The potential use of phytocystatins in crop improvement, with a particular focus on legumes

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Abstract

Phytocystatins are a well-characterized class of naturally-occurring protease inhibitors that function by preventing the catalysis of papain-like cysteine proteases. The action of cystatins in biotic stress resistance has been intensively studied but relatively little is known about their functions in plant growth and defence responses to abiotic stresses, such as drought. Extreme weather events such as drought and flooding will become more frequent as a result of climate change. The concepts that changes in cellular protein content and composition are required for acclimation to different abiotic stresses and that these adjustments are achieved through

regulation of proteolysis are widely accepted. However, the nature and regulation of the protein turnover machinery that underpins essential stress-induced cellular re-structuring remains poorly characterised. Cysteine proteases are intrinsic to the genetic programs that underpin developmental senescence, but their functions in stress-induced senescence are poorly defined. While much remains uncertain regarding the individual cysteine protease targets of endogenous cystatins and their precise functions in the regulation of physiological processes are largely unknown, current evidence suggests that manipulation of cysteine protease activities by engineered cystatin expression might be used for to improve the resilience and quality of crop plants in the face of climate change.

Key words: Cystatin, senescence, protein degradation, soybean, drought, chilling, stress tolerance

Introduction

Since the time when plants first colonized land, they have been forced to evolve mechanisms that enable rapid acclimation to changing environmental conditions in order to survive. It is predicted that hotter summers, milder winters and more frequent severe droughts occur more often over the next 30 years. Similarly, floods and increases in sea levels will favour higher salt levels in arable land. These trends in predicted weather patterns are likely to place increasing limitations on the sustainability of crop production, with negative impacts on food security worldwide **(**Cutforth *et al.*, 2007; Jury and Vaux, 2007; Manavalan *et al*., 2009; Simova-Stoilova *et al*., 2010).

One-third of the world's population already resides in areas that are regularly subjected to water-stress, particularly in Africa. Under field conditions, drought stress is often also associated with high temperatures, factors that together can reduce average crop yields by

more than 50% (Bray *et al.*, 2000). Reductions in crop yields of about 17% have been already predicted for each degree Centigrade increase in growing season temperatures (Lobell and Asner, 2003). A recent analysis of US field trial data, together with meteorological data, information on crop management practices and the adoption of new cultivars between 1994 and 2013 revealed that soybean yields fell on average by around 2.4% for every 1°C rise during growing season temperature (Mourtzinis *et al.*, 2015). Moreover, the combined year-to-year changes in precipitation and temperature have already suppressed the US average yield gain by around 30% over the measurement period, leading to a loss of US$11 billion (Mourtzinis *et al.*, 2015).

Current crop varieties have been largely been bred for increased yield and not stress tolerance. Hence, high yields will not be sustainable under the enhanced stress conditions that are predicated to occur in coming decades. Increases temperature and changes in precipitation patterns with more frequent drought and flooding episodes are predicted, with some features such as intense heat and drought occurring in combination (De Boeck et al., 2010). While such extreme climate events will probably be of short duration, but they could still cause significant yield losses (Ciais et al., 2005) because crops may be challenged beyond their ability to acclimate (Bragazza, 2008; Jentsch et al., 2011). Stress tolerance is a major current target of plant breeding and crop improvement programs (Araus *et al.*, 2008, Parry *et al.*, 2012). The application of classical breeding approaches in recent decades has increased crop productivity by an average of 1% per year (Kucharik and Ramankutty, 2005). In theory, this increase would be sufficient to address the requirements of food supply for an increasing world population in coming years. However, yield increases have to be accompanied by improved stress tolerance traits to prevent the negative impacts of a changing climate.

Enhancing stress tolerance in crop plants, while maintaining high yields remains a challenging task not least because plants often respond to stress by the temporary or long-term cessation of vegetative growth. Application of classical breeding approaches in recent decades

has already increased the productivity by an average of 1% per year (Kucharik and Ramankutty, 2005). In theory, this increase would be sufficient to address the requirements of food supply for an increasing world population in coming years. However, such obtained yield increases have to be accompanied in the future by improved stress tolerance traits to prevent the negative impacts of a changing climate. Breeding for such new traits will thereby not only depend on genetic variability, but also on the duration and severity of the imposed stress, as well as the age and developmental stage of the plant when the stress occurs (Bray, 1997). Despite many recent claims that, for example, drought-tolerant plants have been generated, specifically by genetic modification of single gene targets, no major breakthroughs have been reported as yet in the literature concerning such engineered drought-resistant crop plants. The majority of such genetically engineered plants that exhibit a delayed onset of drought stress effects, do so due to morphological changes better preventing water loss (Lawlor, 2013). It is therefore highly unlikely that a simple single gene fix will solve the curial problem of stress tolerance.

Stress tolerance and protein turnover

Plant survival in the face of climate change depends on the ability to sense stress and make rapid adjustments to cell structure and physiology as well as growth, development and cell suicide programs. Many of these responses are common to various stresses, including the accumulation of reactive oxygen species (ROS), reprogramming gene expression, adjustments in protein content and composition, inhibition of photosynthesis and stimulation of basal respiration as well as other metabolic and structural changes that improve function under the stress conditions (Bohnert and Sheveleva, 1998; Cleays and Inze, 2013; Noctor *et al.*, 2014). They will serve to escape the stress effects or to mitigate the stress impacts (Hirt, 2009). The general response to drought for example involves closure of stomata, with increased

photorespiration relative to photosynthesis, increased root to shoot ratios, accumulation of carbon metabolites and decreases in nitrogen metabolites (Pinheiro *et al*., 2001; Chartzoulakis *et al*., 2002). Response also includes increased protease-mediated proteolysis, which can be blocked by protease inhibitors (Zhang *et al.*, 2008; Simova-Stoilova *et al*., 2010). These changes are often also linked to the onset of premature stress-induced leaf senescence with protease-mediated proteolysis triggering greatly enhanced protein turnover. This allows rapid degradation of proteins that are no longer functional or useful to ensure enhanced protein turnover for plant reproduction.

The term protease comprises endo-peptidases, acting on the interior of the peptide chain, and exo-peptidases, cleaving peptide bonds on the termini of the peptide chains (Barrett, 1994). Proteases, which fulfil a broad range of functions in plants (Beers *et al*., 2000; 2004), are often classified according to the amino acid residue in their reactive site, such as serine, cysteine, aspartic acid and threonine proteases, or as metallo-proteases. The proteases involved in stress-induced proteolysis can now be identified relatively easily because of the availability of whole genome sequences and functional genomics tools in many plant species. The *Arabidopsis thaliana* genome for example is estimated to contain at least 743 protease sequences, representing all the five catalytic classes i.e. serine, cysteine, aspartic acid, metallo and threonine proteases (MEROPS, peptidase database, http://merops.sanger.ac.uk/).

Regulation of protease activity by, for example, manipulation of the cysteine protease inhibitor system, been already successfully used as a tool for pest control (Christou *et al.*, 2006; Kiggundu *et al.*, 2010; Benchabane *et al*., 2010). These cysteine protease inhibitors, also called cystatins, are proteins that contain a Gln–Xaa–Val–Xaa–Gly motif in the centre of the polypeptide chain (where Xaa is any amino acid), a Pro–Trp (or Leu–Trp) dipeptide motif in the C-terminal region, and a conserved Gly residue in the N-terminal (Benchabane *et al*., 2010). Cystatins bind to the active site of their cysteine protease targets and so inactivate enzyme activity in an irreversible manner (Fig. 1). Currently 366 cystatin-like sequences have

