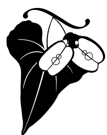

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Review Article

Proteases and their endogenous inhibitors in the plant response to abiotic stress

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ABSTRACT: Controlled protein degradation and activation of proenzymes is required for the growth and development of plants and for them to survive abiotic and biotic stresses. Uncontrolled proteolysis, that is often induced by stress, is however deleterious for plants. Proteases are essential for carrying out and regulating protein breakdown, functions that are regulated by specific endogenous protein inhibitors. General information on proteases and their inhibitors is reviewed, followed by descriptions of some of the increasing numbers of reports on their involvement in the plant response to abiotic stress. Particular emphasis is laid on drought, which is the most frequently studied abiotic stress. It will be shown that levels of proteases are increasingly seen to be associated with tolerance and sensitivity to abiotic stress, and a more complete picture is steadily emerging. The main hindrance to further understanding is the lack of knowledge of the natural substrates ofproteases. Further definition of their role in plant stress will lead, not only to an understanding of tolerance to stresses such as drought, but also provide an important basis for crop improvement.

Key words: protease, protease inhibitor, drought, salinity, cold, stress

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INTRODUCTION

The abiotic stresses most frequently encountered by plants include drought, heat, cold, salinity, extremes of light, and various metals. They exert a strong influence on the life and development of plants, at levels from the morphological and physiological to cellular and molecular. Proteins are involved in structural and functional aspects at all these levels. They are one of the targets of stressors and, at the same time, the main players in plant responses to stress. Under stress, proteins are often attacked by reactive oxygen species (ROS), causing structural changes that jeopardise their functions (Berlett & Stadtman 1997). Such aberrant proteins are frequently misfolded and aggregated and can be degraded by plant proteolytic enzymes (Vierstra 1996). Protein degradation by proteolysis, induced by stress and provoking dehydration of cells, can also be a consequence of disruption of cell membranes and release of hydrolytic enzymes, exhibiting features in common with senescence (McKersie & Leshem 1994).

It is of the utmost importance for their survival that plants maintain protein homeostasis, i.e. the balance between protein biosynthesis and degradation, under both optimal and stressing environmental conditions (Fig. 1). Controlled protein degradation is essential for the functioning of plants in all phases of their development and subsequent life. It is the source of amino acids required for *de novo* synthesis of proteins during recovery from stress, and for the limited hydrolysis of specific peptide bonds that results in posttranslational modification, including activation, of proteins. Well known examples are the breakdown of seed storage proteins during germination and of leaf proteins during senescence, and

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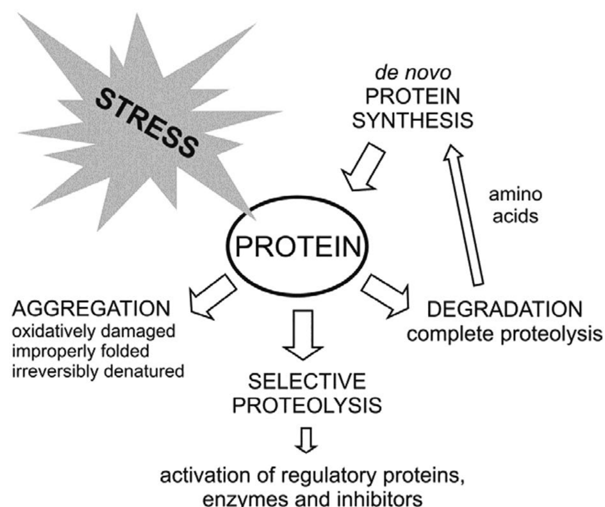

Fig. 1. Proteins under stress. Proteases and protease inhibitors are essential for maintenance of protein homeostasis.

the breakdown of damaged, and hence non-functional, proteins. Specific proteolysis is essential for the activation of the many enzymes and regulatory proteins needed during plant development and diverse environmental challenges. Uncontrolled protein degradation, often induced by biotic or abiotic stress, is deleterious for plant cells. Controlled protein hydrolysis has therefore been recognized as essential for the adaptation of plants to environmental conditions (Vierstra 1996).

Proteases, together with the specific endogenous inhibitors that regulate their activities, are the main players in carrying out and regulating intracellular protein breakdown for the maintenance of protein homeostasis. Plant proteases and their inhibitors have long ago been isolated from plant lattices and juices, the latter mainly from seeds. Some of them, such as papain, a protease isolated from papaya latex, leaves and roots, have been used as models for related proteases in order to study their structure, function and interactions with other proteins (Brzin & Kidrič 1995). Many important endogenous roles of proteases and protease inhibitors (PIs) are known. Recent technological developments, enabling genome sequencing and proteome analysis, have further disclosed the striking diversity and numbers of proteases and PIs in living organisms. For example, the peptidase database MEROPS (Rawlings *et al.* 2012), in its recent release 9.10 (last access on 11 January 2014), states that 5.21% of the genes in the *Arabidopsis thaliana* genome code for peptidases and 0.42% for PIs. These figures indicate the presence of 783 known and putative peptidases and 117 non-peptidase homologues, 72 known and putative PIs and 20 PI homologues. In rice (*Oryza sativa)*, whose genome was the first to be sequenced from a crop, the count for known and putative peptidases is 1194 and 103 for PIs and 356 for non-peptidase homologues (34 for PIs), representing 3.97 % of all genes for peptidases and 0.35% for PIs. Such numbers of proteases and PIs reflect not only the variety of their natural substrates but also the need for precise regulation of their activity, both spatial and temporal. It also presents a great challenge for research!

Studies aimed at increasing our understanding of proteases and their inhibitors in plants were first focused on the occurrence of proteolysis and on the proteases active during different stages of plant development, such as germination, differentiation, morphogenesis, senescence and programmed cell death. Gradually research has become focused on the involvement of proteases and PIs in response to environmental stress. For a long time the main interest was directed towards biotic stress, such as attack by herbivores and pathogens, as well as the often associated physical wounding. In addition to basic reasons, research in this field has been, and still is, inspired by potential applications in biotechnology (Brzin & Kidrič 1995; Sabotič & Kos 2012). Two decades ago abiotic stress attracted little attention and the relatively small number of studies were focused on the involvement of proteases and their inhibitors in plants under drought, high salinity and at high or low temperatures (Brzin & Kidrič 1995). Recent developments have started to change this picture of the field.

The majority of plants investigated belong to three families – Poaceaea (mainly cereals), Fabaceae (mainly legume plants) and Solanaceae (tomato, potato and tobacco). While these plants are still intrinsically important subjects of investigation, attention has also been focussed on *A. thaliana*, as a model plant. The involvement of proteases in the unique response of resurrection plants, which can recover from complete dehydration, has recently started to emerge as a particularly interesting subject of study.

The aim of this review is to present a general overview of the results of research in the field. As a basis for understanding, types of proteases and PIs will first be presented, together with a survey of their subcellular localization. Problems of handling plant systems relevant to studying proteases and PIs will be described, since they can have an important impact on currently reported results. The review will then be directed to the influence of abiotic stress on gene expression, abundance and activity of proteases and PIs. The focus will be on the response to stresses that commonly involve dehydration, mainly drought. More recent approaches, involving analysis of transcriptomes and proteomes, that have contributed to our understanding of the involvement of proteases and PIs, will be reviewed. Our intention is not to present a complete survey but to point to characteristic and

important cases. Although knowledge of the field is still fragmentary, the emergence of possible roles for proteases and their regulation by endogenous PIs is described. Finally, attention will be drawn to potential applications of the results of this research.

DIVERSITY AND LOCALISATION OF PLANT PROTEASES AND THEIR INHIBITORS

Proteases, by definition, cleave peptide bonds between two amino acid residues and are often referred to as proteolytic enzymes or peptidases. They occur in every living organism and are, structurally, highly diverse, as reviewed for particular plants. This is reflected in their diverse specificities for their natural substrates and in the different mechanisms they use for executing hydrolysis of peptide bonds. In addition, genome sequencing has revealed genes whose sequences are similar to those known to code for proteases – the probability of their being peptidases is based on the degree of homology and the presence of catalytic residues. When a protein encoded by such gene has not been isolated and a corresponding proteolytic activity not demonstrated experimentally, it is often termed a putative protease. The number of such genes further supports the great diversity of these enzymes, even though, at this point in evolution, they may not be transcribed.

