UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS
(CENTRO DE BIOTECNOLOGÍA Y GENÓMICA DE PLANTAS)

Roles of C1A peptidases during barley leaf senescence mediated by abiotic stresses

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ABBREVIATIONS

%	Percentage
ºC	Celsius Degree
Ø	Diameter
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
ABA	Abscisic Acid
AMC	Amido Methyl Coumarin
amiRNA	Artificial Micro RNA
AP	Autophagosome
ATG	Autophagy- related gene
ATP	Adenosine triphosphate
B-FVR	B-Phe-Val-Arg-7-amido-4-methyl coumarin
BiFC	Bimolecular Fluorescence Complementation
bp	Base Pairs
BSA	Bovine Serum Albumin
CCV	Chloroplast Vesiculation-Containing Vesicles
cDNA	Complementary DNA
CH	Carbohydrates
CK	Cytokinin
CV	Chloroplast Vesiculation protein
cv	Cultivar
CysProt	Cysteine Proteases
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DTT	Dithiothreitol
E-64	[1-[N-[(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane]
EDTA	Ethylenediaminetetraacetic Acid
EL	Electrolyte Leakage
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
eQTL	Expression Quantitative Trait Loci
FAO	Food and Agriculture Organization
g/l	Grams per Liter
GA	Gibberellic Acid
Gb	Gigabase
gdw	Grams of Dry Weight

GFP	Green Fluorescent Protein
gfw	Grams of Fresh Weight
gs	Stomata conductance
GS	Glutamine Synthetase
GS1	Cytosolic Glutamine Synthetase
GS2	Chloroplastic Glutamine Synthetase
ha	Hectare
hai	Hours After Imbibition
HSD	Honestly Significant Difference test
IPCC	International Panel on Climate Change
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic Acid
KD	Knock-Down
kDa	Kilodalton
Ki	Inhibition constant
LHCI	Light Harvesting Complex I
LHCII	Light Harvesting Complex II
LSD	Leaf Senescence Database
M	Molar
MAPK	Mitogen-Activated Protein Kinase
mg	Milligram
ml	Milliliter
mM	Millimolar
Mr	Molar mass
mRNA	Messenger RNA
NCC	Non-Fluorescent Catabolite
nm	Nanometers
nmol	Nanomol
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NR	Nitrate Reductase
NUE	Nitrogen Use Efficiency
OD	Optical Density
OE	Overexpressing
PAO	Pheophorbide *a* Oxygenase
PBS	Sodium Perborate
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDB	Protein Database
pFCC	Primary Fluorescent Chlorophyll-Derived Catabolites
PhyCys	PhytoCystatins

PSI	Photosystem I
PSII	Photosystem II
PWC	Aerial Plant Water Content
RCB	RuBisCo Containing Body
RNA	Ribonucleic Acid
RNAseq	RNA sequencing
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
RuBisCo	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
SA	Salicylic Acid
SAG	Senescence Associated Gene
SAV	Senescence Associated Vacuole
SDG	Senescence Down-regulated Gene
SDS	Sodium Dodecyl Sulfate
SGR	Stay Green
ssDNA	Single-Stranded DNA
SWC	Soil Water Content
SWD	Soil Water Deficit
TBO	Toluidine Blue O
TCA	Tricarboxylic Acid
TEMED	Tetramethylethylenediamine
TF	Transcription Factor
ud	Units
v/v	Volume/Volume
VPE	Vacuolar Processing Enzyme
w/v	Weight/Volume
WT	Wild Type
ZFR-AMC	Z-Phe-Arg-7-amido-4-methyl coumarin
ZRR-AMC	Z-Arg-Arg-7-amido-4-methyl coumarin

INDEX

Abstract

Protein breakdown and mobilization from old or stressed tissues, such as leaves, to growing and sink organs, such as grains or tubers, are some of the metabolic features associated with leaf senescence, essential for nutrient recycling. Senescence may be naturally activated by endogenous signals and/or modified by the prevalence of abiotic/biotic stresses, as a survival strategy. Protein breakdown in senescing leaves involves many plastidial and nuclear proteases, regulators, different subcellular locations and a dynamic protein traffic to ensure transformation of high molecular weight proteins into transportable and useful hydrolyzed products. C1A cysteine proteases are the most abundant key players responsible for the proteolytic activity during leaf senescence. Besides, cystatins, as specific modulators of C1A protease activities, exert a regulatory role along the process. In barley (*Hordeum vulgare*), the whole gene family members of C1A cysteine proteases and cystatins have been identified. Elucidating the role of barley C1A proteases in response to abiotic stresses is crucial due to their impact on plant growth and grain yield and quality.

Darkness and nitrogen starvation treatments were used to induce leaf senescence in barley. Both abiotic stresses strongly induced the expression of the *HvPap-1* gene encoding a cathepsin F-like protease. Morphological changes presuming chloroplast dismantling designated darkness as an ideal stressor for inducing and analyzing senescence. Differences in biochemical parameters and C1A gene expression and protein accumulation among wild-type and transgenic barley plants over-expressing or silencing this gene were detected under the stress. Besides, a lifespan-delayed phenotype of *HvPap-1* silenced lines was evidenced, indicating a functional role for this protease along the senescence process.

Proteolysis is likewise essential throughout the mobilization of storage proteins in barley grains during germination. Manipulation of the proteolytic machinery could enhance grain yield and quality through alterations along these stages. Transgenic barley plants silencing or over-expressing *HvPap-1* showed differential accumulation of starch, proteins, and free amino acids in the grain. The phenotype displayed by silencing *HvPap-1* lines, showing a drastic delay in germination, was particularly

striking. Alterations in the proteolytic activities associated with changes in the expression levels of several C1A proteases were also detected. Similarly, down-regulating *Icy-2*, encoding one of the proteinaceous inhibitors of the studied cathepsin F-like protease, also brought about important effects on grain filling.

The cooperative role of cystatins and their functional relationship with cysteine proteases have been highlighted in the current study by the enhanced/reduced tolerance of plants silencing phytocystatins towards drought. Two barley phytocystatins, HvCPI-2 and HvCPI-4, were induced by this stress. Alterations in the proteolytic patterns by silencing these cystatins were concomitant with modifications in the expression of target proteases. As a result, accelerated or delayed leaf senescence, depending on the silenced cystatin, was exhibited. Results support the potential use of these plants to modulate plant responses facing abiotic stress and, at the same time, to maintain or even increase crop yields under the evidenced climate change framework

According to data reported in this thesis, manipulation of C1A proteases-cystatins interactions in barley has the potential to modulate sensitivity towards specific abiotic stresses through modifications over established developmental leaf senescence programs. In addition, the *in vivo* implication of this proteolytic network during remobilization of stored compounds along barley grain germination is demonstrated. As a general remark, caution should be taken when designing related biotechnological tools since the plant tries to compensate the genetic modifications by modulating the expression of some other proteases or inhibitors.