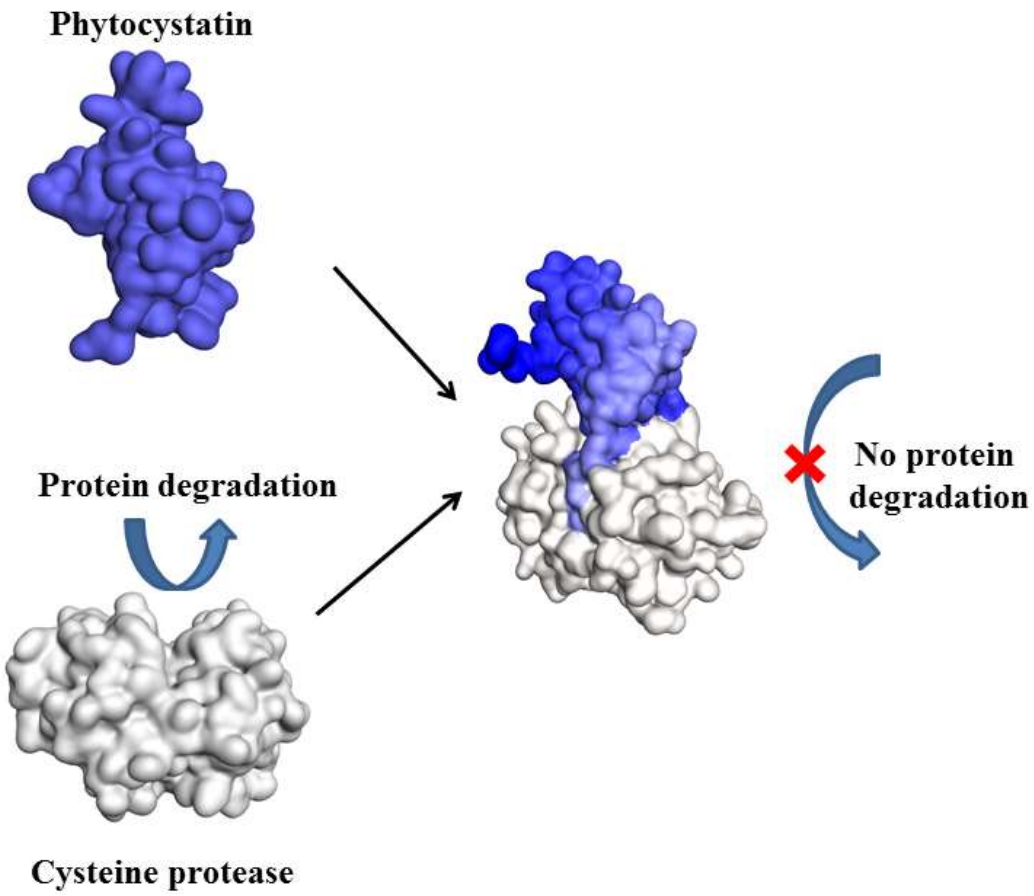

Fig. 1. Inhibition of cysteine protease action on proteins causing protein degradation due to formation of a cysteine protease-cystatin complex.

been identified in the Viridi plantae kingdom, while 957 C1 cysteine protease sequences, which include the papain-like cysteine proteases, have been identified (www.phytozome.net).

Cysteine proteases are strongly expressed upon exposure to abiotic stresses, such as drought, heat and high salt (Seki *et al.*, 2002; Rabbani *et al.*, 2003; Groten *et al.*, 2006). Stress-induced senescence is generally also associated with an increased activity of vacuolar cysteine proteases that are also involved in programmed cell death (PCD; Hara-Nishimura *et al.*, 2005; Beyene *et al.*, 2006, Martinez *et al.*, 2007). We have previously reported that transcripts encoding the tobacco papain-like cysteine protease *Nt*CP2, which is expressed in mature leaves, were increased in plants that have not been watered for 10 days (Beyene *et al.*, 2006).

A recent extensive review has described the roles of proteases and their endogenous inhibitors in plant responses to abiotic stress (Kidric *et al.,* 2014). In addition, the role of the C1A cysteine-protease-cystatin interaction, including participation in a range of specific pathways over the plant life cycle, has been described in detail (Martinez *et al.,* 2012). The following discussion therefore focuses on the evidence to support the view that cystatins are regulated during drought and temperature extremes, addressing the question of whether stress-induced cystatin expression might be linked to abiotic stress tolerance. Specifically, we consider how advanced biotechnological tools might be used to improve the effectiveness of cystatin-targeting against specific endogenous cysteine proteases in cystatin-based strategies for crop improvement.

Phytocystatin expression under abiotic stress

. The discovery of plant cystatin (phyocystatin) gene families in different species, in particular from cDNA libraries, EST collections as well as from recent progress in genome analysis to identify such families by applying bioinformatics tools, have significantly progressed our understanding of cystatin expression and function during abiotic plant stress. In maize, cystatins with distinct functions have been identified. Two members of the cystatin family (CC8 and CC9) are induced by cold stress, while drought stress had the opposite effect, reducing expression of the five maize cystatins CII, CC3, CC4, CC5 and CC9 (Massonneau *et al*., 2005). Various forms of barley cystatins (HvCPI-1 to HvCPI-13) were also identified in different plant parts with different inhibitory capability against barley cathepsin-L like cysteine-proteases (Martinez *et al.*, 2009). Also, gene duplication events might have caused further cystatin structural and functional complexities (Martinez and Diaz, 2008).

Two distantly related Arabidopsis cystatin clusters *AtCYS1* and *AtCYS2,* which show differential expression in response to abiotic stress were identified using cystatin promoter sequence activity with GUS fusions was applied. High temperature stress also enhanced the expression of the two Arabidopsis cystatins, *AtCYS1* and *AtCYS2,* but their induction had different temporal and spatial patterns (Hwang *et al.,* 2010). However, the ultimate function of cystatins under abiotic stress, for example by mutant work or cystatin response following exposure of a plant to abiotic stress, has so far not been greatly explored. Several other cystatin genes are also up-regulated due to drought, salinity, cold and heat. All findings were, however, based on the characterization of a single cystatin gene. Cystatins identified to response to abiotic stress include a chestnut cystatin, which accumulated in response to abiotic stress in both leaves and roots (Pernas *et al*., 2000), a multi-phytocystatin in winter wheat (Christova *et al.,* 2006), a grapevine cystatin (Cramer *et al.,* 2007), a root and stem cystatin of *Amaranthus hypochondriacus* (Valdes-Rodriguez *et al*., 2007), as well as a cowpea multi-cystatin (Diop *et al.,* 2004). Although transcripts of the cowpea cystatin accumulated in two cowpea cultivars, an earlier response was observed in a drought-tolerant cowpea cultivar. The regulation of other genes encoding phtocystatin has been shown to be regulated by drought, salinity, cold and heat stress in different species. These include a stress-inducible chestnut phytocystatin (Pernas*et al*., 2000), a multi-phytocystatin found in winter wheat (Christova*et al.*,2006), a grapevine phytocystatin (Cramer *et al.,* 2007), a root and stem phytocystatin in *Amaranthus hypochondriacus* (Valdes-Rodriguez *et al.*,2007) and a cowpea multi-phytocystatin (Diop*et al.*,2004). Although phytocystatin transcripts accumulated in both drought-sensitive and drought-tolerant cowpea cultivars in response to stress, the response was more rapid in a drought-tolerant cultivar, indicating that this phytocystatin might function in drought tolerance (Diop*et al.*,2004). While cysteine proteases and phytocystatins were amongst the most dorought responsive proteins in lupins, re-watering increased the expression of the phytocystatin even further indicating that this protein might provide protection during

recovery from drought (Pinheiro*et al*., 2005).

Accumulating evidence suggests that cystatins also play an important role in the regulation of protein recycling during stress-induced senescence, in which the abundance of cystatins is generally decreased while the expression and activity of cysteine proteases are increased (Benchabane *et al*., 2010). The *Arabidopsis* cystatin *AtCYS3* (*At2g40880*), induced by both drought and cold treatment, also contains a 9 base pair conserved dehydration responsive element (DRE) in its promoter sequence (Seki *et al*., 2001). DREs are targets for the DRE binding protein DREB1A (Seki *et al.*, 2001). They are important *cis*-acting elements that contribute to the regulation of gene expression that underpins acclimation responses to a range of abiotic stresses including drought, high salt and cold (Shinozaki, 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). The role of DREs in the regulation of cystatin expression is highlighted by the case of the Arabidopsis cystatin *AtCYSa*, which is expressed in Arabidopsis cells and seedlings exposed to abiotic stress (Zhang *et al.*, 2008). The promoter region of *AtCYSa* contains a DRE element, indicating that this cystatin is activated by a DREB pathway (Zhang *et al.*, 2008). Regulation of the expression of the Arabidopsis *PHYTOCYSTATIN 4* (*AtCYS4*) gene in response to heat stress occurs in a similar manner (Je *et al*., 2014). Cystatin expression was also be induced in the absence of stress in protoplasts by expression of the DRE-binding factor 2s (DREB2s) leading to decreased endogenous cysteine peptidase activity (Je *et al*., 2014).