Types of proteases. The most basic division of proteases is between endopeptidases, which cleave peptide bonds within the polypeptide chain, and exopeptidases, which cleave peptide bonds at the termini of polypeptide chains. When the latter act on N-terminal peptide bonds they are called aminopeptidases, and carboxypeptidases when cleaving C-terminal peptide bonds. As a consequence of the multiplicity of their natural substrates, and often the lack of their precise identification, especially in the case of plants (Tsiatsiani *et al.* 2012), proteases are further classified on the basis of their catalytic mechanisms. The IUBMB Enzyme Nomenclature system (http://www.chem.qmul.ac.uk/iubmb) classifies peptidases as hydrolases and combines the position of the cleaved peptide bond and the catalytic type (Table 1).

The MEROPS database (http://merops.sanger.ac.uk/), which reflects the increasing number of proteases identified and is thus very useful for researchers in the field, uses a classification that takes into account only catalytic type (Table 2). They are therefore classified into aspartic (A), cysteine (C), serine (S), threonine (T) and glutamic (G) peptidases and asparagine peptide lyases (N), all based on the amino acid residue at the active site involved directly in peptide bond hydrolysis, and into metallopeptidases (M) that require a divalent metal ion as part of the active site. There are also mixed peptidases (P) of C, S and/or T catalytic type and peptidases of unknown catalytic type for which no active site residues have been determined. Peptidases in the above groups are further classified into 'families' according to similarities in their overall amino acid sequences. Families are grouped into 'clans' according to similarity of primary structure around the active site and similarity of their tertiary structure, both of which provide evidence of their evolutionary relationship. Until recently (Release 9.10) 245 peptidase families have been described in the MEROPS database. Those with available primary and tertiary structures are classified into more than 50 clans (Rawlings *et al.* 2012). Evolutionary and structural similarities are thus basic to the MEROPS classification. Consequently, enzymes with endo- and enzymes with exo-peptidase activity can be found in the same family, and even homologs that do not exhibit peptidase activity (Barrett 2004).

The MEROPS database shows that a large number of proteases have already been isolated from plants. The current situation is that they belong to all catalytic types except G, although the catalytic type is still not known for several proteases (Rawlings *et al.* 2012).

Types of protease inhibitors. The term PIs in this review denotes protease inhibitors that are proteins, usually relatively small, of the order of 2-20 kDa. In addition to these, there are small molecule inhibitors, some of which occur naturally and have been isolated from bacteria and fungi, while many are synthetic. Only protein PIs and their involvement in the plant response to abiotic stress will be reviewed. They can be classified according to their reaction mechanism (competitive, non-competitive, uncompetitive, suicide protease inhibitors) or to their specificity, into those that inhibit various classes of proteases, one class of proteases, one family of proteases or a single protease.

PIs are usually referred to by the type of protease they inhibit, for example as cysteine protease inhibitors. This classification is however approximate, since PIs that target proteases of more than one catalytic type have been isolated and characterized (Barrett 1981; Rawlings *et al.* 2012). As with proteases, the MEROPS database offers a more detailed and appropriate classification and is organised in a way similar to that for proteases (Table 3). Based on sequence homology, PIs are first grouped into families and their tertiary structure is the basis for grouping families into clans. Currently there are 74 families in the MEROPS database (release 9.10), including 22 of plant origin. Of the latter, 10 families include PIs isolated exclusively from plants. The MEROPS database also includes information about well-known and often used small molecule inhibitors.

Table 1. Classification of proteases by the IUBMB Enzyme Nomenclature System (http://www.chem.qmul.ac.uk/iubmb/)

	EC number		Type of classification
Endopeptidase	EC 3.4.21	serine endopeptidases	catalytic mechanism
	EC 3.4.22	cysteine endopeptidases	
	EC 3.4.23	aspartic endopeptidases	
	EC 3.4.24	metalloendopeptidases	
	EC 3.4.25	threonine endopeptidases	
	EC 3.4.99	peptidases of unknown catalytic mechanism	
Exopeptidase	EC 3.4.11	aminopeptidases	cleavage of peptide bonds at C-terminus or N-terminus of the polypeptide chain
	EC 3.4.13	dipeptidases	
	EC 3.4.14	dipeptidyl-peptidases and tripeptidyl-peptidases	
	EC 3.4.15	peptidyl-dipeptidases	
	EC 3.4.16	serine-type carboxypeptidases	
	EC 3.4.17	metallocarboxypeptidases	
	EC 3.4.18	cysteine-type carboxypeptidases	
	EC 3.4.19	omega peptidases	

In general, families of serine protease inhibitors predominate, followed by a few families of inhibitors of cysteine and metallo-proteases, while aspartic protease inhibitors are rare and dispersed in different families. The soybean trypsin inhibitor was the first plant protease inhibitor to be isolated (Kunitz 1945), and similar proteins, subsequently characterized, have been named Kunitz trypsin inhibitors (family I3). They are widespread in plants and encoded by families of genes that are expressed in all plant tissues, but mostly in the seeds of some leguminous plants. They mainly inhibit serine proteases (SPs), while some inhibit aspartic or CPs (Rawlings 2010). Protease inhibitors belonging to the Bowman-Birk family (family I12) are named after the two scientists who isolated and characterized the first member from soya beans (Birk *et al.* 1963; Bowman 1946). They are compound inhibitors, containing one to six inhibitor units that often have different specificities, but all target SPs (Qi *et al.* 2005; Rawlings 2010). The largest family of cysteine protease inhibitors in plants is the cystatin family, also called phytocystatins (family I25). They inhibit papain-like CPs and can be compound inhibitors comprising up to 8 inhibitor units, which are then called multicystatins (Rawlings 2010). The second largest family of CP inhibitors in plants comprises proteins that are homologous to the proregions of papain-like CPs (family I29). They are widespread throughout the plant kingdom and inhibit papain-like proteases with higher selectivity for individual proteases than do cystatins (Rawlings 2010; Wiederanders 2003; Yamamoto *et al.* 2002).

Localisation of proteases and PIs. Plant proteases and their PIs are present within every plant organ and within almost every cell compartment, as well as extracellularly (Fig. 2). It is important to note that they are often not limited to just one organ or cell organelle, so that, for example, a protease abundant in seeds is also found in leaf and root tissues (Rogers *et al.* 1985). Phytocystatins and Bowman-Birk inhibitors are expressed in all plant tissues (Rawlings 2010). In contrast, there are a few examples where some proteases and PIs have been detected in a single organ only.

Our knowledge in this area originates mainly from studies carried out on seeds, tubers and fruits of cereals like barley and rice, legumes like *Vicia sp.* and *Vigna sp.*, and potato (Brzin & Kidrič 1995). These organs are very rich sources of cysteine proteases (CPs), as exemplified by aleurain from barley, which also shows aminopeptidase specificity (Rogers *et al.* 1985), and oryzains from rice

Table 2. Classification of proteases from plants by the MEROPS database (http://merops.sanger.ac.uk/). Only peptidase families with more than 100 counts of homologues found in sequenced plant genomes are included.