Resumen

La degradación y movilización de proteínas desde tejidos maduros o sometidos a estrés, como las hojas, hasta los órganos en desarrollo o sumidero, como los granos de los cereales, son procesos metabólicos inherentes a la senescencia foliar. Los programas de senescencia se activan tanto en respuesta a señales endógenas como a estreses abióticos y bióticos como estrategia de supervivencia. La proteólisis en hojas senescentes implica multitud de proteasas de origen nuclear y plastidial, reguladores, diversas localizaciones subcelulares, así como un tráfico dinámico cuyo fin es asegurar la transformación de proteínas de alto peso molecular en productos hidrolizados que puedan transportarse y reutilizarse. La familia C1A de cisteín-proteasas engloba un buen número de enzimas responsables de la actividad proteolítica asociada a la senescencia foliar. Además, las cistatinas, inhibidores específicos de dichas proteasas, ejercen un papel regulador durante este proceso fisiológico. En cebada (*Hordeum vulgare*), las familias completas de proteasas C1A y cistatinas han sido identificadas. Dilucidar el papel funcional de las proteasas C1A de cebada en respuesta a estreses abióticos es esencial, debido a su impacto sobre el crecimiento de las plantas y la alteración del rendimiento y calidad del grano.

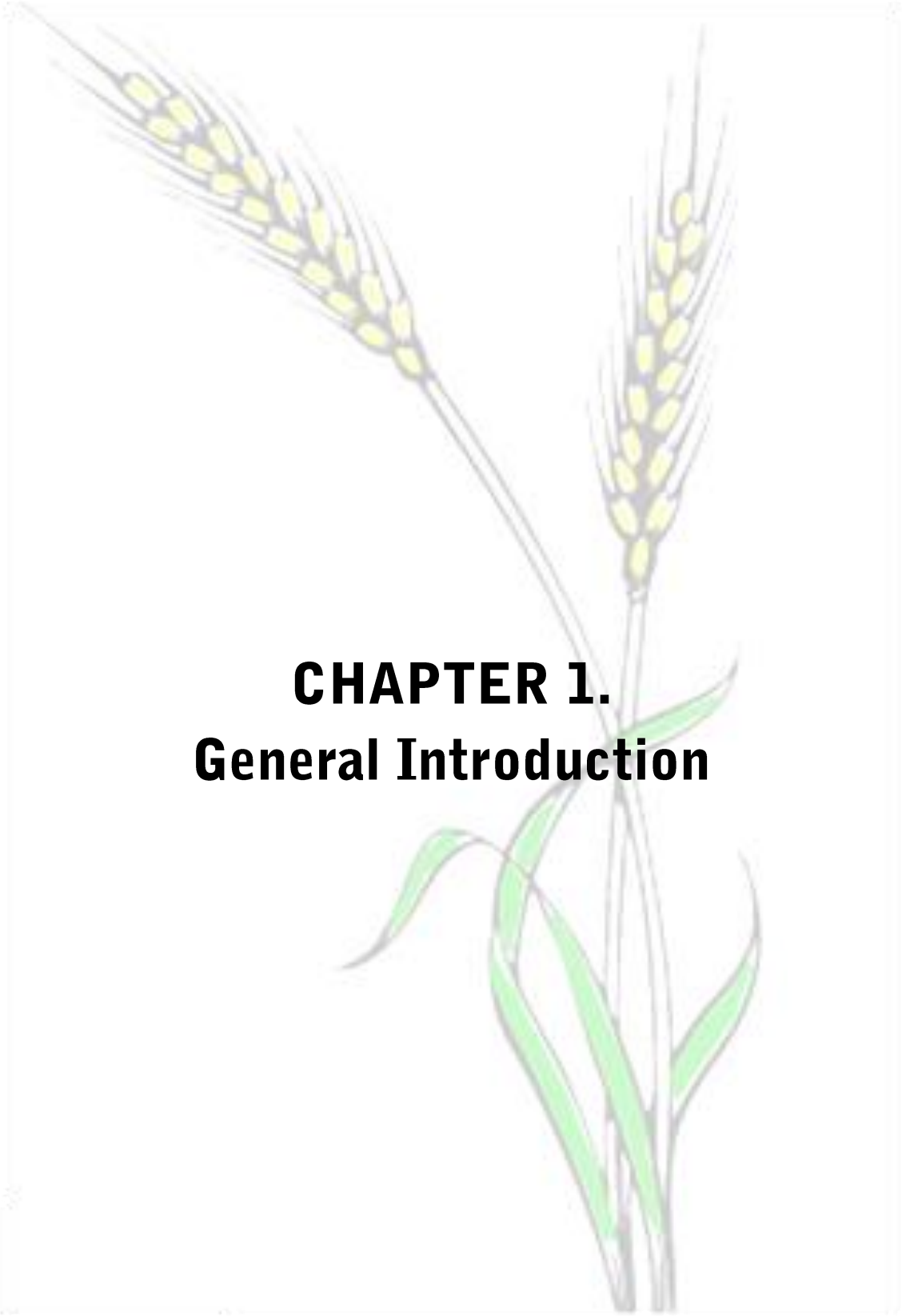
Los tratamientos de oscuridad y de carencia de nitrógeno se utilizaron para inducir senescencia foliar en cebada. Ambos estreses indujeron claramente la expresión del gen *HvPap-1*, que codifica una proteasa tipo catepsina F. Cuando se compararon plantas control frente a líneas transgénicas de sobrexpresión y de silenciamiento para este gen en oscuridad, se observaron alteraciones significativas en parámetros bioquímicos, en patrones de expresión de genes de proteasas C1A, así como en el contenido proteico. Por otro lado, el fenotipo “stay-green” de las líneas de silenciamiento evidenció una vida útil más prolongada en estas plantas, demostrando la implicación funcional de esta proteasa a lo largo del proceso de senescencia.

La proteólisis es asimismo esencial para la movilización de proteínas de reserva del grano durante la germinación. La manipulación de la maquinaria proteolítica durante este proceso fisiológico podría tener un efecto de mejora sobre la calidad del grano y el rendimiento del cultivo. Las líneas transgénicas de sobreexpresión y

silenciamiento del gen *HvPap-1* mostraron una acumulación diferencial de almidón, proteínas y amino ácidos en la semilla. El fenotipo de los granos de las líneas silenciadas evidenció un claro retraso en el proceso germinativo. También se observaron alteraciones en las actividades proteolíticas, asociadas a las variaciones en los niveles de expresión de genes C1A. De forma paralela, al silenciarse el gen *Icy-2* que codifica uno de los inhibidores de la catepsina F estudiada, se observaron efectos en relación con el llenado y calidad del grano.

La interacción y la implicación funcional de cisteín-proteasas y cistatinas en cebada se ha constatado en este estudio, tal y como se infiere de la tolerancia alterada frente a sequía en las líneas de silenciamiento de cistatinas. Dos fitocistatinas, HvCPI-2 y HvCPI-4, se indujeron específicamente por dicho estrés. Las alteraciones en los patrones proteolíticos al silenciar estas cistatinas fueron paralelas a las variaciones en la expresión de genes de sus proteasas diana. En función de la cistatina silenciada, se apreció un retraso o una aceleración en la senescencia. Estos resultados apoyan el uso de estas líneas con el objetivo de modular las respuestas a estreses diversos y mantener, o incluso incrementar, los rendimientos en el marco evidente del cambio climático.