Phytocystatins in nodulation

RNASeq technologies have been applied in the investigation of legume genomics in our lab as well as others (O'Rourke *et al*., 2014). We have used this advanced technology to identify members of the plant cystatin gene family that are specifically expressed in soybean nodules, which house nitrogen-fixing Rhizobia and provide an important source of nitrogen to

support plant growth and yield. The legume-Rhizobia symbiosis drives the plant development by providing reduced nitrogen metabolites to the plant for the synthesis of essential macromolecules, such as proteins (Puppo *et al*., 2005). However, in grain legumes such as soybean that have determinate root nodules, the lifespan of the nodule and duration of the symbiotic interaction are relatively short (11-12 weeks) After this time, nitrogen fixation declines rapidly and leghemoglobin, which is essential for oxygen management during the nitrogen fixing process, degrades as the nodules age and undergo senescence, a process that ends in programmed cell death (Puppo *et al*., 2005). Senescence in determinate soybean nodules generally starts at the center of the organ and extends progressively to the periphery (Puppo *et al*., 2005). Senescence encompasses many changes to nodule function ranging from loss of nitrogen fixation capacity to enhanced protease-mediated protein degradation and translocation of nitrogen remobilized reserves to the plant to support reproductive growth and development. As a result, symbiotic nitrogen fixation has generally almost ceased by the time that pod-filling starts (Puppo *et al*., 2005). In addition, symbiotic nitrogen fixation is also sensitive to abiotic stresses, such as drought, cold, salt or heavy metal stresses. These stresses lead to premature nodule senescence, which shares certain characteristics with developmental senescence (Alesandrini *et al.*, 2003). Interestingly however, the nodules were not the first organs to show drought-induced senescence when nodulated soybean plants were exposed to drought (Marquez-Garcia *et al*., 2005). Drought stress-induced senescence in the oldest leaf ranks preceded nodule senescence, suggesting that leaves of low photosynthetic capacity are sacrificed in favour of nodule nitrogen metabolism during stress (Marquez-Garcia *et al*., 2005). Stress-induced nodule senescence and the accompanying decline in nitrogen fixation, lead to nitrogen deficiency in the plant that negatively affects seed production and crop quality (Puppo *et al*., 2005). Cysteine proteases appear to be important not only in regulating nodule senescence but also in other functions related to nodule biology. For example, transgenic

soybean plants that express the rice cystatin OCI had significantly greater numbers of smaller nodules than wild type controls (Quain *et al*.,.2015)

RNASeq analysis involves the isolation of RNA from appropriate tissue(s), depletion of the ribosomal RNA component followed by deep sequencing. Data are then processed either by alignment to a reference genome, such as the soybean genome (Schmutz *et al.*, 2010), or the transcriptome is assembled *de novo*. In general, the primary advantage of the RNASeq methodology is that no *a priori* selection is required for the genes of interest because all RNAs are covered, including polyA+ and polyA- forms as well as small non-coding RNA sequences. It is important to note, however that such transcriptome information provides an understanding of cellular regulation at only one level of complexity and there is not always a good correlation between mRNA and protein levels, necessitating simultaneous analysis of transcriptome and proteome dynamics (Haider and Pal, 2013):

RNASeq technology has been applied in our group to explore cystatin expression during soybean nodule development. We compared the nodule transcript profile in 4 and 8 week-old nodules, i.e prior to the onset of senescence and in 14 week-old nodules, i.e. after the onset of nodule senescence, which was observed at 11-12 weeks using physiological markers (Fig. 2). All members of the cysteine protease family, which are possible cystatin targets were identified at these time points (Van Wyk *et al.*, 2014).

The application of Next Generation Sequencing technologies has significantly advanced our capacity to analysis the transcriptome profiles of different organs (Mortazavi *et al.*, 2008; Nagalakshmi *et al.*, 2008). Since RNASeq provides large datasets including less abundant transcripts, this technique has rapidly become method of choice for the identification of the complete transcriptome under different developmental or environmental conditions. However, microarray technologies might still be more appropriate in some cases, such as when an analysis of a smaller number of transcripts is required from a large number of samples.

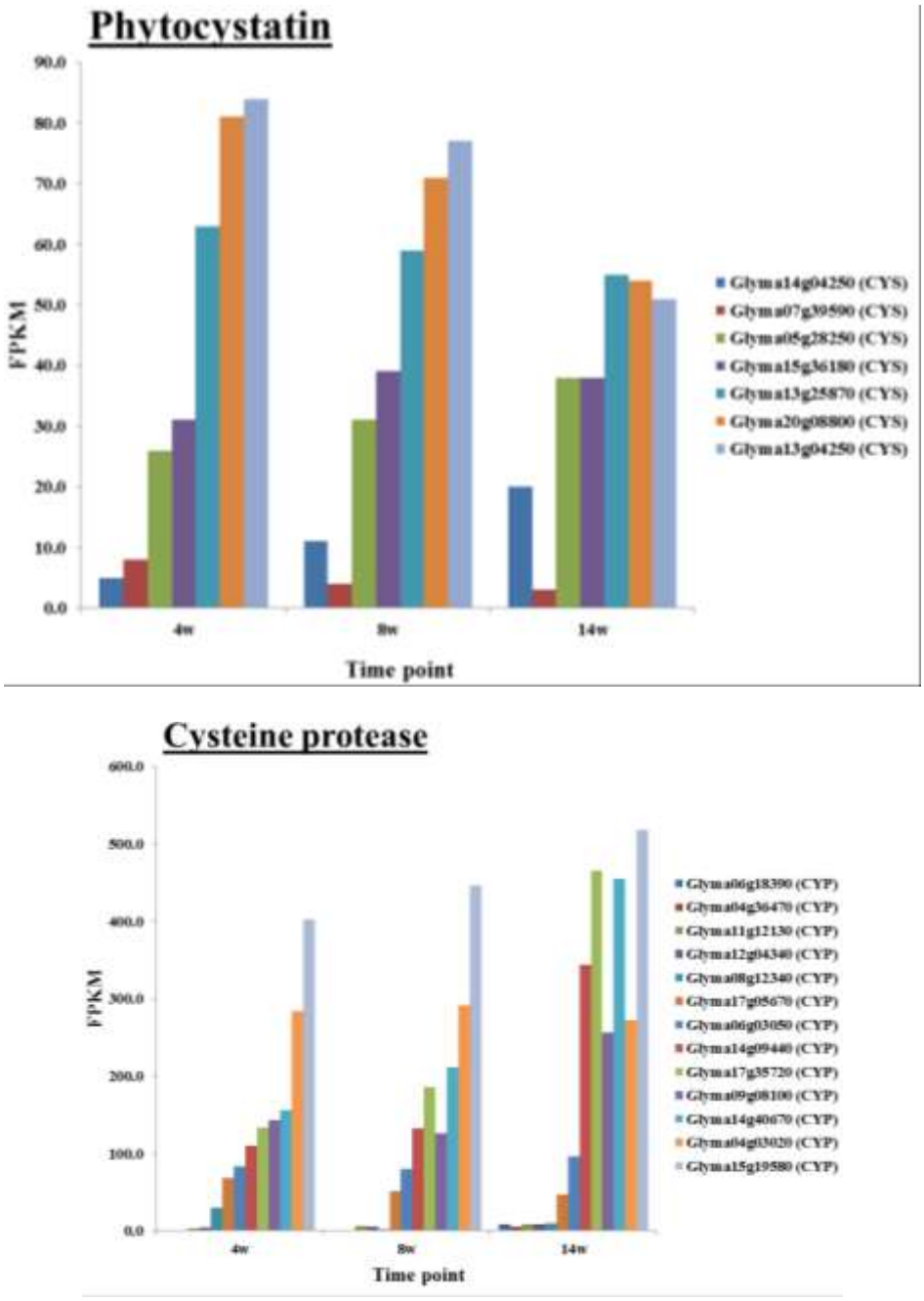

Fig. 2. Transcription of members of the phytocystatin and cysteine protease gene family in soybean determined by RNASeq and measured before onset (4 and 8 weeks) and after onset of nodule senescence (14 weeks) with onset of natural senescence at 11-12 weeks. Transcription expressed as FPKM (transcript abundances in fragments per kilobase of exon per million fragments mapped). Three biological replicates at each time point were pooled for RNA extraction with a Qiagen RNeasy® kit (Qiagen, Germany). The Illumina mRNA-SEQ kit was applied for sample preparations and RNA-Seq libraries were generated with an Illumina Genome AnalyzerIIx. All changes in gene transcription were confirmed by real-time-PCR analysis. Data shown adapted from van Wyk *et al.*, 2014.

Nineteen non-redundant cystatins similar to the rice cystatin OCI with seven actively transcribed cystatins were identified in soybean nodules (Van Wyk *et al.*, 2014). Most of the identified cystatins had a preferential affinity to cathepsin L-like cysteine proteases. Transcripts encoding three cystatins increased during the onset of senescence, a phase which is characterized by a decrease in symbiotic nitrogen fixation. These three cystatins may possibly contribute to the regulation of proteolysis as the nodules senesce. Seventy nine non-redundant soybean cysteine protease gene sequences with homology to papain were also identified (Van Wyk *et al.*, 2014). These cysteine proteases belong to different subfamilies including legumain-like, vacuole-processing enzymes (Hara-Nishimura *et al.*, 2005). Eighteen of these cysteine proteases were actively transcribed during nodule development and senescence (Fig. 2). The identification non-actively transcribed cystatins has also raised the question of whether this cystatin pool might possibly provide a reservoir for response to particular stress situations, for example, when cysteine proteases are accidentally released due to stress-induced premature senescence (Van Wyk *et al.*, 2014), However, one might also hypothesize that simultaneous expression of both a cystatin and a cysteine protease is a strategy by the plant to limit any consequence of protease action due to exposure of stress and onset of premature senescence. We are currently investigating whether cystatin expression under drought can, for example, provide a balance between further protein degradation and easier recovery from stress due to cystatin expression. However, a prolonged stress period might ultimately favor proteolytic processes causing plant death (Fig. 3).