Catalytic mechanism	Clan	Family	Number of homologues	Type peptidase (source)
aspartic peptidases	AA	A1	1544	pepsin A (*Homo sapiens*)
		A11	1267	Copia transposon peptidase (*Drosophila melanogaster*)
		A28	138	DNA-damage inducible protein 1 (*Saccharomyces cerevisiae*)
	AD	A22	200	presenilin 1 (*Homo sapiens*)
cysteine peptidases	CA	C1	1130	papain (*Carica papaya*)
		C19	768	ubiquitin-specific peptidase 14 (*Homo sapiens*)
		C85	209	ubiquitin-specific peptidase 14 (*Homo sapiens*)
	CD	C13	186	legumain (*Canavalia ensiformis*)
		C14	208	caspase-1 (*Rattus norvegicus*)
	CE	C48	286	Ulp1 peptidase (*Saccharomyces cerevisiae*)
	CP	C97	170	DeSI-1 peptidase (*Mus musculus*)
metallo-peptidases	MA	M1	135	aminopeptidase N (*Homo sapiens*)
		M3	101	thimet oligopeptidase (*Rattu norvegicus*)
		M10	146	matrix metallopeptidase-1 (*Homo sapiens*)
		M11	106	gametolysin (*Chlamydomonas reinhardtii*)
		M41	351	FtsH peptidase (*Escherichia coli*)
	ME	M16	354	pitrilysin (*Escherichia coli*)
	MG	M24	311	methionyl aminopeptidase 1 (*Escherichia coli*)
	MH	M20	272	glutamate carboxypeptidase (*Pseudomonas* sp.)
	MP	M67	195	PSMD14 peptidase (*Saccharomyces cerevisiae*)
mixed (C, S, T) catalytic type	PA	S1	319	chymotrypsin A (*Bos taurus*)
	PB	C44	222	amidophosphoribosyltransferase precursor (*Homo sapiens*)
		T1	677	archaean proteasome, beta (*Thermoplasma acidophilum*)
	PC	C26	474	gamma-glutamylhydrolase (*Rattus norvegicus*)
		C56	173	PfpI peptidase (*Pyrococcus furiosus*)
serine peptidases	SB	S8	476	subtilisin Carlsberg (*Bacillus licheniformis*)
	SC	S9	792	prolyl oligopeptidase (*Sus scrofa*)
		S10	1020	carboxypeptidase Y (*Saccharomyces cerevisiae*)
		S28	139	lysosomal Pro-Xaa carboxypeptidase (*Homo sapiens*)
		S33	730	prolyl aminopeptidase (*Neisseria gonorrhoeae*)
	SF	S26	164	signal peptidase I (*Escherichia coli*)
	SJ	S16	109	Lon-A peptidase (*Escherichia coli*)
	SK	S14	871	peptidase Clp (*Escherichia coli*)
		S41	108	C-terminal processing peptidase-1 (*Escherichia coli*)
	ST	S54	175	rhomboid-1 (*Drosophila melanogaster*)

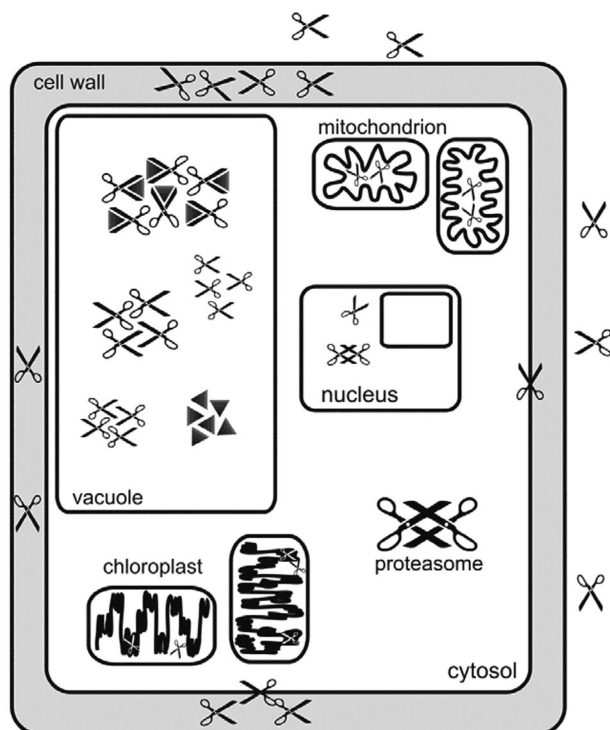

Fig. 2. Localization of proteases in plant cell. Proteases are indicated by scissors, and protease inhibitors by triangles.

(Watanabe *et al.* 1991). Many of them are engaged in the final stages of maturation of major storage proteins or in the degradation of seed storage proteins during germination. These organs are also very rich sources of PIs. Some of the first studied plant inhibitors of phytocystatins, that are involved in germination and whose gene and protein structures have been elucidated, are those from rice. They appear in the seed during the ripening stage and disappear during germination, when oryzains begin to appear (Kondo *et al.* 1990). SPs and aspartic proteases (APs) and their PIs appear to be less abundant here (Brzin & Kidrič 1995).

Comparison of our present knowledge with that from two decades ago (Brzin & Kidrič 1995) shows that the greatest advance in understanding cellular proteolysis has been achieved by research focused on the proteases and PIs in leaves, in particular those involved in the specific, targeted proteolysis that operates in the proteinaceous milieu of the cytoplasm and in close cooperation with molecular chaperones (Vierstra 1996). They require metabolic energy in the form of ATP for their activity and are termed ATP-dependent (Adam 2007). In general, such energy coupling is typical of the large multi-subunit protease complexes. The proteases detected in different cellular compartments are adapted to specific functions and to the local environments of the cytosol and cellular organelles.

Most of the proteolytic activity measured in crude plant extracts originates from proteases localised in vacuoles. These organelles contain proteases of all classes, optimally active at acidic pH (Müntz 2007). Probably they are either APs, CPs such as papain-like proteases mostly active at acidic pH, meta-caspases or legumains, the vacuolar processing enzymes that are highly selective in cleaving after specific residues (Rawlings *et al.* 2012). It should be noted here that CPs are among the few proteases so far detected in a resurrection plant (Blomstedt *et al.* 1998; Rodriguez *et al.* 2010). Other catalytic types are also present but they are less abundant – for example, carboxypeptidases belonging to SPs – but which are distinct from other SPs in that they are active only at acidic pH (van der Hoorn 2008). The acidic environment in the vacuoles could facilitate degradation of target proteins. Vacuoles in leaves, that contain high proteolytic activity, would not be expected to contain significant amounts of PIs, particularly of APs and CPs. But they are the storage place of an array of proteases and PIs such as wound-inducible proteinase inhibitors and proteases that target the proteins and/or proteases of herbivores and phytopathogens (Müntz 2007). Proteases acting in the vacuoles or in extracellular space are in general energy independent; however, vacuolar degradation may require energy for trafficking substrates to this lytic compartment (Vierstra 1996).

In contrast to vacuoles, in which complete proteolysis predominates, proteolysis in the cytosol and nucleus is mainly selective. The main proteolytic system in the cytosol is the ubiquitin/26S proteasome pathway that involves threonine proteases (TPs). The structure and activities of proteasomes are highly conserved among eukaryotes, suggesting essential functions in protein homeostasis. This system provides an extremely large and complex route for protein degradation, involving not only a complex structure formed by several proteases but also a whole array of enzymes needed for covalent binding of proteins targeted to ubiquitin for degradation. The system accounts for nearly 6% of the *Arabidopsis thaliana* transcriptome (Vierstra 2009). Phytocalpain, belonging to the CP calpains, also resides in the cytosol (Croall & Ersfeld 2007).

The optimal pH for activity of the majority of SPs lies within the neutral to alkaline region, so it is not surprising that few have been found in mitochondria, chloroplasts and peroxisomes, where other proteases also reside, notably metallopeptidases (MPs), which are rarely found in plants. Mitochondria and chloroplasts possess their own conserved proteolytic systems that are very similar to those of the prokaryotes. Although these proteases contain

Table 3. Classification of protein protease inhibitors from plants in the MEROPS database (http://merops.sanger.ac.uk/). Only families with more than 10 counts of homologues found in sequenced plant genomes are included.