De acuerdo con los resultados obtenidos, la manipulación de las interacciones entre proteasas C1A y cistatinas en cebada permitiría modular la sensibilidad frente a estreses abióticos concretos en base a modificaciones sobre los programas de senescencia endógenos. Se confirma asimismo, la importancia *in vivo* de esta compleja red proteolítica durante la germinación. Como observación general, cuando se diseñen estrategias biotecnológicas basadas en estos mecanismos moleculares se han de considerar los efectos de compensación derivados de la expresión de otros inhibidores y/o proteasas de la planta.



CHAPTER 1.
General Introduction

1.1. LEAF SENESCENCE: A NATURAL EVENT MODULATED BY STRESSES

1.1.1. A GENERAL LEAF SENESCENCE OVERVIEW

Senescence is a natural process which occurs in all plants when the maturity phase is coming to its end, leading to the death or completion of a life cycle. Senescence-like processes occur in angiosperm and non-angiosperm land plants, algae and photosynthetic prokaryotes (Gan and Amasino, 1997; Lim *et al*., 2007; Thomas *et al*., 2009). This developmental phase is illustrated by the striking changes in leaf color observed during the autumn for trees and other perennial plants in temperate regions. In annual crops such as cereals, something similar is observed when the green color changes to golden as the grain ripens (Buchanan-Wollaston *et al*., 2003). It is noteworthy that these color changes evidence chlorophyll degradation or/and *de novo* synthesis of anthocyanins (Rapp *et al*., 2015), among other protective compounds.

In an overall view, the main goal of this complex physiological event can be compared to the three R´s theory of the environment (reduce, reuse and recycle), in this case with an extra R (remobilize). After `*Reducing´* the photosynthetic rate in response to the activation of a senescence program, a massive `*Recycling´* of nutrients that will be `*Reutilized´* as scaffolds for new macromolecule biosynthesis and insurance of the next generation survival begins. This implies an important `*Remobilization´* of nutrients through the phloem, from the source plant parts, such as senescent leaves, towards sink organs such as emergent leaves, grains, tubers or fruits. This strictly controlled event is integral to the flowering plant life-cycle and is determined by endogenous developmental signals governed by the reproductive age (Ghanem *et al*., 2012). In many monocarpic plants the developing reproductive structures often govern the timing and onset of leaf senescence, thereby affecting all organs of a given plant (Munné-Bosch, 2008).These intrinsic cues are continuously modulated by external factors (abiotic environmental stresses, like drought or flooding, high irradiance or darkness, extreme temperatures, salinity, wounding or accumulation of pollutants; and biotic stresses, i.e., pathogens and pests) which modify, to some extent, the natural

senescence programs of the plant (Fig.1.1). The degree of influence of such stresses will determine if it causes an impact on the yield (Breeze *et al*., 2011).

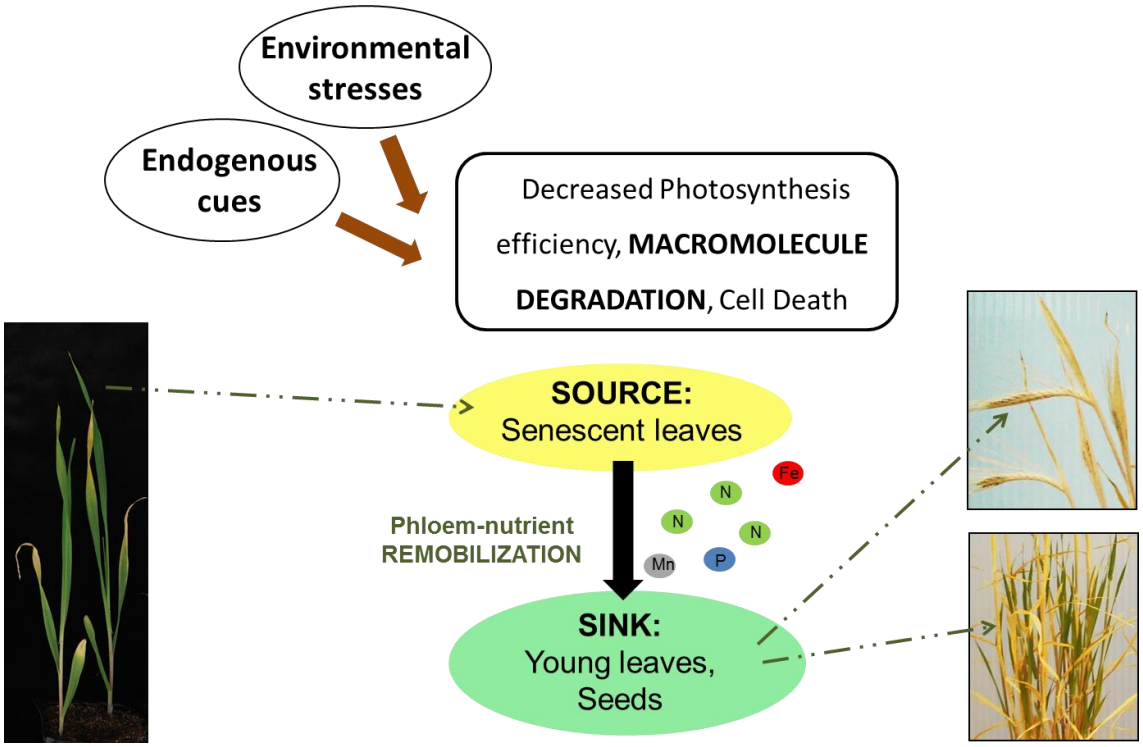


Fig. 1.1. Schematic representation of source to sink nutrient recycling favored by leaf senescence.

All the dramatic changes undergoing along senescence are finely tuned and do not constitute a mere chaotic event. Genetic and epigenetic mechanisms regulating phase change from juvenility to maturity directly influence the capacity for responding to senescence signals (Thomas, 2013). The endogenous signals and the environmental stresses perceived by a plant are integrated into the natural senescence program and subsequently transmitted, forming complex interactions of regulatory pathways among plant hormonal routes, transcription factors (TF), signaling transduction cascades of calcium, phosphatases, kinases and others, to control the onset and progression of senescence. These sophisticated networks somehow channel the impacts from the environment and determine multiple changes in gene expression patterns during senescence (Schippers *et al*., 2007). The orderly and orchestrated sequential changes in cellular physiology, biochemistry and metabolism are strongly triggered by a rapid reprogramming in the expression of an important battery of

Senescence Associated Genes (SAGs; He *et al*., 2001; Breeze *et al*., 2008, 2011). The degree of effectiveness in the response of the plant after the detection of a stressor factor will determine the degree of reversibility, delimiting a narrow border between degenerative cell death and senescence as a recycling process.