Since RNASeq analysis detects all genes expressed, the data can be interrogated to answer the question as to whether RNASeq analysis can also identify cystatins uniquely expressed during abiotic stress. The data from an RNASeq study in our group, designed to provide a first insight what type of cystatins and cysteine proteases are particularly expressed in soybean after drought treatment, have already provided strong evidence that at least one soybean cystatin, Glyma05g2850, and two papain-like cysteine proteases are specifically

expressed under drought. These genes are only slightly, or not at all, expressed during natural senescence (Du Plessis, unpublished results). Such genes might allow the development of new senescence protein markers that can be used to monitor the onset of stresses such as drought.

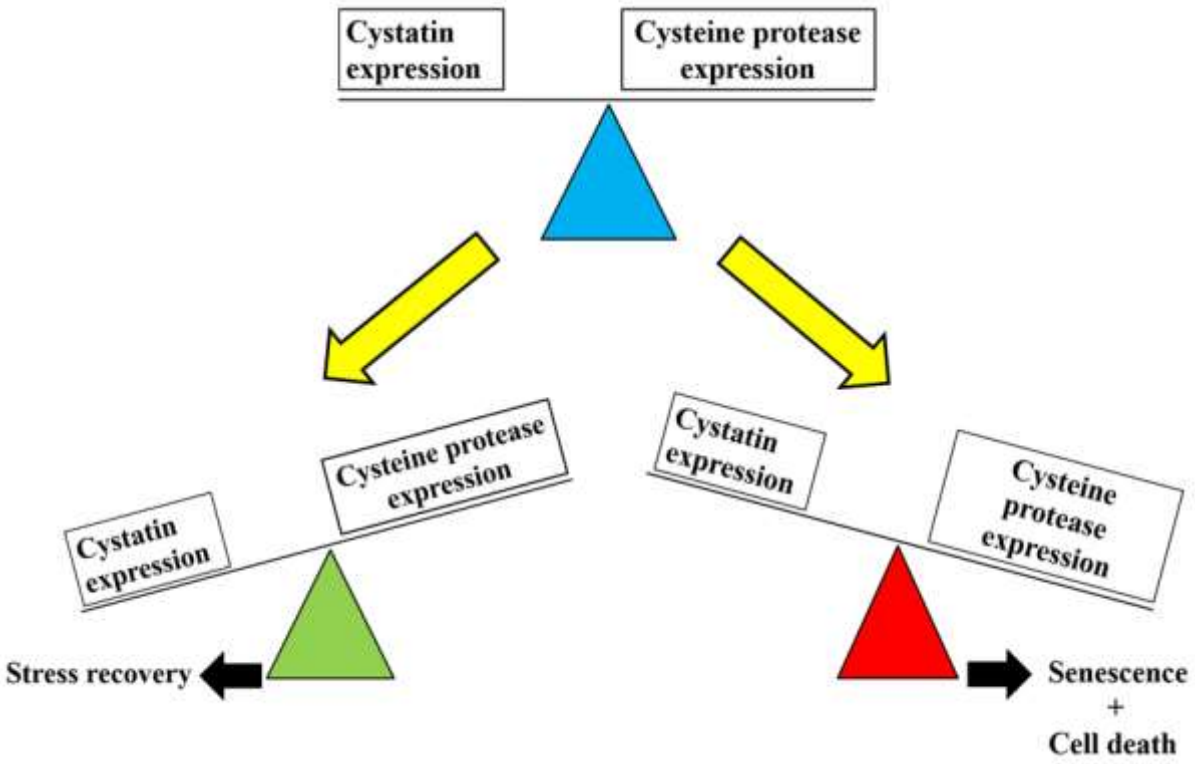

Fig. 3. Proposed action of stress-induced cystatins providing a balance between easier recovery from stress due to protein protection and protein degradation caused by a prolonged stress period ultimately favor proteolytic processes causing plant death.

Phytocystatin-cysteine protease interactions

A key question concerns whether the cystatins that are identified in gene expression studies can interact with cysteine proteases *in vivo*, and so limit proteolytic processes. Little is currently known about the interactions between cystatins and their target proteases *in vivo*. First evidence of possible *in vivo* interactions was provided by a sub-cellular localization study when a cystatin and cathepsin L-like cysteine protease were fused to a green fluorescent

protein (Martinez *et al.*, 2009). Both co-localized throughout the endoplasmic reticulum and the Golgi complex, which would allow their interaction *in vivo*. The coordinated expression of transcripts encoding cysteine protease and cystatin interacting partners and formation of a cysteine protease–cystatin complex has recently also been found in senescent spinach leaves (Tajima *et al.*, 2011). In a first step to study such interaction, we also tagged the papain-like cysteine protease proteome with the inhibitor DCG-04. This inhibitor, an analogue of the irreversible papain-like cysteine protease inhibitor E-64, carries a biotin residue allowing tagging of cysteine proteases and biotin detection with a peroxidase-labelled streptavidin. By applying two-dimensional polyacrylamide gel electrophoresis, several papain-like cysteine proteases with a molecular mass of about 30 kDa were identified (Vorster *et al.*, 2013). Results indicated that a variety of papain-like cysteine proteases are produced in the early stages of nodule development but a much smaller number during senescence.

The expression of cystatins and cysteine proteases has been recently demonstrated in soybean nodules (van Wyk *et al*. 2014). The expression of two such genes in the same tissue does not allow us to conclude that these proteins interact *in vivo.* Single cell transcriptomics (Shalek *et al.*, 2013) can be applied to identify cells expressing both types of genes unambiguously. Proteomic studies that directly demonstrate interactions between specific cystatins and their proteases targets are also required to identify interacting partners. The yeast two-hybrid system has proved to be a versatile tool in the characterization of cellular interactomes. Variations of the yeast two-hybrid technologies, including high-throughput systems, and single cell proteomics contribute to the growing repertoire of available tools for analyzing proteomic profiles and protein-protein interactions (Stasi *et al.*, 2014: Willison and Klug, 2013),

Phytocystatin-cysteine protease interactions explored in transgenic plants

The analysis of transgenic plants that have been engineered to constitutively express cystatins provides evidence that these proteins interact with cysteine proteases in the cell to limit proteolytic processes occurring in plants exposed to abiotic stress (Van der Vyver *et al.*, 2003). Transgenic tobacco plants expressing the rice cystatin OCI were better protected against the negative impacts of chilling on photosynthesis (Van der Vyver *et al.,* 2003). The beneficial action of a cystatin in enhancing stress tolerance is likely to be the direct result of the inhibition of cysteine protease targets (Van der Vyver *et al.*, 2003; Zhang *et al.*, 2008). The cystatin technologies might therefore be applied in crop improvement, even though any 'pleiotropic' effects are often considered only as unintended metabolic interference (Schlüter *et al*., 2010; Benchabene *et al.*, 2010). Also, OCI expression in transgenic Arabidopsis and soybean plants provided a higher tolerance to drought (Prins *et al.*, 2008; Quain *et al.*, 2014). In particular, the recovery of photosynthesis, was more rapid in OCI-expressing plants than controls during the recovery phase upon re-watering after a period of drought. Similarly, over-expression of *AtCYS3* and *AtCYS6* in transgenic Arabidopsis plants enhanced resistance to high salt, drought, cold and oxidative stress (Zhang *et al.*, 2008).

Proof of the beneficial effects of cystatin expression has been obtained in broccoli heads, where overexpression of BoCPI-1 led to a decrease in total protease activity and delayed the onset of post-harvest senescence, as measured by changes in chlorophyll conten (Eason *et al.*, 2014t. The increased expression of BoCPI-1 was accompanied by decreases in the accumulation of transcripts encoding several senescence-associated cysteine protease). The expression of stress-related cystatins and other types of protease inhibitors, such as the *oryza sativa* chymotrypsin inhibitor-like 1 *(*OCPI1*)*, might have agronomical benefits. The enhanced stress tolerance observed in protease inhibitor-expressing plants might contribute to the sustainability of grain yield and seed set under fluctuating environmental conditions

=(Van der Vyver *et al.*, 2003; Huang *et al.*, 2007; Shan *et al.*, 2008; Srinivasan *et al.*, 2009; Munger *et al.*, 2010).