Clan	Family	Number of homologues	Type inhibitor (source)
IC	I3	327	soybean Kunitz trypsin inhibitor (*Glycine max*)
ID	I4	192	alpha-1-peptidase inhibitor (*Homo sapiens*)
IE	I7	37	trypsin inhibitor MCTI-1 (*Momordica charantia*)
IF	I12	213	Bowman-Birk inhibitor unit 1 (*Glycine max*)
IG	I13	182	eglin c (*Hirudo medicinalis*)
IH	I25	207	cystatin A (*Homo sapiens*)
IJ	I6	68	ragi seed trypsin/alpha-amylase inhibitor (*Eleusine coracana*)
JB	I63	13	pro-eosinophil major basic protein (*Homo sapiens*)
JD	I18	19	mustard trypsin inhibitor-2 (*Sinapis alba*)
JE	I51	186	serine carboxypeptidase Y inhibitor (*Saccharomyces cerevisiae*)
JF	I29	176	cytotoxic T-lymphocyte antigen-2 alpha (*Mus musculus*)
JO	I20	142	potato peptidase inhibitor II inhibitor unit 1 (*Solanum tuberosum*)
unassigned	I55	23	squash aspartic peptidase inhibitor (*Cucumis sativus*)

several subunits, forming rather complex structures, they are less complex than those in the ubiquitin/26S proteasome pathway. It is notable that each of the major chloroplast compartments contains defined proteases, among them being SPs and metallopeptidases. The former include the ATP-dependent Clp proteases in stroma, ATP-independent DegP proteases within the thylakoid lumen and on both sides of thylakoid membranes, and the SppA protease on the stromal side of the thylakoid. The ATP-dependent FtsH proteases (M-type) are found in stroma-exposed thylakoid membranes (Adam & Clarke 2002). They are the best known plant MPs, and it is interesting that one of them has been detected in a resurrection plant (Ingle *et al.* 2007). Another SP found in mitochondria and chloroplasts is a hexameric, ATP-dependent SP named Lon protease, which also resides in peroxisomes (Adam 2007; Janska *et al.* 2010). Other proteases are also present in peroxisomes (Palma *et al.* 2002). About 70% of the total proteolytic activity in these organelles can be assigned to SPs.

Cell walls and intercellular space also contain proteolytic activities that mostly belong to SPs (Brzin & Kidrič 1995). The apoplast is the site of the first line of proteolytic defence against pathogens. Furthermore, extracellular proteases that catalyse the hydrolysis of proteins into smaller peptides and amino acids for subsequent absorption into the cell, constitute a very important step in nitrogen metabolism (Lopez-Otin & Bond 2008; Vierstra 1996).

Natural substrates of plant proteases. Knowledge of natural substrates of plant proteases is very limited, due to the difficulties in identification. A remarkable set of results exists concerning the occurrence and localisation of plant proteases, their experimentally proven involvement in various processes during different stages in the life of plants and in the response to changes in their environment. Despite this, less than 40 of their natural substrates have been identified (Tsiatsiani *et al.* 2012). Among them are the seed storage proteins β-conglycinin and β-type phaseolin, both substrates of SP subtilases C1 and C2 from *Glycine max*; the components of photosystem II, that are substrates of DegP proteases from *A. thaliana* chloroplasts; ORF239 protein that is associated with cytoplasmic male sterility, substrate of Lon protease from *Phaseolus vulgaris* mitochondria; and, probably, Rieske Fe-S, a protein of the cytochrome b_6-*f* complex, substrate of FtsH from chloroplasts. The recently established degradomics technologies should enable proteome-wide studies of plant proteases (Tsiatsiani *et al.* 2012).

Problems in handling plant systems relevant to the study of proteases and PIs. Several aspects influence the validity of conclusions drawn from experiments studying proteases and PIs from plants. In addition to physiological aspects, particular care needs to be taken in the choice of the plant species and its cultivars, plant parts and the conditions under which they are cultivated. Furthermore, the proper choice and preparation of materials and adequate sampling are very important in investigating proteases and their PIs, especially when quantification of changes of their activities is the aim, as in studies directed towards impact of environmental stress. Studies carried out on different cultivars of the same plant can give non-negligibly different results (Brzin & Kidrič 1995; Hieng *et al.* 2004). Even when dealing with a single cultivar, appreciable variation has been observed between plants grown under slightly different conditions (Brzin & Kidrič 1995). When materials are obtained from plants grown in growth chambers, greenhouses or in the field, special attention has to be paid to any changes in conditions and to possible infection, since many proteases and PIs are markedly sensitive to changes in environment (Kidrič *et al.* 2014). Sampling is a very critical point: the stage in plant development and circadian expression should be taken into account (Brzin & Kidrič 1995; Martinez *et al.* 2003). In the case of leaves, their age and/or position on the plant can have an important impact on levels of proteolytic activities (Budič *et al.* 2013). All these factors are also critical in preparing samples for proteome analysis, since only small differences in growth conditions can influence the abundance of some proteins (P. Jamnik, unpublished results).

The nature of plant material itself presents some specific problems, such as tough cell walls, heavy pigmentation and the presence of polyphenols that can induce protein aggregation and can cause interference in detecting proteolytic activity by spectroscopic methods. Very large amounts of proteinaceous and carbohydrate materials, usually in the form of inactive storage materials, are another 'obstacle'. It is useful and often essential therefore to separate subcellular fractions in order to be able to identify target proteases and/or PIs that could otherwise be 'covered' by a multitude of others. Due to rigid cell walls, plant materials often require rather severe homogenisation procedures, thus reducing the yield of intact organelles (Brzin & Kidrič 1995). Unwanted proteolysis in extracts may occur during longer isolation procedures, since general proteolytic inhibitors cannot be used.

Last, but not least, there is the problem of the substrates appropriate for use in studies. Many specific proteases and PIs can be overlooked, especially when they are present in small quantities, by using non-physiological substrates and not considering specific pH and metal ion requirements (Brzin & Kidrič 1995).

PROTEASES AND THEIR INHIBITORS IN THE PLANT RESPONSE TO ABIOTIC STRESS

The number of proteases and PIs and their diversity described above, their appearance in a variety of plant organs and subcellular organelles and their evident functional redundancy is striking. Coupled with the lack of knowledge of their endogenous substrates, this makes identification of their involvement in plant responses to abiotic stress and elucidation of their roles very difficult. The application of new techniques, coupled with useful older ones, has nevertheless led to marked advances in the field. More and more proteases and PIs involved in stress have been detected and some of them identified and characterised. Consequently, the puzzle is starting to fill, though far from complete by.

The suggestion that proteolytic enzymes are specifically involved in the response of plants to abiotic stress originated in observations that stress conditions often bring about senescence of plant tissue and that the senescence is closely connected with enhanced proteolysis that involves several proteases in the same tissue (Huffaker 1990). This complicates differentiation between the different possible causes of change in protease activity. Further, in the natural environment, factors that induce a state of stress seldom act individually. However, at the same time, different stresses can have the same effect at the cell level and responses to them may share common molecular mechanisms. Such a relationship between drought, salt stress and cold is well known (Bartels & Nelson 1994).

Degenerative changes in cell membranes are common to different kinds of stress and senescence. They lead to degradation of membrane structures and leakage of solutes (McKersie & Leshem 1994). This not only changes the local environment of the plant cell and thus denatures and/or degrades some molecules, but also enables pathogens, another stress for plants, to invade such tissue. Moreover, many kinds of abiotic stress induce production of reactive oxygen species (ROS), a common secondary stress, whose levels can increase dramatically, damaging cell structures, including proteins. Many of these events are known to induce proteolysis, uncontrolled or regulated and limited, but their complexity makes it difficult to establish clear correlations between a particular stress and the corresponding response at the level of proteases and PIs. Nevertheless, it has been established that levels of expression of some proteases and PIs, as well as of their activities, are involved in the plant response to abiotic stress.

Abiotic stress can execute its influence on the activity of proteases and their inhibitors at a number of fundamentally different levels – gene expression, translation of transcripts to proteins, often in the form of pre-pro-proteins, posttranslational modification leading to their activation, and by direct action on the molecule itself. Although these levels are closely related and interdependent, changes in protease gene expression do not necessarily lead to changes in protease activity nor do changes in the latter necessarily indicate changes in the former, so knowledge of both is essential. The majority of studies have been directed to one of these levels only, so the following sections will focus separately on changes in transcriptome, proteome and proteolytic activity. Results in the field are still limited to observation of, individually, changes in gene expression, abundance of proteins and changes in their activity, and only seldom have these observations been linked to a specific pathway. They lead to the impression that both proteases and PIs play important roles in responses to different abiotic stresses but much more research will be needed to link them together.