The senescent phase is reversible in the green mesophyll cells until almost all macromolecules have been recycled and exported to the rest of the plant (Thomas, 2013). Senescing leaves can, under certain conditions, re-green and regain their photosynthetic capacity (Rapp *et al*., 2015). Cells within the same organ can be at different stages in the progression from senescence to death (Thomas, 2013). Leaf senescence is thus a type of Programmed Cell Death (PCD) but some key hallmarks make it distinguishable from other PCD (van Doorn, 2004; Lim *et al*., 2007; Avila-Ospina *et al*., 2014). It proceeds at the organ-level whereas other PCD occur in limited tissues and cell types; it shows a slower rate than other PCD; and, regarding the physiological goal, leaf senescence fulfills the essential role of recycling cellular nutritional components for plant survival and productivity (Breeze *et al*., 2008).

The participation of hormones during the regulation of leaf senescence is becoming evident through characterization of genetic mutants and global gene expression analysis. In general, senescence is accelerated by brassinosteroids, abscisic acid (ABA), ethylene, jasmonic acid (JA), and salicylic acid (SA), and slowed down by auxin, cytokinins (CK) and gibberellic acid (GA; Podzimska-Sroka *et al*., 2015). One of the signals during the onset of leaf senescence involves cell sugar status alterations as a consequence of the initial dismantling of the photosynthetic apparatus. There are some lines of research demonstrating that accumulation of sugars compromises the photosynthetic capacity and accelerates leaf senescence (Lim *et al*., 2007). In addition, the production and accumulation of reactive oxygen species (ROS), derived from alterations in the cell machineries, has also been proposed as an important promoting signal during natural and altered senescence. Albeit ROS production is known to have harmful effects upon diverse biomolecules, it has been proven that a certain level is required to trigger the activation of genetically programmed pathways of gene

expression during leaf senescence (Khanna-Chopra, 2012; Zhang and Zhou, 2013; Noctor *et al*., 2014, 2016).

Considering that within the leaf the main source of nitrogen-containing molecules is located inside the chloroplasts, it is not surprising that the earliest structural, biochemical and metabolic changes are observed inside these organelles. All enzymes required for carbon fixation and nitrogen assimilation, such as ribulose bisphosphate carboxylase/oxygenase (RuBisCo), as well as most of the proteins that plants can use for nitrogen recycling and mobilization, are inside this organelle (Masclaux-Daubresse and Krupinska, 2014; Havé *et al*., 2016). Leaf cells require a certain energy status until late stages of senescence; thus, nucleus and mitochondria, essential for gene expression and power generation, are the last organelles being degraded (Yoshida, 2003; Lim *et al*., 2007). Furthermore, during senescence, most of the fatty acids from membranes are oxidized to provide energy. An evident drop in the nucleic acid content, especially total RNA, has also been documented (Lim *et al*., 2007). A decrease in the overall protein anabolism is one of the best studied markers for the leaf senescence progress (Lim *et al*., 2007; Díaz-Mendoza *et al*., 2014), besides the decline in photosynthesis and chlorophyll content. As the amount of polysomes and ribosomes has been observed to decrease fairly early, it clearly reflects a cessation in protein synthesis (Lim *et al*., 2007). The bulk macromolecule degradation mainly relies on proteolysis. Among proteases, serine, and mostly cysteine proteases (CysProt) participate during important events related to senescence and stress (Roberts *et al*., 2012; Kidric *et al*., 2014; Velasco-Arroyo *et al*., 2016).

During senescence and stress, many genes related to anabolism, mainly those related to photosynthesis, are down-regulated. These are usually referred as “senescence down-regulated genes” (SDG). On the opposite side, those genes that are induced along this process belong to the SAGs group (Ay *et al*., 2014). These genes fall into different categories according to their function as they may be participating in protein, lipid and nucleic acid turnover, in transport of nutrients, amino acids, sugars, and in defense mechanisms. However, not all SAGs are induced by external cues and some stress-associated genes are not influenced by natural senescence (Buchanan-

Wollaston *et al*., 2005), evincing a complex crosstalk between and among the routes drawn by developmental- or stress-induced senescence (He *et al*., 2001).

Senescence has been intensively studied in the model plant Arabidopsis (Quirino *et al*., 2000; Guo *et al*., 2004; Buchanan-Wollaston *et al*., 2005), with more than 800 genes identified as SAG. This reflects the dramatic alteration in cellular physiology that underlies this plant stage (Lim *et al*., 2007). Probably, the best well-known SAG gene is *SAG12* from Arabidopsis. SAG12 is a CysProt specifically induced during the last stages of developmentally-controlled senescence (Gan and Amasino, 1997), being widely used as a leaf senescence-associated molecular marker. Another well-known Arabidopsis SAG marker corresponds to the WRKY53 transcription factor which, in contrast, is activated at the onset of the process (Zentgraf *et al*., 2010). In crop plants, knowledge related to molecular mechanisms driving leaf senescence is not sufficiently extensive. Some sporadic reports have broadened the information concerning this field in maize, wheat or barley (Smart *et al*., 1995; Kleber-Janke and Krupinska, 1997; Uauy *et al*., 2006). Interestingly, Jukanti *et al*. (2008) found a new regulatory SAG in senescing primary barley leaves consisting on a transmembrane protein kinase.

Many investigations in the field of plant senescence and stress can be integrated into two different but complementary areas: research based upon dilucidation of the molecular basis underlying this crucial event at different layers; and translation of basic research to design tools through biotechnological approaches in combination with conventional breeding to manipulate senescence for agronomic advantages; i.e., translating laboratory bench findings to practical projects (Gan and Hörtensteiner, 2013). Special emphasis is being undertaken in the maintenance or improvement of acceptable yields in important crops for human feed such as cereals, in a context of an evident climate change scenario. It is of pivotal importance to invest efforts to interpret the processes behind the decrease in productivity under adverse situations, which substantially relies on a deeper knowledge of chloroplast dismantling mechanisms in both model and crop species. Accordingly, there exists a continuous effort in updating the resources related with leaf senescence information, as

evidenced the last releases of the Leaf Senescence Database (LSD; Liu *et al*., 2011; Li *et al*., 2014). A growing senescence community continuously sheds light on some relevant and particular aspects concerning senescence and stress, as it is evidenced by the elevated number of reports and reviews related to this fascinating topic, mainly focused in signaling and regulatory pathways, nutrient management and nitrogen use efficiency (NUE), chlorophyll and chloroplast degradation mechanisms, with a key participation of proteases and protease inhibitors (Masclaux-Daubresse and Krupinska, 2014; Díaz-Mendoza *et al*., 2014, 2016b).

1.1.2. LEAF SENESCENCE, GRAIN QUALITY AND YIELD

1.1.2.1. Nitrogen economy in plants: The `Dilemma´ of Senescence and The Stay-Green trait

The timing of the senescence process affects the length of the photosynthetic period, thus influencing the grain filling in the case of cereals and therefore determining the yield and/or the quality. In 2008, Gregersen *et al*. raised the concept of `Dilemma of senescence´ questioning wether is better to delay or to accelerate senescence (Fig. 1.2). When late senescence occurs, looking at the grain, a higher carbohydrate (CH) content and a lower protein accumulation are observed, whereas when senescence arrives earlier the opposite trend is appreciated. It is paramount to understand the molecular mechanisms behind senescence in order to improve these traits, depending on the end-product usage. For instance, applied to barley, delayed senescence would be desirable for malting purposes, since a higher amount of CH is necessary for fermentation. Conversely, early senescence would be ideal for manufacturing meals for animal feeding, since a high content in proteinaceous components is required.