Several studies have reported negligible phenotypic effects for protease inhibitor expression in transgenic plants based on macroscopic evaluation (Masoud *et al.*, 1993; Brunelle *et al.*, 2004; Badri *et al.*, 2009). However, ectopic expression of the rice cystatin OCI alters the growth and development of various plant species (Van der Vyver *et al.*, 2003; Prins *et al.*, 2008; Quain *et al.*, 2014). By exploring in more detail in our group a possible mechanism by which the inhibition of endogenous plant cysteine proteases by protease inhibitors leads to altered plant development and enhanced stress tolerance, an effect on strigolactone-mediated growth regulation was identified (Quain *et al.*, 2014). However, the exact association with strigolactone pathways, which can function in the regulation of natural and stress-induced leaf senescence and also in drought responses, has not been thoroughly investigated (Stirnberg *et al.*, 2002; Woo *et al.*, 2001, 2004).

Chloroplast proteins, such as ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) and Rubisco activase, are more stable in tobacco plants over-expressing an OCI transgene (Prins *et al.*, 2008). While it is still unclear how the stability of chloroplast proteins is achieved by cystatin expression, the Rubisco protein has been detected inside different types of vesicle in the cytosol of senescent leaves. For example, the Rubisco protein has been observed in Rubisco Containing Bodies (RCB) and Senescence Associated Vacuoles (SAVs), which are produced from the chloroplast. Such vesicles are probably a type of autophagosome, which are delivered to the vacuole (Prins et al., 2008). While cysteine proteases are considered to present in RCB and SAV, cysteine proteases have been only proven in SAVs, which contain the senescence-associated protease SAG12 (Carrión et al., 2013). In addition, Rubisco has been detected in Chloroplast Vesiculation-Containing Vesicles (CVVs) that are produced in plants exposed to stress (Wang and Blumwald, 2015).

CVVs appear to be involved in the degradation of all types of chloroplast proteins (Wang and Blumwald, 2015).

While cysteine proteases, which could directly interact with cystatins in chloroplasts, have not been identified, Bayer *et al.* (2011) identified an Arabidopsis protein with cysteine protease activity belonging to the OTU-like superfamily. This was the first identified chloroplast-localized cysteine protease in Arabidopsis. Future research is required to demonstrate whether such OTU cysteine proteases can interact with cystatins. A proteomic comparison of OCI-overexpressing tobacco plants and controls provides further evidence that chloroplast proteins, which are nuclear-encoded, are more abundant in plants expressing the

Table 1: Proteins whose abundance changed in response to drought in 3-weeks old OCI (NOCI) expressing *Nicotiana tabacum* plants relative to a wild-type (NWt) control grown under non-drought conditions.

Protein identifier	Protein description	Ratio NOCI/NWt	Mascot protein score	Total number of peptide identification events
ATPB_TOBAC	ATP synthase subunit beta, chloroplastic; EC=3.6.3.14; AltName: F-ATPase subunit beta; AltName: ATP synthase F1 sector subunit beta; [Nicotiana tabacum]	10.8	6,016	9
ATPD_TOBAC	ATP synthase delta chain, chloroplastic; AltName: F-ATPase delta chain; Flags: Precursor; [Nicotiana tabacum]	2.38	330	5
Q53UI6_TOBAC	PsbQ; [Nicotiana tabacum]	2.02	286	5
CYF_TOBAC	Apocytochrome f; Flags: Precursor; [Nicotiana tabacum]	1.97	330	10
ATPF_TOBAC	ATP synthase subunit b, chloroplastic; AltName: ATPase subunit I; AltName: ATP synthase F(0) sector subunit b; [Nicotiana tabacum]	1.95	301	10
Q14TB1_TOBAC	Chloroplast pigment-binding protein CP24; [Nicotiana tabacum]	1.87	398	8

RBS_TOBAC	Ribulose bisphosphate carboxylase small chain, chloroplastic; Short=RuBisCO small subunit; EC=4.1.1.39; AltName: TSSU3-8; Flags: Precursor; [Nicotiana tabacum]	1.73	3,553	32
Q84QE5_TOBAC	Ribulose bisphosphate carboxylase small chain; EC=4.1.1.39; [Nicotiana tabacum]	1.62	3,109	28
Q84QE7_TOBAC	Putative photosystem I subunit III; [Nicotiana tabacum]	1.61	584	14
Q9ZP50_TOBAC	FtsH-like protein Pftf; Flags: Precursor; [Nicotiana tabacum]	1.53	543	14
PSBA_TOBAC	Photosystem Q(B) protein; AltName: 32 kDa thylakoid membrane protein; AltName: Photosystem II protein D1; Flags: Precursor; [Nicotiana tabacum]	1.40	464	10
Q84QE8_TOBAC	Oxygen evolving complex 33 kDa photosystem II protein; [Nicotiana tabacum]	1.39	949	5
ATPA_TOBAC	ATP synthase subunit alpha, chloroplastic; EC=3.6.3.14; AltName: F-ATPase subunit alpha; AltName: ATP synthase F1 sector subunit alpha; [Nicotiana tabacum]	1.33	1,047	23
Q71V35_TOBAC	ATP synthase subunit beta; EC=3.6.3.14; Flags: Fragment; [Nicotiana tabacum (Common tobacco).]	1.32	414	6
G3PB_TOBAC	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic; EC=1.2.1.13; AltName: NADP-dependent glyceraldehydephosphate dehydrogenase subunit B; Flags: Precursor; Fragment; [Nicotiana tabacum]	1.30	878	19
RBS_TOBAC	Ribulose bisphosphate carboxylase large chain; Short=RuBisCO large subunit; EC=4.1.1.39; Flags: Precursor; [Nicotiana tabacum]	1.23	10,458	268
Q14TB1_TOBAC	Heat shock protein 90; [Nicotiana tabacum]	1.16	423	11
Q9XG67_TOBAC	Glyceraldehyde-3-phosphate dehydrogenase; EC=1.2.1.12; [Nicotiana tabacum]	1.16	448	6
C3RXI5_TOBAC	Plastid transketolase; [Nicotiana tabacum]	1.08	963	22

EFTU_TOBAC	Elongation factor Tu, chloroplastic; Short=EF-Tu; Flags: Precursor; [Nicotiana tabacum]	0.83	285	7
PGKH_TOBAC	Phosphoglycerate kinase, chloroplastic; EC=2.7.2.3; Flags: Precursor; [Nicotiana tabacum]	0.81	1,207	20
O82077_TOBAC	Glycolate oxidase; EC=1.1.3.15; Flags: Fragment; [Nicotiana tabacum]	0.68	193	6
ATPG_TOBAC	ATP synthase gamma chain, chloroplastic; AltName: F-ATPase gamma subunit; Flags: Precursor; [Nicotiana tabacum]	0.65	410	7
Q8W183_TOBAC	Carbonic anhydrase; EC=4.2.1.1; [Nicotiana tabacum]	0.57	1,943	8
PSBS_TOBAC	Photosystem II 22 kDa protein, chloroplastic; AltName: Full=CP22; Flags: Precursor; [Nicotiana tabacum]	0.56	314	7
Q7M242_TOBAC	Glutamate synthase (Ferredoxin) (Clone C(35)); EC=1.4.7.1; Flags: Fragment; [Nicotiana tabacum]	0.53	1,163	35
CAHC_TOBAC	Carbonic anhydrase, chloroplastic; EC=4.2.1.1; AltName: Carbonate dehydratase; Flags: Precursor; [Nicotiana tabacum]	0.52	2,281	8
O24511_TOBAC	Catalase; EC=1.11.1.6; [Nicotiana tabacum]	0.48	263	5
Q5QJB2_TOBAC	Harpin binding protein 1; [Nicotiana tabacum]	0.44	159	5
PSAA_TOBAC	Photosystem I P700 chlorophyll a apoprotein A1; Short=PsaA; Short=PSI-A; [Nicotiana tabacum]	0.28	668	21

The youngest fully expanded leaves were harvested from 3.weeks old plants exposed for seven days of drought. Metabolism was arrested in each leaf disc by immediate freezing in liquid nitrogen. Protein reduction/alkylation, trypsin digestion, isobaric tagging, off-gel fractionation of the labelled peptides, and LC-MS/MS analysis were performed essentially as described by Diaz Vivancos *et al*. (2011). Labelling was carried out with a TMT Isobaric Mass Tagging Kit (Thermo-Fisher) and proteins were labeled with TMT reagents 126, 127 and 128. The experiment was repeated once and data present the mean from both experiments. Only proteins with the correct molecular weight size were used for analysis.

cystatin (Table 1). Moreover, the OCI-dependent increase in chloroplast protein levels was more pronounced when the plants were exposed to drought (Table 1). In particular, the abundance of subunits of the chloroplast ATP synthetase protein were up to 10-fold higher in OCI-expressing leaves exposed to drought than well-watered controls. Given that impaired photophosphorylation and ATP synthetase activity are considered to be major factors limiting photosynthesis, even under mild drought conditions (Lawlor, 2002), the enhanced abundance of chloroplast ATP synthetase proteins might serve to alleviate the adverse effects of drought on photophosphorylation. The OCI-dependent protection of chloroplast proteins, particularly those involved in photosynthesis, from degradation is likely to be responsible for the observed protection of photosynthesis against the inhibitory effects of drought and other stresses (Van der Vyver *et al*., 2003, Prins *et al*., 2008, Quain *et al*., 2014).