Changes in gene expression. The first studies concerning the influence of abiotic stress on the expression of genes suggested to encode proteases appeared at the beginning of 1990. All the proteases were CPs, but belonged to different families and were detected in a variety of plants under different stresses such as tomato under low temperature and pea under drought, *A. thaliana* under drought and/or salinity (Brzin & Kidrič 1995; Ingram & Bartels 1996). Since then, differential expression, mainly increased, of genes coding for different putative proteases affected by drought has been shown in *Arabidopsis* and in other plants (Bartels & Sunkar 2005; Seki *et al.* 2002). Changes in expression of genes of several PIs in response to abiotic stress have also been observed.

Abiotic stress influences the expression of many genes encoding putative CPs, usually resulting in their up-regulation. For example, genes coding for CP vacuolar processing enzyme in *Arabidopsis* under heat shock (Li *et al.* 2012), a cathepsin B-like CP in barley leaves stressed by cold shock (Martinez *et al.* 2003), a CP in poplar cells under osmotic stress (Bae *et al.* 2010) and a putative CP in the resurrection grass *Sporobolus stapfianus* during drying (Blomstedt *et al.* 1998). It is remarkable that analysis of transcriptomes from the desiccation tolerant resurrection plant *Craterostigma plantagineum* showed a great accumulation of transcripts for several types of CPs in completely desiccated plants (Rodriguez *et al.* 2010). Low water deficit induced a decrease in the accumulation of transcripts of a gene encoding a putative CP in leaves of two peanut cultivars, while more severe drought led to an increase, at an earlier stage than in a more tolerant cultivar (Drame *et al.* 2007). Post-harvest chilling induced down-regulation of the CP bromelain in pineapple fruits, most dramatically in the resistant variety (Raimbault *et al.* 2013). On the other hand, accumulation of transcripts of two wheat CPs was supressed in a drought-tolerant cultivar and unchanged or augmented in a less resistant one (Simova-Stoilova *et al.* 2010). The expression of genes coding for CPs was down-regulated in shoots of foxtail millet in response to dehydration (Zhang *et al.* 2007).

Expression of genes coding for APs are also influenced by abiotic stress. They are highly up-regulated in early grain development in wheat under combined drought and heat (Szűcs *et al.* 2010) and in buckwheat leaves under the influence of drought, dark and UV-B light (Timotijević *et al.* 2010). They are, however, down-regulated in roots of foxtail millet in response to dehydration (Zhang *et al.* 2007). Expression of the gene encoding the precursor of phytepsin-like aspartic acid protease in common bean leaves was significantly up-regulated in the drought-susceptible cultivar under mild drought stress, but only under stronger stress in the more drought-tolerant cultivar. In cowpea, expression was constitutive, and up-regulated in senescence (Contour-Ansel *et al.* 2010; Cruz de Carvalho *et al.* 2001; Cruz de Carvalho *et al.* 2004), but up-regulated in pineapple fruit under postharvest chilling stress in a resistant cultivar and down-regulated in a susceptible cultivar (Raimbault *et al.* 2013).

Expression of the gene coding for chloroplast serine endopeptidase DegP2 increased in *A. thaliana* under stresses induced by high salt, desiccation or high light (Haussühl *et al.* 2001). In flowers and young leaves of this plant, the expression of genes encoding several subtilisin-like proteases was increased following treatment with cadmium (Golldack *et al.* 2003). Expression of the gene coding for a subtilisin-like SP was down-regulated in leaves of two peanut cultivars, the decrease being more pronounced in the tolerant one (Drame *et al.* 2007). In leaves of a common bean cultivar, expression did not change (Budič *et al.* 2013). Decline of the expression of genes encoding SPs in wheat occurred earlier in early grain development under combined drought and heat treatment than under control conditions (Szűcs *et al.* 2010).

Transcription of one member of a gene family encoding ATP-dependent FtsH in maize leaves was markedly up-regulated by water deficit and abscisic acid (ABA) treatment, but its overexpression did not improve the drought tolerance of the plant (Yue *et al.* 2010). Short-term moderate and severe drought, high salt, cold, heat, oxidation, wounding and high light exerted no influence on the expression of chloroplast ATP-dependent Clp proteases in *Arabidopsis*, although increases in transcripts

and in the content of encoded proteins did occur during long-term high light and cold acclimation (Zheng *et al.* 2002).

Ubiquitin transcripts in the resurrection plant *Sporobolus stapfianus*, almost completely absent in the desiccated stage, accumulated during dehydration and rehydration, indicating involvement of the proteasome during entry into anhydrobiosis and subsequent rehydration (O'Mahony & Oliver 1999). On the other hand, the abundance of the 20S proteasome α6 subunit was reduced (Oliver *et al.* 2011). Ubiquitin genes were up-regulated in drought-tolerant cultivars of sugarcane (Jangpromma *et al.* 2010).

Expression of the gene coding for methionine aminopeptidase in barley was induced by low temperature and ABA treatment (Jeong *et al.* 2011). A significant increase in the transcript levels of genes coding for prolyl aminopeptidase, an SP, was rapidly induced by salt and drought stresses in *Arabidopsis* (Sun *et al.* 2013), in the shoots of triticale plants under drought and saline conditions, and in the presence of cadmium and aluminium ions (Szawlowska *et al.* 2012). Levels of transcript of the gene coding for hexameric leucine aminopeptidase LAP-A increased in cadmium treated tomato roots, together with a corresponding increase in LAP-A protein levels (Boulila-Zoghlami *et al.* 2011).

Changes in gene expression of various PIs have also been observed (Seki *et al.* 2002). Various cystatin genes in *A. thaliana* show different patterns of expression during development and in their responses to abiotic stresses, suggesting that individual cystatins have distinct functions in response to abiotic stress (Hwang *et al.* 2010). Expression of two cystatin genes was strongly induced in *Arabidopsis thaliana* by a number of abiotic stresses, including high salt and drought (Zhang *et al.* 2008). Up-regulated genes include the phytocystatin gene in *Arabidopsis* under heat stress (Je *et al.* 2014) and the gene coding for cystatin in roots and stems of *Amaranthus hypochondriacus* in response to water deficit, salinity, cold and heat stresses, the last also inducing a rapid and transient accumulation of its transcripts in leaves (Valdes-Rodriguez *et al.* 2007). The cystatin gene in grapevine was also induced under comparative drought and salt stress (Cramer *et al.* 2007). A multicystatin in winter wheat, whose expression is induced during cold acclimation, is also induced by drought, mostly in roots (Christova *et al.* 2006). A multicystatin in leaves of cowpea is also induced under the influence of drought, shown at the levels of both gene expression and protein content (Diop *et al.* 2004; Shui *et al.* 2013). However, the transcript accumulation patterns observed in the two cowpea cultivars differed with an earlier response in the tolerant one (Diop *et al.* 2004). Post-harvest chilling had the opposite effect, causing up-regulation of the gene encoding for the endogenous inhibitor of the CP bromelain in pineapple fruits in the resistant variety and down-regulation in the susceptible one (Raimbault *et al.* 2013). Massonneau *et al.* (2005) observed down-regulation of some cystatin genes in maize in response to severe water deficit.

Expression of a Kunitz-type SP inhibitor, BnD22, which moonlights as a water-soluble chlorophyll binding protein (WSCP), was induced in young leaves of oilseed rape (*Brassica napus*) in response to drought. (Desclos *et al.* 2008; Downing *et al.* 1992; Ilami *et al.* 1997). Constitutive expression and increased expression in expanded mature leaves of the trypsin specific protease inhibitor SPLTI from sweet potato (*Ipomoea batatas*) was observed (Wang *et al.* 2003). A trypsin inhibitor (29 kDa) in the leaves of *A. hypochondriacus* is expressed constitutively while the expression of two smaller trypsin inhibitors (2 and 8 kDa) is induced by water and salt stresses (Sanchez-Hernandez *et al.* 2004). Water deficit induced up-regulation of the gene coding for a Bowman-Birk inhibitor of SPs in peanut leaves. The patterns of transcript accumulation during water deficit differed in tolerance between two cultivars – transcripts accumulated earlier and more strongly in the tolerant one (Drame *et al.* 2013). This type of inhibitor was also induced at the gene level in aluminium induced oxidative stress in *Arabidopsis* (Richards *et al.* 1998), as well as in wheat roots exposed to salt, drought or oxidative stress (Shan *et al.* 2008). An SP inhibitor was induced in drought-tolerant sugarcane cultivars (Jangpromma *et al.* 2010). Expression of the gene for an inhibitor of SP chymotrypsin was strongly up-regulated in rice under dehydration stress (Huang *et al.* 2007). However, expression of the gene encoding inhibitor 2, that inhibits SPs subtilisin and chymotrypsin, was down-regulated in shoots of foxtail millet in response to dehydration (Zhang *et al.* 2007).