Improvement of cereal cultivars requires a delicate balance among senescence timing, grain nutrient content, NUE, and yield (Distelfeld *et al*., 2014). The physiological stage at which a plant faces a given stress will largely influence upon the activation and progression of the senescence programs, determining the efficiency in the remobilization of nutrients as a strategy for survival.

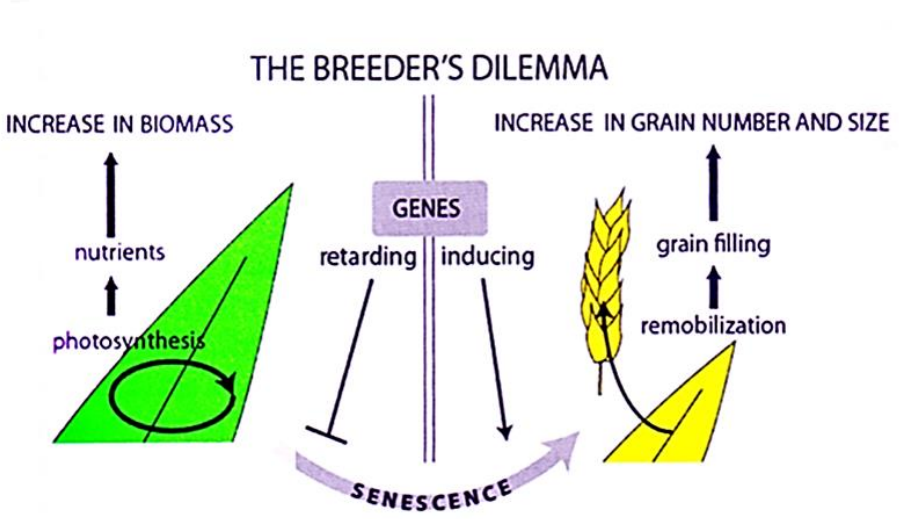


Fig. 1.2. Schematic hypothesis for the breeder´s dilemma. Delayed senescence leads to an extended photosynthetic period resulting in an increased biomass accumulation whereas accelerated senescence provokes a faster remobilization of nutrients translated into an increase in the grain number and size. Gregersen *et al.*, (2014).

"Stay-green" crops have the potential for higher plant yields due to an extended photosynthetic period. There exists a comprehensive classification for these mutants: those which present the ability of extending the vegetative phase which results in higher yields are designated as ''functional stay-green'' mutants. These mainly include Type A and Type B. In the first case, senescence proceeds at a normal rate but its initiation is retarded. In the Type B, the process begins on schedule but is somehow slowed down, while the active photosynthetic stage is usually prolonged. "Non-functional stay-green" mutants or cosmetic mutants (type C) are those that remain green due to impaired chlorophyll catabolism, but lack photosynthetic competence. These last ones were very helpful when elucidating the enzymes and steps involved in the route of chlorophyll catabolism (Thomas and Howarth, 2000). In these mutants, the stay-green character is heritable (Thomas and Ougham, 2014), and it renders a greener phenotype due to a mutation in the Mendel's green cotyledon gene *sgr*, encoding a chloroplast protein required to initiate chlorophyll degradation (Sato *et al*., 2007). Stay-green mutants have been used to understand the correlation between senescence, yield and total nitrogen content of the grain in several crop species (Kichey *et al*., 2007). A wheat stay-green variety presented a reduced harvest

index even after a prolonged grain filling period. It was postulated that remobilization of carbon was inefficient and that extra photoassimilates remained in the vegetative parts instead of being translocated to the grain. Nitrogen concentration in the straw of a stay-green line of wheat remained higher than in controls. Extended photosynthesis did not mean an increase in grain yield as expected; instead, these plants necessitated more nitrogen uptake to achieve a grain protein content comparable to that for wild-type (Chen *et al*., 2011). Another wheat mutant, *tasg1*, showing delayed leaf senescence, was identified as a functional stay-green (Hui *et al*., 2012). The explanation for the last examples in which extended photosynthesis did not result in higher harvest index relies on the fact that sink tissues may have a limitation in their capacity, which is in term influencing a major trait, the growth and size of the seed (Serrago and Miralles, 2014). In a detailed transcriptome study performed in barley, Sreenivasulu *et al*. (2008) analyzed late seed maturation and initial germination stages. They concluded that during maturation, the barley grain stores all required compounds and regulators, among them many TF, meaning that plant seeds prepare for germination already during seed maturation. This leads to conclude that maturation of the grain is a crucial developmental stage, and apparently alterations in source/sink communication influenced by modifications along senescence timing may have negative effects upon the accumulation of valuable elements required for a later and successful germination during next generation (Fig. 1.3).

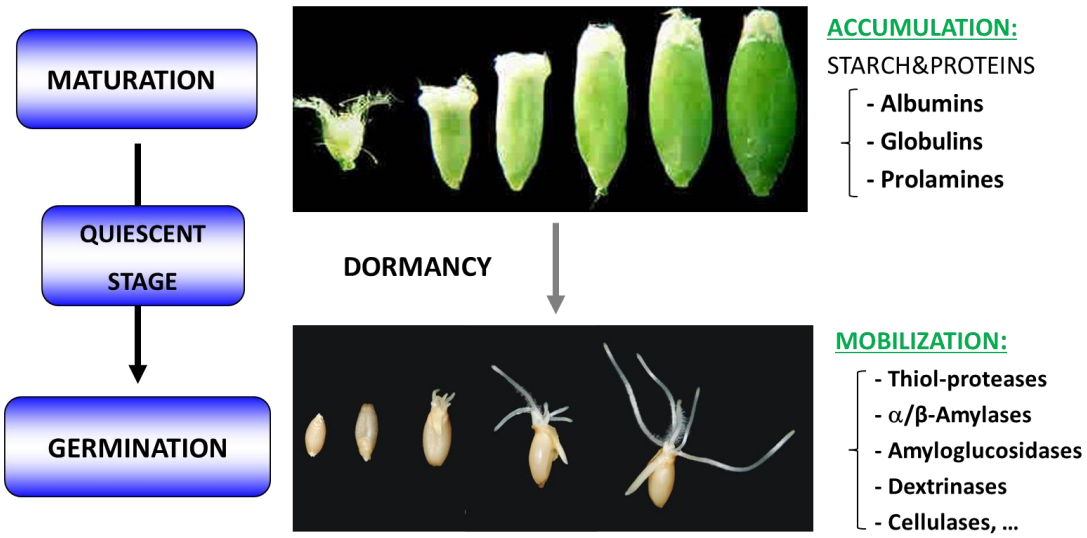


Fig. 1.3. Barley seed developmental stages and prevalent compounds accumulation.