Engineering phytocystatins for improved activity

The potential to engineer improved cystatins with enhanced binding properties with regard to their cysteine protease targets provides an added value to their prospects as tools for crop improvement. This might be particularly true for transgenic plants in which the cystatin structure might be tailored to target any specific cysteine protease. Strengthening the interaction between the protease target and its inhibitor would allow better regulation of specific proteolytic processes. It is feasible to engineer cystatins to have a high specificity against target proteases expressed under specific environmental conditions and that would also be less effective against non-target plant proteases. Such engineering would be especially important in the targeted regulation of stress-induced proteases while prevent interference with non-target proteases responsible for housekeeping functions. It has already been demonstrated that changes in the amino acid sequence, specifically amino acids under positive selection, alter cystatin inhibition (Kiggundu *et al.*, 2006). To date, research related to

changing cystatin amino acid sequences has focused almost exclusively on cystatins targeting insect pest digestive systems, particularly altering inhibitory spectrum and strength (Goulet *et al*., 2008). While there is still a dearth of information on plant systems, the demonstration of cystatin modification in plant-insect systems provides already valuable clues for applications in plant stress tolerance. We have already identified positions with general decisive influence on cystatin potency. For example, a glutamine adjacent to the conserved Gln-Xaa-Val Xaa-Gly in the cystatin motif of the first inhibitor loop has a decisive role in potency (van Wyk, unpublished result).

Applying *in silico* tools to improve our understanding of structure-function relationships is also an important first step in the rational design of cystatins with better targeted specificities. Homology modelling and protein-protein docking algorithms have been applied to simulate the interactions between different plant cystatins and papain, highlighting the relative importance of different phytocystatin regions during the inhibition of plant cysteine proteases (Vorster *et al.,* 2010). Combining this approach with *in silico* modelling may lead to the creation of cystatin variants in functionally important regions, such as the N-terminal, which has already been shown to alter the specificity of cystatins against specific cysteine protease families in complex biological systems, such as those found in the insect gut (Sainsbury *et al.,* 2012). These approaches can easily be applied to plants in order to target specific families, or members of a cysteine protease family that are induced by specific environmental stress conditions. The *in silico* identification of binding sites between cysteine protease and cystatin partners, together with subsequent analysis of the variation in these binding sites between the different members of the cysteine protease family, will also provide new targets for improving cystatin-protease specificity.

Conclusion and future prospects

In the above discussion, we have considered the potential use of cystatins in crop improvement to mitigate against the negative impacts of a changed climate. While our current knowledge is still too fragmented to provide a definitive answer, accumulating evidence already suggests that cystatin functions and endogenous interactions with cysteine proteases are important in limiting stress-induced proteolysis. Hence there is considerable potential for application of this system in strategies to improve cops to withstand the stresses associated with future changes in climate. We have highlighted the current lack of knowledge concerning cystatin function in plants, particularly in important crop species. However, comparative genomic analyses may provide valuable insights into the conservation and evolution of cysteine proteases and their inhibitors, which will help to further clarify the function of the different cysteine protease-cystatin systems in crop species exposed to different environmental conditions. We consider, for example, that cystatins may play a vital role in protecting the photosynthetic processes from degradation in plants exposed to stress or recovering from stress.

Our understanding of the regulated functions of plant proteolytic processes also remains in-complete. These systems have been largely ignored by the plant research community until recently, and therefore relatively little is known about the added value of ectopic cystatin over-expression, over and above previously described effects on resistance to insect pests. Constitutive overexpression of cystatins has the potential to have a positive influence on crop quality and performance. The recent findings using transgenic plants over-expressing the rice cystatin OCI open new avenues for the use of cystatins in plant protection against abiotic stresses that are predicted to occur as a result of climate change. However, such studies also highlight how little is known about the impact of such manipulations leading to a plethora of metabolic effects that might ultimately influence plant ecosystems. Cystatins

are considered to be a safe technology with regard to humans (Atkinson *et al.*, 2004). Nevertheless, the widespread use of transgenic plants with engineered cystatins against pest digestive systems has the potential to shift pest populations.

The above discussion has also focused on the value of transcript profiling data that provide useful signposts for plant stress responses. Such studies indicate that the prevention of premature, and precocious, senescence induced by stress may be key facet in engineering stress tolerance. The control stress-induced senescence is likely to require a highly dynamic process. More precise information is required about time dependence of specific cystatin expression, together with details concerning the endogenous cysteine protease targets. Identification of the cystatins that are uniquely expressed under specific stress conditions and how they contribute to the regulation of the balance between recovery from stress, and excessive protein degradation resulting in PCD (Fig. 3), will be crucial to manipulating the plant response to stressful environmental conditions.

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References

Alesandrini F, Frendo P, Puppo A, Hérouart D. 2003. Isolation of a molecular marker of soybean nodule senescence. *Plant Physiology and Biochemistry* **41**,727-732.

Araus JL, Slafer GA, Royo C, Serret MD. 2008. Breeding for yield potential and stress adaptation in cereals. *Critical Reviews Plant Sciences* **27,** 377–412.

Atkinson HJ, Johnston KA, Robbins M. 2004. Prima facie evidence that a phytocystatin for transgenic plant resistance to nematodes is not a toxic risk in the human diet. *Journal Nutrition* **134,** 431-434.

Badri MA, Rivard D, Coenen K, Michaud D. 2009. Unintended molecular interactions in transformed plants expressing clinically-useful proteins–The case of bovine aprotinin travelling the potato leaf cell secretory pathway. *Proteomics* **9,** 746–756.

Barrett AJ. 1994. Classification of peptidases. *In*: Barrett AJ, ed. *Methods in Enzymology. New York, Academic Press* **244,** 1-15.

Bayer RG, Stael S, Csaszar E, Teige M. 2011. Mining the soluble chloroplast proteome by affinity chromatography. *Proteomics* **11**, 1287–1299.

Beyene G, Foyer CH, Kunert KJ. 2006. Two new cysteine proteinases with specific expression patterns in mature and senescent tobacco (Nicotiana tabacum L.) leaves. *Journal of Experimental Botany* **57,** 1431–1443.

Beers EP, Woffenden BJ, Zhao C. 2000. Plant proteolytic enzymes: possible roles during programmed cell death. *Plant Molecular Biology* **44,** 399-415.

Beers EP, Jones AM, Dickerman AW. 2004. The S8 serine, C1A cysteine and A1 aspartic protease families in Arabidopsis. *Phytochemistry* **65,** 43-58.

Benchabane M, Schlüter U, Vorster J, Goulet MC, Michaud D. 2010. Plant cystatins. *Biochimie* **92,** 1657-1666.

Bohnert HJ, Sheveleva E. 1998. Plant stress adaptations– making metabolism move. *Current Opinion Plant Biology* **1,** 267-274.

Boyer JS. 1982. Plant productivity and environment. *Science* **218,** 443-448.

Bragazza L. 2008. A climatic threshold triggers the die-off of peat mosses during an extreme heat wave. *Global Change Biology*, **14**, 2688–2695.

Bray EA. 1997. Plant responses to water deficit. *Trends in Plant Science* **2,** 48-54.

Bray EA, Bailey-Seres J, Weretilnyk E. 2000. Responses to abiotic stresses. *In*: BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry & Molecular Biology of Plants, Split edition. American Society of Plant Physiologists, Rockville, MD, pp. 1158-1203.

Brunelle F, Cloutier C, Michaud D. 2004. Colorado potato beetles compensate for tomato cathepsin D inhibitor expressed in transformed potato. *Archives of Insect Biochemistry and Physiology* **55,** 103–113.

Carrión, CA, Costa ML, Martínez, DE, Mohr C, Humbeck K, Guiamet JJ . 2013. In vivo inhibition of cysteine proteases provides evidence for the involvement of 'senescence associated vacuoles' in chloroplast protein degradation during dark-induced senescence of tobacco leaves. *Journal of Experimental Botany* **64,** 4967–4980.

