, unpublished results) and Kunitz type SPI from giant taro (Bradbury and Hammer, 1990).

Maybe the most typical feature of plants that a protein biochemist should be aware

of, is the non-negligible cultivar variation. In potato tubers, the number of multiple isoforms of inhibitors of CPs, APs and SPs was constant with respect to the age of the tuber, but varied in their relative proportions (Brzin, Meško and Kregar, 1995) in selected cultivars. Still more pronounced variation of content of potato inhibitors PI-I and PI-II was observed in wild and cultivated tomato species (Wingate, Franceschi and Ryan, 1991).

Even when dealing with identical cultivars, appreciable variation was observed when plants were grown under slightly different conditions. Thus elevated SPI activity was measured in the leaves of tobacco plants grown in the growth chamber and in the greenhouse, all other treatments being identical (Jongsma *et al.*, 1994). Similarly, tobacco plants grown in a greenhouse, although not intentionally wounded, show some of the responses obtained in wounded plants (McManus *et al.*, 1994). Important seasonal variation has been observed for the relative abundance of multiple forms of CP such as asclepain (Tablero *et al.*, 1991). It is far from clear what is the sensitivity of the environmental switches to which plants may respond with induced synthesis of gene products which neutralise the effect of the harmful stress condition.

Insufficient attention has been paid to the fact that in normal plants each organ, and not just the whole plant, has its own ontogenetic development, and that each event in development is a gradient of changes following one another quite fast, perhaps within days and hours, because plants are limited in their growth period. Huangpu and Graham (1995) recently demonstrated that the content of a MP and a CP in soybean leaves changed dramatically during the first 15 days of leaf growth to be gradually stabilised in mature leaves, and again changed in senescing leaves (Huffaker, 1990). Similarly a specific processing subtilisin-like SP is present in initiating stages of nodules, to be gradually replaced by a CP at the onset of nodule senescence (Ribeiro *et al.*, 1995). Even faster changes were observed for the content of two CPs in tobacco leaves, following circadian expression (Linthorst *et al.*, 1993b).

In recent years many inappropriate approaches towards studying plant enzymes and inhibitors have been used. Not only have they resulted in instability, due to harsh conditions of purification, but in addition they often showed low specific activities, explained by use of inappropriate substrates. Thus chloroplast Rubisco degrading activity cannot be observed when assayed with usual methods (Ragster and Chrispeels, 1981). This is an indication that many specific enzymes such as calpains may have been overlooked by using unphysiological substrates, and by not considering specific pH and metal ion requirements.

The customary test enzymes in screening for plant SPIs have for a long time been trypsin and chymotrypsin. Their use led to detection of many inhibitors that are today believed to be involved in plant protection rather than endogenous control. They were, by definition, unable to detect possible specific inhibitors of the subtilisin family of SPs, which are today recognised as the sole plant SPs (Rawlings and Barrett, 1993). Similarly, papain as the test enzyme is unable to detect potential inhibitors of asparagine-specific CP legumain (Kembhavi *et al.*, 1993), soybean leaf atypical CP (Huangpu and Graham, 1995), stem bromelain (Ritonja *et al.*, 1989), and papaya glycyl endopeptidase (Buttle *et al.*, 1990), which in spite of being CPs, all have different requirements for their specific inhibitors.

No endogenous plant inhibitor has yet been described for APs. The potato Kunitz family API has so far been assayed using only mammalian APs, and found effective

solely against human cathepsin D. The recently identified soybean leaf MP, plant counterparts of vertebrate MP (McGeehan *et al.*, 1992), still await their endogenous inhibitors, which are well defined in animals.

Conclusions and prospects

Much is now known, in a general sense, about endogenous proteases and inhibitors, their tissue and intracellular localisation, their substrate and inhibitor specificity. In order to build on the more qualitative description in terms of percent inhibition under defined *in vitro* conditions, three approaches stand out.

First there is the need for data on the velocity of interaction between protease and inhibitor, the stability of the complexes, and the ratio between inhibitor concentration *in vitro* and the inhibition constant, K_i. In this way, following the principles proposed by Bieth (1980) for mammalian systems, the real physiological role of inhibitors could be evaluated, remembering that an inhibitor will play its role only in the conditions where it can fully and rapidly inhibit its target protease.

In attempts to engineer plant resistance to the target proteins or proteases of insects, fungi, etc., it will be essential to characterise fully the interaction of the inserted or existing plant protease or inhibitor with the target protein. Identification of a single target protein whose inhibition or inactivation will result in effectively combating the target organism will also require extensive knowledge of that organism.

Secondly, existing techniques need to be developed and applied to allow separate quantitation of levels of actual protease and inhibitor, as well as activities. This will require more effective ways of plant cell fractionation to allow quantitation of levels and activities in each compartment.

Thirdly, changes in levels of proteases and inhibitors and of activities need, for their interpretation, quantitative assessment of expression levels of the relevant genes. In this way, a more complete picture can be built up of unstressed growth and of the response of the plant to stress.

With these three sets of tools, it will be possible to investigate and evaluate the signals and signal pathways involved in the response to stress.

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