Changes in the proteome. Changes in transcript levels do not necessarily reflect changes in protein abundance, so the study of proteomes provides another level of information. More than 1000 transcripts were differentially regulated in *Arabidopsis* subjected to mild drought but levels of only about 1.5% of more than 2008 proteins were changed significantly (Baerenfaller *et al.* 2012). This suggests that, under environmental perturbation, the stability of the proteome is greater than that of the transcriptome.

One of the drawbacks of proteomics is that many proteins that may be differentially regulated during the plant response to stress cannot be detected because of their low abundance. Some proteases and PIs could be among them. One way of overcoming this problem is to combine sub-proteome enrichment with depletion of highly-abundant proteins characteristic of plants, such

as Rubisco or some storage proteins. A further problem, especially for proteases, is that changes in abundance may not be indicative of their roles, since many proteases need posttranslational modification for activation. A global approach to the proteolytic system, termed degradomics, has recently been introduced. It combines the application of both genomic and proteomic approaches to identification of the protease and protease-substrate repertoires in an organism, with the aim of revealing the specific functions of these enzymes (Lopez-Otin & Overall 2002).

Studies of the proteome have contributed significantly to understanding the plant response to abiotic stress (Kosova *et al.* 2011) by revealing changes in the abundance of proteases. For example, up-regulation of different subunits of the 20S proteasome has been detected in desiccation-tolerant maize embryos during desiccation (Huang *et al.* 2012), in leaves of drought-treated *Medicago sativa* plants (Aranjuelo *et al.* 2011), in wheat stems under drought (Bazargani *et al.* 2011), and during dehydration of desiccation tolerant grass *Sporobolus stapfianus* (Oliver *et al.* 2011). On the other hand, in response to dehydration, proteins involved in proteolysis carried out by the proteasome were found to be expressed to constant levels in nuclei in the desiccation tolerant *Xerophyta viscosa*, probably to help maintain minimum viability in cells under stress (Abdalla & Rafudeen 2012). In addition, subunits of the proteasome are down-regulated in leaf symplast of legume *Vigna unguiculata* under excessive manganese nutrition and up-regulated in deficient iron nutrition in tomato, in deficient potassium nutrition in seedlings of *A. thaliana*, in wounded potato tubers (Kosova *et al.* 2011) and in apoplast in rice shoot stems under salinity (Song *et al.* 2011).

Another ATP-dependent protease showing changes in abundance under abiotic stress is chloroplast FtsH protease. It is up-regulated in leaves during dehydration of the resurrection plant *Xerophyta viscosa* (Ingle et al. 2007) and in leaves of barley under heat stress (Rollins *et al.* 2013), but down-regulated in the nuclear proteome of *Cicer arientum* seedlings under dehydration (Pandey *et al.* 2008).

The FtsH-like protein Pftf precursor was shown to be responsive to low temperature stress in rice seedlings (Cui *et al.* 2005). ATP-dependent Clp protease is down-regulated in leaves of barley under heat stress (Rollins *et al.* 2013), but expressed in nuclei to constant levels in desiccation tolerant *Xerophyta viscosa* in response to dehydration (Abdalla & Rafudeen 2012). ATP-dependent Clp protease ATP-binding subunit responds to low temperature stress in rice seedlings (Cui *et al.* 2005), and ATP-dependent Clp protease proteolytic subunit is up-regulated in drought-induced senescence in wheat stem (Bazargani *et al.* 2011).

Changes in the protein expression of ATP-independent proteases have also been detected by proteome analysis. A putative zinc dependent protease is down-regulated in leaves of barley under heat stress (Rollins *et al.* 2013), while the precursor of the CP aleurain in wheat stem is significantly up-regulated under drought (Bazargani *et al.* 2011). CPs in heat-exposed peach fruits are similarly up-regulated (Lara *et al.* 2009). Differential analysis of the leaf proteome of common bean under drought indicates higher abundances of CP precursors in stressed samples (Zadražnik *et al.* 2013). CP from tomato roots under waterlogging is also up-regulated (Ahsan *et al.* 2007). AP precursor and serine carboxypeptidase 1 are up-regulated, and subtilisin-like proteases down-regulated in apoplast in rice shoot stems under salinity (Song *et al.* 2011).

Analysis of the stem proteome of *Lupinus albus* subjected to water deficit showed simultaneous *de novo* expression of a senescence-associated CP and up-regulation of a putative subtilisin-like SP (Pinheiro *et al.* 2005). Interestingly, this study also showed concomitant up-regulation of several PIs.

Changes in expression of PIs have been detected in this type of study, often those inhibiting SPs such as serpins which increased in amount in wheat seeds subjected to heat stress (Laino *et al.* 2010; Yang *et al.* 2011). In the elongation zone of the soybean and in maize primary root, trypsin inhibitors are up-regulated under water stress, together with a CP inhibitor (Yamaguchi *et al.* 2010). The abundance of a putative cystatin increases in desiccation tolerant maize embryos during desiccation (Huang *et al.* 2012), and a multidomain cystatin is also up-regulated in wheat grain under high temperature (Yang *et al.* 2011). In contrast, a CP inhibitor is down-regulated in stem of wheat stressed by drought (Bazargani *et al.* 2011). Another drought responsive PI, detected in wheat grain, is the WSCI protease inhibitor (Hajheidari *et al.* 2007). Protease inhibitors are up-regulated in the proteome of potato tubers subjected to mechanical wounding (Kosova *et al.* 2011).

C***hanges in proteolytic activities.*** Two approaches to the study of the involvement of proteases in the plant response to abiotic stress are described in previous sections. Since activity is the operative feature involved when dealing with proteases, a further approach is needed that focuses directly on changes in their activities as a consequence of stress. This can also lead to identification and characterisation of the proteases whose changes in activity are detected. Few investigations at the levels of gene expression and protein abundance have incorporated determination of activity, although the reverse was true for many of the early studies in the field.

The changes observed in proteolytic activity in response to abiotic stress are often similar to those that occur in senescence. Increased acidic proteolytic activity in leaves of plants subjected to prolonged drought stress is well documented in plants at the vegetative growth stage (Cruz de Carvalho *et al.* 2001; Simova-Stoilova *et al.* 2010; Zagdanska & Wisniewski 1996), as well as at the reproductive stage, at which accelerated senescence is observed (Simova-Stoilova *et al.* 2009; Srivalli & Khanna-Chopra 1998). Senescence of wheat leaves induced by waterlogging led to increased endopeptidase activities at pH 5, and later at pH 9, while aminopeptidase activities decreased (Stieger & Feller 1994). Furthermore, some differences have been observed between the composition of drought-induced proteases and of those up-regulated in natural senescence (Khanna-Chopra *et al.* 1999).

Several endoproteolytic activities, differing in their pH optima, many of which are vacuolar CPs and APs, are increased significantly by drought in leaves of common bean and cowpea, the levels being higher in sensitive than in the more tolerant cultivars (Roy-Macauley *et al.* 1992). These results were confirmed by Cruz de Carvalho et al. (2001) and Hieng et al. (2004) and, in the latter study, induced activities were assigned to CPs and SPs. Drought has been shown to induce large increases in acid protease activity in leaves of a susceptible wheat cultivar, probably associated mainly with CPs, while the corresponding drought-resistant cultivar showed relatively little increase (Simova-Stoilova *et al.* 2010). Similarly, endoproteolytic activity, mainly of CPs, was enhanced in winter wheat seedlings of sensitive cultivars, but much less in seedlings of a tolerant cultivar. Acclimation of seedlings to low temperatures depressed the activity in the latter cultivar but had no effect in the former (Grudkowska & Zagdanska 2010). CP activities in frost-acclimated seedlings, visualized in-gel, did not differ from those in dehydrated, non-acclimated seedlings. In wheat under long-term field drought, increased protease activities, mainly APs and CPs, were observed related to the drought sensitivity of cultivars (Vassileva *et al.* 2012). The activity of several vacuolar CPs was increased in leaves of wheat plants subjected to water deficit and in senescing leaves (Martinez *et al.* 2007). A CP vacuolar processing enzyme activity increased in *Arabidopsis* under heat shock (Li *et al.* 2012). In pea roots, herbicides induced activities of papain-like CPs, of several putative SPs and of the ubiquitin-26S proteasome system, but activities of vacuolar processing proteases were reduced (Zulet *et al.* 2013).