Chartzoulakis K, Patakas A, Kofidis G, Bosabalidis A, Nastou A. 2002. Water stress affects leaf anatomy, gas exchange, water relations and growth of two avocado cultivars. *Scientia Horticulturae* **1778,** 1-13.

Christou P, Capell T, Kohli A, Gatehouse JA, Gatehouse AMR. 2006. Recent developments and future prospects in insect pest control in transgenic crops. *Trends in Plant Sci*ence **11,** 302–308.

Christova PK, Christov NK, Imai R. 2006. A cold inducible multi-domain cystatin from winter wheat inhibits growth of the snow mold fungus, Microdochiumnivale. *Planta* **223,** 1207–1218.

Ciais PH, Reichstein M, Viovy N, Granier A, Ogée J, Allard V, Aubinet M *et al.* 2005. Europe-wide reduction in primary productivity caused by the heat and drought in 2003. *Nature*, **437**, 529–533.

Claeys H, Inzé D. 2013.The agony of choice: how plants balance growth and survival under water-limiting conditions. *Plant Physiology* **162,** 1768–1779

Crafts-Brandner SJ, Salvucci ME. 2000. Rubiscoactivase constrains the photosynthetic potential of leaves at high temperature and CO_2. *Proceedings of the National Academy of Science USA* **97,** 13430-13435.

Cramer GR, Ergül A, Grimplet J, Tillett RL, Tattersall EA, Bohlman MC, Vincent D *et al*. 2007. Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. *Functional and Integrative Genomics* **7,** 111-34.

Cutforth HW, McGinn SM, McPhee KE, Miller PR. 2007. Adaptation of pulse crops to the changing climate of the northern Great Plains. *Agronomy Journal* **99,** 1684-1699.

De Boeck HJ, Dreesen FE, Janssens IA, Nijs I. 2010. Climatic characteristics of heat waves and their simulation in plant experiments. *Global Change Biology* **16**, 1992–2000.

Diaz Vivancos P, Driscoll SP, Bulman CA, Ying L, Emami K, Treumann A, Mauve C, Noctor G, Foyer CH. 2011. Perturbations of shikimic acid metabolism modulate cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration. *Plant Physiology* **157**, 256-268.

Diop NN, Kidric M, Repellin A, Gareil M, d'Arcy-Lameta A, Pham Thi AT, Zuily-Fodil Y. 2004. A multicystatin is induced by drought-stress in cowpea (*Vigna unguiculata* (L.) Walp) leaves. *FEBS Letters* **577,** 545-550.

Eason JR, West PJ, Brummell DA, Watson LM, Somerfield SD, McLachlan ARG. 2014. Overexpression of the protease inhibitor*BoCPI-1* in broccoli delays chlorophyll loss after harvest and causes down-regulation of cysteine protease gene expression. *Postharvest Biology and Technology* **97,** 23–31.

Goulet MC, Dallaire C, Vaillancourt LP, Khalf M, Badri AM, Preradov A, Duceppe MO *et al.* 2008. Tailoring the specificity of a plant cystatin toward herbivorous insect digestive cysteine proteases by single mutations at positively selected amino acid sites. *Plant Physiology* **146,** 1010-1019.

Groten K, Dutilleul C, van Heerden PDR, Vanacker N, Bernard S, Finkemeier I, Dietz KJ, Foyer CH. 2006. Redox regulation of peroxiredoxin and proteinases by ascorbate and thiols during pea root nodule senescence. *FEBS Letters* **580,** 1269-1276.

Haider S, Pal R. 2013. Integrated analysis of transcriptomic and proteomic data. *Current Genomics* **14,** 91–110.

Hara-Nishimura I, Hatsugai N, Nakaune S, Kuroyanagi M, Nishimura M. 2005. Vacuolar processing enzyme: an executor of plant cell death. *Current Opinion Plant Biology* **8,** 404-408.

Hirt H. 2009. Plant Stress Biology: From Genomics to Systems Biology (Wiley, West Sussex).

Huang YM, Xiao BZ, Xiong LZ. 2007. Characterization of a stress responsive proteinase inhibitor gene with positive effect in improving drought resistance in rice. *Planta* **226,** 73-85.

Hwang JE, Hong JK, Lim CJ, Chen H, Je J, Yang KA, Kim DY *et al.* 2010. Distinct expression patterns of two Arabidopsis phytocystatin genes, AtCYS1 and AtCYS2, during development and abiotic stresses. *Plant Cell Reports* **29,** 905-915.

Je J, Song C, Hwang JE, Chung WS, Lim CO. 2014. DREB2C acts as a transcriptional activator of the thermo tolerance-related phytocystatin 4 (AtCYS4) gene. *Transgenic Research* **23,** 109.

Jentsch A, Kreyling J, Elmer M, Gellesch E, Glaser B, Grant K, Hein R *et al*. 2011. Climate extremes initiate ecosystem-regulating functions while maintaining productivity. *Journal of Ecology*, **99**, 689–702.

Jury WA, Vaux HJ. 2007. The emerging global water crisis: managing scarcity and conflict between water users. *Advances in Agronomy* **95,** 1–76.

Kiggundu A, Goulet MC, Goulet C, Dubuc JF, Rivard D, Benchabane M, Pépin G *et al*. 2006. Modulating the proteinase inhibitory profile of a plant cystatin by single mutations at positively selected amino acid sites. *The Plant Journal* **48,** 403-413.

Kiggundu A, Muchwezi J, van der Vyver C, Viljoen A, Vorster J, Schlüter U, Kunert K, Michaud D. 2010. Deleterious effects of plant cystatins against the banana weevil Cosmopolites sordidus. *Archives of Insect Biochemistry and Physiology* **73,** 87-105.

Kidrič M, Kos J, Sabotič J. 2014. Proteases and their endogenous inhibitors in the plant response to abiotic stress *Botanic Serbica* **38,** 139-158.

Kucharik CJ, Ramankutty N. 2005. Trends and variability in U.S. corn yields over the 20th century. *Earth International* **9,** 1–29.

Lawlor DW. 2002. Limitation to photosynthesis in water-stressed leaves: stomata vs metabolism and the role of ATP. *Annals of Botany* **89,** 871-885.

Lawlor DW. 2013. Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations and possibilities. *Journal Experimental Botany* **64,** 83–108.

Lobell DB, Asner GP. 2003. Climate and management contributions to recent trends in U.S. agricultural yields. *Science* **299,** 1032.

Manavalan LP, Guttikonda SK, Tran LSP, Nguyen HT. 2009. Physiological and molecular approaches to improve drought resistance in soybean. *Plant Cell Physiology* **50,** 1260-1276.

Martínez DE, Bartoli CG, Grbic V, Guiamet JJ. 2007. Vacuolar cysteine proteases of wheat (Triticum aestivum L.) are common to leaf senescence induced by different factors. *Journal Experimental Botany* **58,** 1099–1107.

Martinez M, Diaz I. 2008. The origin and evolution of plant cystatins and their target cysteine proteinases indicate a complex functional relationship. *BMC Evolutionary Biology* **8,** 198.

Martinez M, Cambra I, Carrillo L, Diaz-Mendoza M, Diaz I. 2009. Characterization of the entire cystatin gene family in barley and their target cathepsin L-Like cysteine-proteases, partners in the hordein mobilization during seed germination. *Plant Physiology*, **151,** 1531-1545.

Martinez M, Cambra I, Gonzalez-Melendi P, Santamaria ME, Diaz I. 2012. C1A cysteine-proteases and their inhibitors in plants. *Physiologia Plantarum* **145,** 85-94.

Masoud SA, Johnson LB, White FF, Reeck GR. 1993. Expression of a cysteine proteinase inhibitor (oryzacystatin-I) in transformed tobacco plants. Plant Molecular Biology **21,** 655–663.

Márquez García B, Shaw D, Cooper J, Karpinska B, Quain MD, Makgopa EM, Kunert K, Foyer CH (2015) Redox markers for drought-induced nodule senescence, a process occurring after drought-induced senescence of the lowest leaves in soybean (*Glycine max* Merr.) *Annals of Botany.* 10.1093/aob/mcv030

Massonneau A, Condamine P, Wisniewski JP, Zivy M, Rogowsky, PM. 2005. Maize cystatins respond to developmental cues, cold stress and drought. *Biochimica et BiophysicaActa (BBA) - Gene Structure and Expression* **1729,** 186-199.

Mourtzinis S, Specht JE, Lindsey LE, Wiebold WJ, Ross J, Nafziger ED, Kandel HJ *et al.* 2015. Climate-induced reduction in US-wide soybean yields underpinned by region- and in-season-specific responses. *Nature Plants* **1,** Article Number 1402, doi:10.1038/nplants.2014.26

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods* **5,** 621-628.