Senescence might reduce crop yield when is prematurely induced under adverse environmental conditions. One of the most common approaches to achieve stay-green varieties through biotechnology is based upon the expression of isopentenyltransferase (IPT), an enzyme that catalyzes the rate-limiting step in CK synthesis (Gan and Amasino, 1997), under the control of senescence-associated promoters. Binding of WRKY family members, among others, to the *cis*-elements on these promoters, is regulated by ABA. These constructs determine an increased biomass in the crops in most of the cases, but this is not very commonly translated into an improved seed yield. On the other hand, it was demonstrated a better performance of these transformed plants under certain adverse environmental stresses, such as drought (Gregersen *et al*., 2013). Accelerated senescence was also achieved in several plant species by means of classical breeding and, in many cases, this was correlated with higher protein content in the seeds. The *Gpc-B1* locus was linked with accelerated flag leaf senescence in wheat and with a shorter grain filling period (Uauy *et al*., 2006). In barley, a similar locus was previously characterized (See et al., 2002). Gpc-B1 belongs to the NAC family of TF, which seems to be up-regulated in many expression studies in response to senescence in both cereals (Gregersen, 2011), pointing these members as ideal candidates involved in senescence regulation (Gregersen, 2011;

Distelfeld *et al*., 2014; Christiansen *et al*., 2016). In fact, it was proposed that NAC TF might be associated with ABA signaling in plants (Jensen *et al*., 2007).

1.1.2.2. Coordinated Carbon and Nitrogen assimilation during remobilization events

In cereal species, senescence is predominantly controlled at the level of the individual leaf, and remobilization usually begins in the older leaves towards the younger ones and the flag leaf, this last making important contribution to the remobilization of the major part of photoassimilates during seed filling (Wiedemuth *et al*., 2005). As opposed to dicotyledonous species, cereal leaves have a basal meristem, and the leaf tip consists on the oldest cells while the youngest are at the leaf base (Gregersen *et al*., 2008). Therefore, cereals represent suitable models to study the progression of senescence. Even though interactions between senescence associated-remobilization and grain filling are complex and poorly understood (Thomas and Howarth, 2000; Gregersen *et al*., 2013), a wealth of literature evidence supports the importance of the remobilization during natural or induced senescence, making special emphasis on nitrogen remobilization, since nitrogen starvation is a well-known trigger of accelerated senescence in many crops (Havé *et al*., 2016). Thereby, several transcriptomic, proteomic and ultra-structural reports, as well as large and properly documented revision works, discuss about the topic, focusing on cereals (Gregersen *et al*., 2013) and other crop species such as *Brassica napus* (Avice and Etienne, 2014). Some authors propose the thorough study of tissue-specific structural modifications in order to determine possible links with NUE and remobilization during a stress episode, as observed in the changes of palisade and spongy parenchyma in oilseed rape leaves during senescence (Sorin *et al*., 2014).

Phloem-specific metabolic compounds might signal high grain demands for N to distantly located plant organs (Kohl *et al*., 2012). Schiltz *et al*. (2004) analyzed protein variations during nitrogen mobilization from leaves to filling seeds in pea (*Pisum sativum*), proving that a chloroplastic protease (FtsH) increased during N mobilization. They proposed that a better understanding of the processes occurring during grain filling from senescing leaves required an estimation of protein turnover by means of

[35S] Met- or [35S] Cys-labeled proteins. Several CysProt and N transporter genes of the AAT family appeared to play a role in remobilization and accumulation of nitrogen as observed in a RNAseq analysis of flag leaves, glumes and developing grains from barley (Kohl *et al*., 2012). In addition, transcriptomes of flag leaves from field experiments subjected to variable levels of nitrogen supply were analyzed (Hollmann *et al*., 2014). *HvPAP-14* and *HvPAP-20* encoding CysProt, and *SCPL51* encoding a serine protease, were differentially expressed. *HvPAP-20* encodes a cathepsin-B-like CysProt (Martinez and Diaz, 2008) also known to be upregulated during barley grain germination (Sreenivasulu *et al*., 2008; Diaz-Mendoza *et al*., 2016a).

More than 75% of the potentially remobilizable reduced nitrogen in plants is located inside the chloroplasts and mainly assembled into Rubisco (Hörtensteiner and Feller, 2002) and other stromal components, such as glutamine synthetase (GS). Chlorophyll-apoprotein complexes from thylakoids represent the second major fraction. Likewise, it was estimated that around 70% of the nitrogen from senescing vegetative organs is exported during seed development in most annual crop plants (Peoples and Dalling, 1988). Although a part of ammonia is evaporated from leaves, the bulk ammonium content is exported from the senescing leaf and utilized to build new amino acids. An intense traffic of amino acids occurs along the phloem during developing and maturation grain stages. The major phloem-exported amino acid in barley and wheat is glutamate (Forde and Lea, 2007), followed by aspartate, glutamine, threonine and serine (Kichey *et al*., 2007). Two forms of GS have been identified in plants, the cytosolic GS1 and the chloroplastic/mitochondrial GS2 (Swarbreck *et al*., 2011). In non-senescing leaves, GS2 is the abundant isoform in the mesophyll cells, where it assimilates ammonium originating from nitrate reduction and photorespiration. During leaf senescence GS1 fulfills a key function in the assimilation and recycling of ammonium generated from various catabolic processes (Masclaux-Daubresse *et al*., 2010). This role is particularly important after anthesis and during grain development and filling when nitrogen is remobilized to the reproductive sinks (Kichey *et al*., 2007; Brauer *et al*., 2011). Schildhauer *et al*. (2008) followed the expression patterns of two genes involved in nitrogen metabolism in barley during reversal of senescence after supply with nitrogen: GS2 and lysine-ketoglutarate

reductase/saccharopine dehydrogenase (LKR⁄SDH). LKR⁄SDH catalyzes the first two steps in the degradation of the important amino acid lysine. In Arabidopsis, a higher total amino acid content in shoots of plants grown under continuous N limitation was observed in comparison to control conditions; authors explained that there was a reduced utilization of amino acids for protein synthesis (Tschoep *et al*., 2009), possibly as a consequence of a slowdown in the tricarboxylic acid (TCA) cycle, which determined a general down-regulation of biosynthetic metabolism (Balazadeh *et al*., 2014).

A rapid reversion in the cytosolic carbon to nitrogen (C⁄N) ratio is required to revert leaf senescence. In both barley and *Arabidopsis thaliana*, senescence can be completely reversed when additional nitrate is resupplied after a nitrogen starvation period (Schildhauer *et al*., 2008). A situation of carbon feast (high CH levels) undergoing in source senescing organs may act as a first signal to start remobilization of nutrients; but a state of carbon starvation in the sink organs may also represent the initial stimulus for beginning the maintenance of molecules (Parrott *et al*., 2005). Importantly, a set of proteases were induced under these conditions.

Given the complexity and the lack of precise descriptions on the events taking place during senescence, either developmental or stress-induced, there is a need to discern which is the main mechanism involved. Since amino acid and nutrient transport are usually the main hallmarks, it seems very likely that, in general, proteolysis represents the ruling process.