Of the activities optimal at acidic pH, one ascribed to AP activity was strongly induced on water deficit in common bean and *Vigna*. It was investigated in more detail, leading to a new plant AP being characterised (Contour-Ansel *et al.* 2010; Cruz de Carvalho *et al.* 2001). The sequence of its precursor has all the features characteristic of phytepsins, typical plant APs. Proteolytic processing of the precursor form was shown to be induced by drought. This, together with the effect of stress on the level of its transcript, led to the suggestion that water deficit regulates its activity at both the transcriptional and post-transcriptional levels. This response occurred earlier and was stronger in the cultivar more susceptible to drought.

Activities of some extravacuolar SPs have been shown to increase under stress, as in the cases of chloroplast serine endopeptidase DegP2 in *A. thaliana* under high salt, desiccation and light stress (Haussühl *et al.* 2001), of subtilase in leaves of a sensitive cultivar of common beans exposed to drought (Budič *et al.* 2013; Hieng *et al.* 2004) and of an alkaline SP in leaves of spinach under salinity stress (Srivastava *et al.* 2009). A strong increase of activity, similar to that of the latter, was observed in desiccated leaves of the resurrection plant *Ramonda serbica* (Kidrič *et al.* 2014).

Aminopeptidase activities have also been observed to increase in response to drought, as in the cases of metallo- and serine aminopeptidases from common bean (Hieng *et al.* 2004) and aminopeptidase activities in wheat (Miazek & Zagdańska 2008; Simova-Stoilova *et al.* 2010). Leucine aminopeptidase A activity and aminopeptidase activities hydrolysing methionine-, arginine-, proline- and lysine-*p*NA substrates increased in tomato roots under drought and treatment with cadmium (Boulila-Zoghlami *et al.* 2011; Chao *et al.* 1999). Prolyl aminopeptidase activity increased in shoots of triticale plants grown under conditions of salinity, drought-stress and the presence of cadmium and aluminium ions (Szawlowska *et al.* 2011). Interestingly, activities of several aminopeptidase activities are much higher in desiccated leaves of the resurrection plant *R. serbica* than in those of well-watered plants (Kidrič *et al.* 2014).

Cross-talk between ATP-dependent and ATP-independent protein degradation, coupled with compensation of the lower activity of vacuolar proteases by increased ATP-dependent activity have been shown, especially under acclimation to dehydration stress and drought tolerance (Grudkowska & Zagdanska 2010; Wisniewski & Zagdanska 2001). The activity and amount of the 20S proteasome changed on salt stress of wheat root tips (Shi *et al.* 2011).

In conclusion, it is important to note that some of the studies quoted in this and earlier sections demonstrate a connection between the proteases whose activities changed on stress and the expression of genes encoding them, in some cases, however, only with high probability. Prolyl aminopeptidase from shoots of triticale plants (Szawlowska *et al.* 2012; Szawlowska *et al.* 2011), leucine

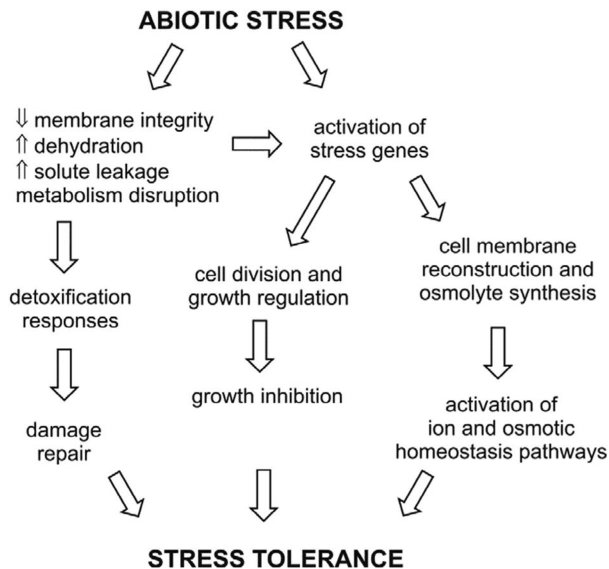

Fig. 3. Pathways to stress tolerance in plants. Several pathways are activated upon abiotic stress to enable a plant's adaptation and survival. Proteases and protease inhibitors play important roles in many steps of all the pathways indicated.

aminopeptidase-A from tomato (Boulila-Zoghlami *et al.* 2011) and vacuolar processing enzyme belonging to CPs in *Arabidopsis* (Li *et al.* 2012) are examples. The case of a newly discovered SP that plays a role in response to drought in common bean started with identification of proteolytic activities influenced by drought in its leaves. This was followed by isolation and characterization of the protein whose activity changed on water deficit. The sequence of the gene and hence of the protein was then determined. The protein was classified as a subtilisin-like SP of the S8 family of clan SB, having either a signal peptide or a mitochondrial targeting peptide. Finally it was found that, despite the fact that increased proteolytic activity was observed, the expression of the gene did not change on withdrawal of water, indicating that the protease is most probably regulated at the posttranslational level (Budič *et al.* 2013; Hieng *et al.* 2004). The complete picture awaits the discovery of its natural substrate(s).

An example of a comprehensive investigation is that of DegP2, a trypsin-like SP from chloroplast (Haussühl *et al.* 2001). It started from the predicted genomic sequence of a protease, moved to isolation of the gene and production of the corresponding protein in a bacterial expression system. The protein was then characterised biochemically, its subcellular localisation identified and changes in its expression under various stress conditions determined at the levels of transcript, protein and activity. Finally its physiological target, the substrate, was identified.

To realise a more precise picture of the mediation of the effects of abiotic stress by proteases, similar studies are needed that include determination of the gene sequence, the pattern of expression in time and space, the protein accumulation, its plant and subcellular localization, together with the regulation of its activity levels and its natural substrates.

The role of proteases and PIs. Proteases and PIs have been suggested to play many roles in the plant response to abiotic stress (Fig. 3). It is generally accepted that adaptation to stress requires the active involvement of regulated proteolysis and the inhibition of uncontrolled proteolysis. Different stresses can act on a common degradative pathway. Many of the studies quoted above, that report stress induced changes in expression of protease and PI genes, protein content and/or proteolytic activity, also suggest possible functions for such changes. Suggestions are based on, and positioned within, the general outline of possible roles of proteases already described in the Introduction. They have been founded on the evidence concerning functions of proteolytic enzymes and PIs in many of aspect of plant physiology and development (Adam & Clarke 2002; Feller 2004; Palma *et al.* 2002; Schaller 2004; Vierstra 1996, 2009). An example is the suggestion that vacuolar enzymes are involved in the cellular degradation induced by developmental senescence and by senescence induced by stress, both of which may converge on a single degradative pathway (Martinez *et al.* 2007). Another example is the 26S proteasome that is involved in the control of regulated proteolysis of functional proteins and in the removal of misfolded and damaged proteins (Vierstra 2009).

A number of recent studies, using overexpression of genes encoding proteases and PIs in transgenic plants, offer additional proof for their positive involvement in the plant response to abiotic stress. Examples include the overexpression of an AP gene that confers drought avoidance in *Arabidopsis* (Yao *et al.* 2012), the overexpression of the prolyl aminopeptidase gene that, in the same plant, enhances plant tolerance to salt and drought stress (Sun *et al.* 2013), and the greater heat shock tolerance of transgenic *Arabidopsis* overexpressing the thermo tolerance-related phytocystatin gene that possesses canonical ABA (ABREs) and dehydration-responsive elements (DREs) (Je *et al.* 2014). An example of a damaging effect is the overexpression of sweet potato CP in transgenic *Arabidopsis* plants that enhances drought-stress sensitivity (Chen *et al.* 2013).