Munger A, Coenen K, Cantin L, Goulet C, Vaillancourt LP, Goulet MC, Tweddell R,Sainsbury F, Michaud D. 2012. Beneficial unintended effects of a cereal cystatin in transformed lines of potato, Solanum tuberosum. *BMC Plant Biology* **12,** 198.

Nagalakshmi U, Wang Z, Waern K, Shou C, Raha D, Gerstein M, Snyder M. 2008. The transcriptional landscape of the yeast genome defined by RNA Sequencing. *Science* **6,** 1344-1349.

Noctor G, Mhamdi A, Foyer CH. 2014.The roles of reactive oxygen metabolism in drought: not so cut and dried. *Plant Physiology* **164,** 1636–1648.

O'Rourke JA, Bolon Y-T, Bucciarelli B, Vance CP 2014. Legume genomics: understanding biology through DNA and RNA sequencing *Annals of Botany* **113**, 1107–1120.

Parry MAJ, Wang J, Araus JL. 2012. New technologies, tools and approaches for improving crop breeding. *Journal Integrative Plant Biology* **54,** 210–214.

Pastori GM, Foyer CH. 2002. Common components, networks and pathways of Cross tolerance to stress. The central role of "redox", and abscisic acid-mediated controls. *Plant Physiology* **129,** 460-468.

Pernas M, Sánchez-Monge R, Salcedo G. 2000. Biotic and abiotic stress can induce cystatin expression in chestnut. *FEBS Letters* **467,** 206-210.

Pinheiro C, Chaves MM, Ricardo CP. 2001. Alterations in carbon and nitrogen metabolism induced by water deficit in the stems and leaves of *Lupinus albus* L. *Journal Experimental Botany* **52,** 1063-1070.

Pinheiro C, Kehr J, Ricardo CP. 2005. Effect of water stress on lupin stem protein analysed by two-dimensional gel electrophoresis. *Planta* **221,** 716-728.

Puppo A, Groten K, Bastian F, Carzaniga R, Soussi M, Lucas MM, de Felipe MR, Harrison J, Vanacker H, Foyer CH. 2005. Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. *The New Phytologist* **165**, 683-701.

Prins A, Van Heerden PDR, Olmos E, Kunert KJ, Foyer CH. 2008. Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. *Journal Experimental Botany* **59,** 1935-1950.

Quain MD, Makgopa ME, Marquez-Garcıa B, Comadira G, Fernandez-Garcia N, Olmos E, Schnaubelt D, Kunert KJ, Foyer CH. 2014. Ectopic phytocystatin expression leads to enhanced drought stress tolerance in soybean (*Glycine max*) and Arabidopsis thaliana through effects on strigolactone pathways and can also result in improved seed traits. *Plant Biotechnology Journal* **12,** 903-913.

Quain MD, Makgopa ME, Cooper J, Kunert KJ, Foyer, CH 2015. Ectopic phytocystatin expression alters nodule numbers and the nitrogen deficiency-dependent expression of

cysteine proteases and their inhibitors in soybean (*Glycine max*). *Phytochemistry* doi.org/10.1016/j.phytochem.2014.12.027

Rabbani MS, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2003. Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiology* **133,** 1755-1767.

Sainsbury F, Rhéaume AJ, Goulet MC, Vorster J, Michaud D. 2012. Discrimination of differentially inhibited cysteine proteases by activity-based profiling using cystatin variants with tailored specificities. *Journal of Proteome Research* **11,** 5983-5993.

Schlüter U, Benchabane M, Munger A, Kiggundu A, Vorster J, Goulet MC, Cloutier C, Michaud D. 2010. Recombinant protease inhibitors for herbivore pest control: A multitrophic perspective. *Journal of Experimental Botany* **61,** 4169-4183.

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J *et al*. 2010. Genome sequence of the palaeopolyploid soybean. *Nature* **463**, 178-183.

Shan L, Li C, Chen F, Zhao S, Xia G. 2008. A Bowman-Birk type protease inhibitor is involved in the tolerance to salt stress in wheat. *Plant Cell and Environment* **31,** 1128–1137.

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K. 2001. Monitoring the expression pattern of 1300 Arabidopsis

genes under drought and cold stresses by using a full-length cDNA microarray. *The Plant Cell* **13,** 61-72.

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A *et al*. 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant Journal* **31,** 279-292.

Shalek AK, Satija R, Adiconis X, Gertner RS, Gaublomme JT, Raychowdhury R, Schwartz S *et al*. 2013. Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. *Nature* **498**, 236 – 240.

Shinozaki K, Yamaguchi-Shinozaki K, Seki M. 2003. Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion Plant Biology* **6,** 410-417.

Simova-Stoilova L, Vaseva I, Grigorova B, Demirevska K, Feller U. 2010. Proteolytic activity and cysteine protease expression in wheat leaves under severe soil drought and recovery. *Plant Physiology and Biochemistry* **48,** 200–2006.

Srinivasan T, Kumar KRR, Kirti PB. 2009. Constitutive expression of a trypsin protease inhibitor confers multiple stress tolerance in transgenic tobacco. *Plant Cell Physiol*ogy **50,** 541-553.

Stasi M, De Luca M, Bucci C. 2014. Two-hybrid-based systems: Powerful tools for investigation of membrane traffic machineries. *Journal of Biotechnology* pii: S0168-1656(14)01043-8. doi: 10.1016/j.jbiotec.2014.12.007.

Stirnberg P, van de Sande K, Leyser HM. 2002. MAX1 and MAX2 control shoot lateral branching in Arabidopsis. *Development* **129,** 1131‑1141.

Tajima T, Yamaguchi A, Matsushima S, Satoh M, Hayasaka S, Yoshimatsu K, Shioi Y. 2011. Biochemical and molecular characterization of senescence-related cysteine protease–cystatin complex from spinach leaf. *Physiologia Plantarum* **141,** 97-116.

Valdés-Rodríguez S, Guerrero-Rangel A, Melgoza-Villagómez C, Chagolla-López A, Delgado-Vargas F, Martínez-Gallardo N, Sánchez-Hernández C, Délano-Frier J. 2007. Cloning of a cDNA encoding a cystatin from grain amaranth (*Amaranthus hypochondriacus*) showing a tissue-specific expression that is modified by germination and abiotic stress. *Plant Physiology and Biochemistry* **45,** 790–79.

Van der Vyver C, Schneidereit J, Driscoll S, Turner J, Kunert K., Foyer CH. 2003. Oryzacystatin I expression in transformed tobacco produces a conditional growth phenotype and enhances chilling tolerance. *Plant Biotechnology Journal* **1,** 101-112.

Van Wyk SG, Du Plessis M, Cullis CA, Kunert KJ, Vorster BJ. 2014. Cysteine protease and cystatin expression and activity during soybean nodule development and senescence. *BMC Plant Biology* **14,** 294.

Vorster BJ, Tastan-Bishop Ö, Schlüter U, Coetzer N, Michaud D. 2010. New insights towards the understanding of plant cystatin–papain interactions. *Aspects of Applied Biology* **96,** 403-408.

Vorster B, Schlüter U, du Plessis M, van Wyk S, Makgopa M, Ncube I, Quain M, Kunert K, Foyer CH. 2013. The cysteine protease–cysteine protease inhibitor system explored in soybean nodule development. *Agronomy* **3,** 550-570.

Wang S, Blumwald, (2015) Stress-induced chloroplast degradation in Arabidopsis is regulated via a process independent of autophagy and senescence-associated vacuoles. *Plant Cell*. doi/10.1105/tpc.114.133116

Willison KR, Klug DR. 2013. Quantitative single cell and single molecule proteomics for clinical studies. *Current Opinion in Biotechnology* **24,** 745-751.

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG. 2001. Ore 9, an F-box protein that regulates leaf senescence in Arabidopsis. *Plant Cell* **13,** 1779-1790.

Woo HR, Kim JH, Nam HG, Lim PO. 2004. The delayed leaf senescence mutants of Arabidopsis, ore1, ore3, and ore9 are tolerant to oxidative stress. *Plant Cell Physiology* **45,** 923‑932.

Yamaguchi-Shinozaki K, Shinozaki K. 2005. Organization of cis-acting regulatory elements in osmotic- and cold-stress responsive promoters. *Trends in Plant Science* **10,** 88-94.

Zhang XX, Liu SK, Takano T. 2008. Two cysteine proteinase inhibitors from Arabidopsis thaliana, AtCYSa and AtCYSb, increasing the salt, drought, oxidation and cold tolerance. *Plant Molecular Biology* **68,** 131-143.