1.2. SENESCENCE AND ABIOTIC STRESS

1.2.1. CLIMATE CHANGE SCENARIO

According to recent estimations, world population is increasing at an alarming rate and is expected to reach about 9 billion by the end of 2050 (http://www.fao.org). These striking predictions coincide with the pessimistic data concerning climate change. Rainfall frequency and distribution patterns are expected to vary in most of the

regions. Precipitations will come in the form of severe storms, with irregular trends followed by scarcity periods, which will be translated into flooding and drought episodes, closely linked to the loss of soil fertility and increased salinity. In addition, extreme temperatures are also predicted to represent important threats for agriculture (Fig. 1.4), either due to increased temperatures or chilling and freezing events (Cutforth *et al*., 2007; Jury and Vaux, 2007; Manavalan *et al*., 2009; Simova-Stoilova *et al*., 2010). Such trends in predicted weather patterns are seriously threatening plant development and seed production in agricultural lands (United Nations Convention to Combat Desertification, FAO, 2014), with drought representing the most harmful stressor limiting crop yield, thus being one of the major constraints to global food security (Godfray *et al*., 2010). Moreover, apart from abiotic environmental cues, the occurrence of biotic stresses may be also triggered under these conditions, stimulating the expansion of plant diseases and pests into new geographical areas, wreaking havoc on crop performance (Fig. 1.5). For instance, the invasive insect and acari species are expected to cause severe damage on plants under climate change episodes (Atkinson and Urwin, 2012; Ximénez-Embún *et al*., 2016).

To meet the increasing nutritional demands for this sharp growing population and avoid a food crisis, there is an urgent need to design strategies in order to reach productions around 70% higher than the current ones by the year 2050 (Mahajan and Tuteja, 2005). This ideal framework should be achieved without land surface expansions and with a reduction in the inputs based on inorganic fertilizers, which damage soil fertility (Comadira *et al*., 2015).

This background leads the scientific community to implement projects focused in ameliorating resistance, tolerance or acclimation in relevant crops, with a special focus on drought, temperature and/or salinity among other relevant constraints, by means of plant biotechnology.

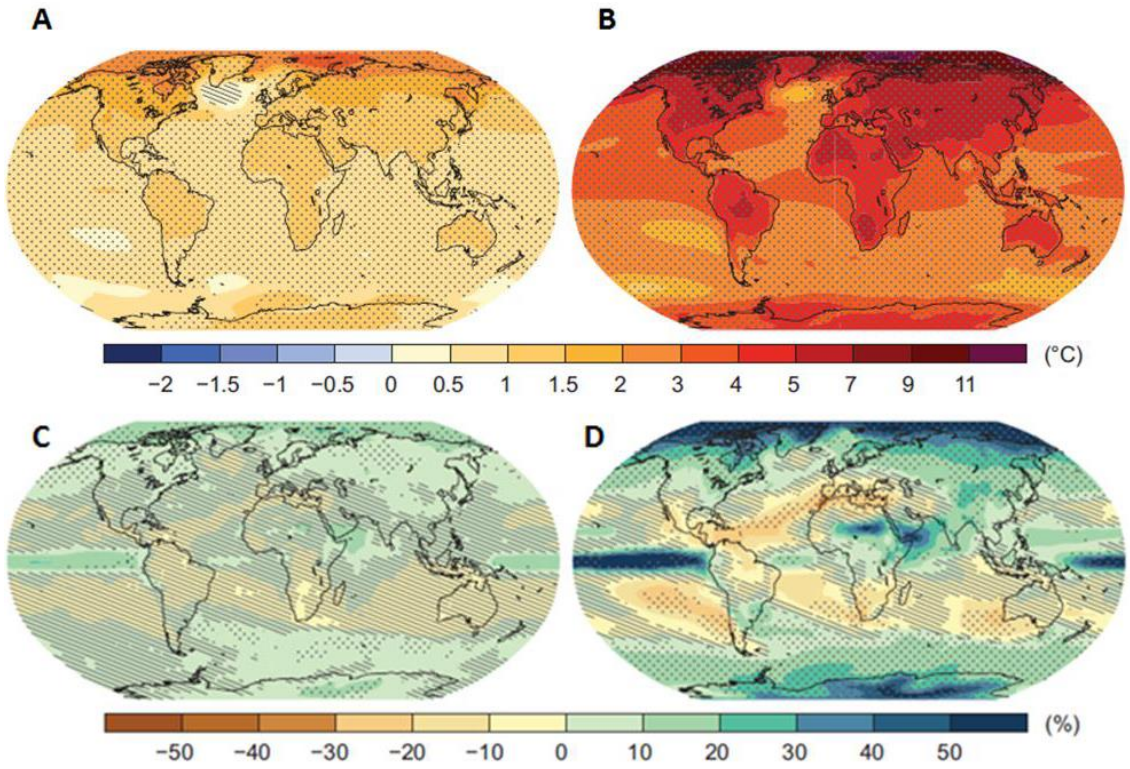


Fig. 1.4. Predicted change in average surface temperature for the interval (A) 1986-2005 and (B)2081-2100; and predicted change in average precipitation for the interval (C) 1986-2005 and (D) 2081-2100 (IPPC, Synthesis Report for Climate Change, 2014). https://www.ipcc.ch/report/ar5/syr/

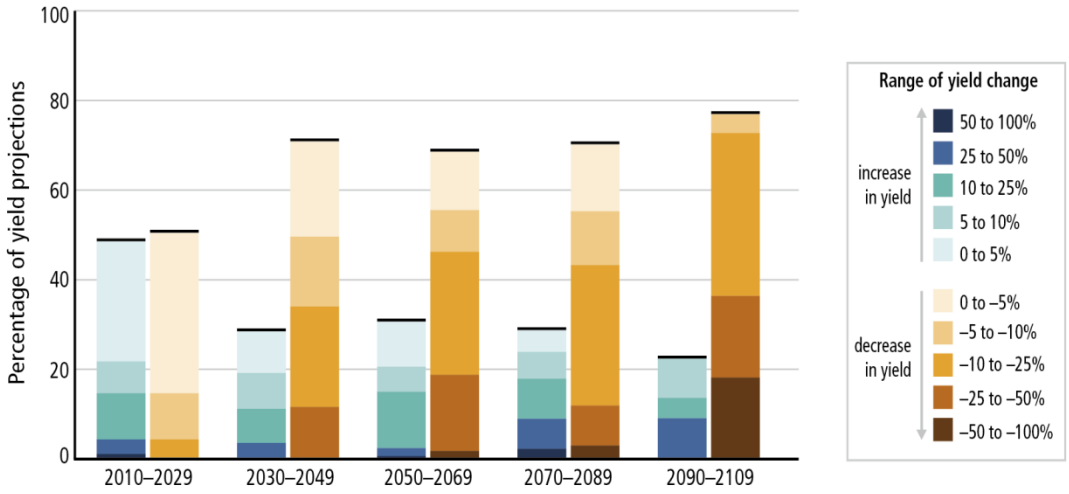


Fig. 1.5. Estimated risks for food production (% of yield projection, increased or decreased) posed by climate change for different year intervals (IPPC, Synthesis Report for Climate Change, 2014). https://www.ipcc.ch/report/ar5/syr/