Only a few precisely determined roles of proteases with identified natural substrates have so far been described. They include vacuolar processing enzymes belonging to CPs that reside in the lytic vacuoles of vegetative organs and

whose genes are up-regulated under stress (Tsiatsiani *et al.* 2012). Probably the best described are the ATP-dependent proteases in organelles, such as several DegP and FtsH enzymes, that eliminate proteins damaged by photo-oxidation and exert protein qualitycontrol. Some of their natural substrates are also known (Tsiatsiani *et al.* 2012).

The complexity of determining the roles of proteases is great. Protease DegP2, described in the previous section, is an example. The D1 reaction centre of photosystem II (PSII) was identified as its substrate *in vitro* (Haussühl *et al.* 2001). However, DegP2 appears not to be required for D1 degradation *in vivo*. As a consequence it has been proposed that it could play a role at higher light intensities to facilitate the FtsH-mediated processive degradation of D1 (Nixon *et al.* 2010). This protease could therefore, by degrading damaged proteins, be involved in the repair mechanism of PSII crucial for plant survival under light stress.

Proteolytic enzymes are responsible for a variety of physiological functions under stress. Control of their activities, notably by protein inhibitors, therefore has considerable physiological consequences, including those relevant to reaction to abiotic stress. The regulation by protein PIs of proteases activated as a result of water deficit is important for fine-tuning the stress response and assists the survival of plants. Further, based on their highly hydrophilic nature, PIs probably play an important role in osmoprotection.

In addition to their role in maintaining protein homeostasis during the response to abiotic stress, proteases and PIs have been shown to provide a defence strategy against pathogenic, parasitic and herbivorous organisms.They target the important proteolytic virulence factors of phytopathogenic bacteria, fungi, parasites and viruses, preventing their roles in nutrient acquisition and evasion of host defence. Furthermore, they target digestive proteases of herbivorous pests (e.g. insects, mites, slugs), preventing the utilization of food-derived organic nitrogen for their growth and development (Haq *et al.* 2004; Ryan 1990). Thus, proteases and PIs most probably play multiple roles and, in addition to their contribution to stress tolerance, also counter biotic stress exerted during periods of reduced growth under conditions of abiotic stress.

APPLICATIONS IN IMPROVEMENT OF CROP PLANTS

The first thing that comes to mind when considering the possibility of applying such findings as are described in this review is the number of cases where changes in gene expression, abundance and/or activity of proteases and/or PIs correlate with the degree of the plant/cultivar's resistance to drought. Not only is the possible application of proteases and inhibitors evident as biochemical markers for assessing drought tolerance, but also their possible application in conventional breeding for cultivars that inhibit uncontrolled and enhance beneficial proteolysis. In addition, the possibility can be envisaged of the application of their genes as candidates for characterizing and cloning quantitative trace loci (QTLs). They could also be applied for saturation mapping of QTL regions, as in the case of the identification of putative candidate genes for drought tolerance in rice (Nguyen *et al.* 2004). Moreover, knowledge of individual proteases and PIs underlies their use as transgenes in the development of plants with improved stress tolerance.

Recently, transgenic plants have been prepared that overexpress genes encoding proteases previously shown to be induced by abiotic stress. Such transgenic plants have been reported to exhibit enhanced tolerance to stress. Overexpression of an AP gene similarly confers drought avoidance in *Arabidopsis* (Yao *et al.* 2012). Transgenic *Arabidopsis* plants overexpressing a gene for a plant CP show stronger drought tolerance and higher CP activity under conditions of water stress than wild type plants (Zang *et al.* 2010). Those plants with constitutive expression of a gene coding for a putative papain-like CP are more tolerant to salt and drought stress than the control plants (Chen *et al.* 2010). Further, overexpression of a barley gene coding for methionine aminopeptidase confers strong freezing tolerance on transgenic *Arabidopsis* plants (Jeong *et al.* 2011). On the other hand, sweet potato CP, overexpressed in transgenic *Arabidopsis* plants, enhances sensitivity to drought stress (Chen *et al.* 2013). These last examples emphasize the importance of detailed knowledge of the individual proteases involved in stress-tolerance processes, since, depending on the regulation of their activity, they are capable of playing opposing roles.

It is well known that plant PIs are involved in one of the plant defence strategies against pathogenic, parasitic and herbivorous organisms (Sabotič & Kos 2012). Plants stressed by abiotic stressors often become targets of biotic stressors. Improvement of crop plants for better defence against the latter can therefore be beneficial for their survival under the former. Some investigations aimed at augmenting endogenous crop resistance have used conventional breeding, with the aim of increasing expression of PIs. In addition, several PIs of plant origin, that inhibit the digestive proteases of aggressors, have been used to prepare transgenic crop plants that exhibit enhanced resistance to biotic stress. These attempts, however, often face evolutionary pressure on biotic stressors, leading to the development of resistance to the products of overexpressed genes. Genes coding for PIs up-

regulated in plants under abiotic stress have been used for genetic modification of plants. For example, transgenic plants of tobacco with constitutive expression of trypsin PI show tolerance to a wide range of pHs (Srinivasan *et al.* 2009). Overexpression of the phytocystatin gene enhances heat shock tolerance of *Arabidopsis* more than that of the wild type (Je *et al.* 2014).

CONCLUSIONS

A mechanism or, more likely, mechanisms involving proteases and PIs, that account for tolerance of plants to drought and other forms of abiotic stress and, in some spectacular cases, recovery from complete desiccation, have yet to appear above the horizon.

There are three elements involved in the search for a solution. The substantial body of knowledge of the biochemistry and physiology of the life and death of plants already provides a rather accurate overview of what changes at metabolic and structural levels must occur. This complex picture involves, at many points, the synthesis and cleavage of peptide bonds and the body of knowledge and understanding of proteolytic enzymes, acquired over much of the preceding century, is already at hand to explain how such changes can occur.

Coupled with these two areas of current knowledge is the exponential increase in studies of changes in level of proteolytic enzymes and their inhibitors and of their more detailed characterisation. They make it inescapable that proteolytic activity is central to any explanation of the sensitivity and tolerance of plants to stress. This needs to be further established by more comprehensive studies, in which a greater range of the approaches described in this review are applied to individual systems.

A, or perhaps the, crucial next step is to identify the natural substrates for these proteases. This is the *sine qua non* to establishing the link to the first two elements. The overall goal is to establish our understanding of the processes affording plants stress tolerance, which will provide the basis for producing stress resistant crops and help to tackle a major hindrance to feeding the world.

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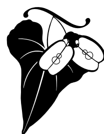

REZIME

Proteaze i njihovi endogeni inhibitori u odgovoru biljaka na abiotski stres

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Kontrolisana razgradnja proteina i aktivacija proenzima su neophodni za rast i razviće biljaka i za njihovo preživljavanje abiotskog i biotskog stresa. Medjutim, nekontrolisana proteoliza koju stres često indukuje, je za biljke veoma škodljiva. Proteaze su bitne za sprovođenje i regulaciju razgradnje proteina. Te njihove funkcije regulišu specifični endogeni inhibitori proteinske prirode. U članku su prvo pretstavljene opšte informacije o proteazama i njihovim inhibitorima. Sledi opis nekih od istraživanja posvećenih njihovoj upletenosti u odgovor biljaka na abiotski stres, čiji se broj stalno povećava. Poseban naglasak je dat na sušu, koja je najčešće ispitivani abiotski stres. Biće pokazano da je sve jasnije da su nivoji proteaza povezani sa stepenom tolerancije i osetljivosti na abiotski stres i da slika postaje sve kompleksnija. Najveća prepreka daljem razumevanju je nedostatak poznavanja prirodnih substrata proteaza. Bolje odredjivanje uloge proteaza u biljnom stresu će voditi ne samo razumevanju tolerancije na stresove kao što je suša, već će obezbediti i neophodnu osnovu za usavršavanje poljoprivrednih biljaka.

Ključne reči: proteaze, inhibitori proteaza, suša, slanost, hladnoća, stres