1.2.2. STRESS CONCEPT

Very recently, Gilbert and Medina (2016) published a useful document in which they give proper definitions related to stress, adaptation and drought. The home message indicates that it is essential to think about the precise mechanism on which the research will be focused on when initially designing an experiment. This is sometimes obviated and results from various investigations are somehow uncertain and confusing. They define stress `as a negative change in the physiology of a plant away from a reference state as a result of the action of an external stress factor or internal stress´. They consider 'stress factors' as external and 'stresses' as physiological responses. Albeit the manuscript is based on drought, we could make a general assumption for all environmental factors. Hence, they describe four hypothetical cases for drought: *"SWD (Soil Water Deficit) avoidance"* refers to a mechanism that, for instance, includes plants that explore deeper soils or match phenology to the wet season; *"stress avoidance"* requires more specialized mechanisms such as succulence; those adaptations that allow plants to tolerate some negative external factor are included in *"damage avoidance"* term, for example through changes in leaf orientation or altered root to shoot ratios; lately, *"damage tolerance"* refers to the state at which plants may tolerate damage through recovery mechanisms, for instance during the night-time or by generating new conductive tissue. All those mechanisms rely on physiological, metabolic and biochemical changes determined by alterations in gene expression. For this reason, knowledge about adaptation mechanisms at the macromolecular level is essential, but it needs to be intimately linked to a perfect comprehension of molecular interplays. A rising number of research papers shows, in most of the cases, adaptation mechanisms responding to the last definition listed above: damage avoidance through recovery mechanisms (Simova-Stoilova *et al*., 2010; Dinakar and Bartels, 2013).

1.2.3. OVERLAPS, SIMILARITIES AND DIVERGENCES AMONG DEVELOPMENTAL LEAF SENESCENCE, ABIOTIC AND BIOTIC STRESSES

Responses to abiotic stresses resemble, in many molecular and phenotypic aspects, the plant senescence syndrome. According to recent studies, plant stress tolerance, apart from crop yield and nutritional values, may be modified through manipulating the timing of senescence (Gepstein and Glick, 2013). Much investment has been made towards identification of stress-protective or adaptation-related genes activated during abiotic stress (Bray *et al*., 2000). Overexpression of these genes could help plants to increase tolerance. A wide spectrum of reports demonstrates the potential of abiotic stresses to trigger leaf senescence by reprogramming specific subsets of SAGs differentially expressed in distinct tissues and several species, including crops. For instance, in order to detect senescence-associated physiological changes involving SAG expression in wheat, detached leaves were subjected to several abiotic and hormonal treatments. *TaSAG3* and *TaSAG5* were expressed in natural senescent leaves and showed differences in expression patterns depending upon the treatment, although both were upregulated immediately after leaf detachment (Zhao *et al*., 2012). In sweet potato, the calmodulin gene *SPCAM* is NaCl-inducible and participates in salt stress-mediated leaf senescence regulating the expression of specific SAGs (Chen *et al*., 2012). Evident effects upon modulation of salt- and osmotic-induced leaf senescence in *Capsicum annuum* L. were likewise observed when downregulating the *CaCP* gene in this species (Xiao *et al*., 2014).

Light is essential for photosynthesis and acts as the main signal for natural development and interactions with the environment. Its deprivation potentially leads to sugar starvation, which is already known to be one of the signals promoting senescence (Parrott *et al*., 2005). Dark-induced senescence results in chlorophyll loss, slowdown of photosynthetic activity and dismantling of cellular constituents, in a similar manner to that observed during age-dependent natural senescence (Fujiki *et al*., 2001; Buchanan-Wollaston *et al*., 2005). Variations in the light intensity modulate the timing of senescence and, under certain conditions, the senescence process may be reversed (Humbeck and Krupinska, 2003). Although darkness cannot be considered

as a true abiotic stress in nature, apart from those events regarding extreme shading of the lower parts in dense canopies, it has been extensively used to analyze mechanisms of leaf senescence in plants based on its immediate effects on the photosynthetic machinery (Gan, 2007). Sometimes, the dark treatment was applied on entire and intact plants (Buchanan-Wollaston *et al*., 2005), but in most cases it was used in detached leaves (Fischer and Feller, 1994; Chiba *et al*., 2003; Thoenen *et al*., 2007; Zhao *et al*., 2012), provoking rapid genome-wide alterations and metabolic responses, which helped to elucidate specific gene functions. Dark-induced senescence has been extensively used since several decades both in Arabidopsis (Keech *et al*., 2007; Niu and Guo, 2012) and in crops, mostly in cereals (Kleber-Janke and Krupinska, 1997; Chrost *et al*., 2004). These experiments have shed light about regulatory networks, as demonstrates the darkness-induced transcription of *AtWRKY22* that suggests its participation in the signal transduction pathway mediated by this abiotic stress in Arabidopsis (Zhang and Zhou, 2013). More recently, it was reported that Phytochrome-Interacting Factors 4 and 5 from Arabidopsis promoted dark-induced and natural senescence by directly activating the expression of typical SAG like ORESARA1 (ORE1) and ETHYLENE INSENSITIVE3 (EIN3) (Piao *et al*., 2015). The implication of autophagy-related pathways during senescence was recently demonstrated using darkness (Avila-Ospina *et al*., 2016). Besides, in a broad range of darkness-based studies, overexpressed SAGs corresponded to CysProt (Parrott *et al*., 2005; Thoenen *et al*., 2007; Watanabe *et al*., 2009; Carrión *et al*., 2013).

Drought stress is produced when evapotranspiration rate exceeds the amount of absorbed water through root tissues (Lawlor and Cornic, 2002). In other words, it involves `a decrease in water inputs into an agro/ecosystem over time that is sufficient to result in SWD´ (Kramer, 1983). At field capacity, this situation is associated with high temperature episodes which results in a negative synergic effect causing severe damage to crops and production losses up to 50% (Bray *et al*., 2000). The severity of drought stress, besides its duration and intensity, will produce a greater or lesser impact depending upon the plant developmental phase at which the stress is faced. If this occurs along the vegetative growth phase, the stress is mostly transient and plant growth slows down to be restored after a new rainfall period, as exemplified in pre-

summer drought events. The plants may even start wilting and yield is normally impacted since a lower number of ear-bearing tillers per plant are often quantified (Sreenivasulu *et al*., 2008). Premature leaf senescence, causing acceleration of the whole-plant maturation, is usually detected when drought episodes arise during the generative plant development, i.e., around flowering (Gan, 2007). A considerable set of research is focused on the roles of proteases and their inhibitors in relation with drought-induced senescence, since enhanced expression of genes coding for proteases is a common event both in senescence and under various environmental stresses (Simova-Stoilova *et al*., 2010.) As reported in an expression profiling of genes in juvenile barley, significant correlations exist within the group of genes involved in drought stress and those acting in leaf senescence (Wehner *et al*., 2016). Neverthless, in some cases the proteases expressed during drought episodes may substantially differ from those expressed during senescence (Simova-Stoilova *et al*., 2010). Drought-induced and natural senescence were monitored in the cowpea leaf, with a focus on CysProt, concluding that the abiotic stress induces many forms of these proteases not observed during developmental senescence (Khanna-Chopra *et al*., 1999). The involvement of acidic proteases in soil drought response of winter wheat in three cultivars differing in water stress tolerance was likewise studied. Results suggested that lower proteolytic activity and decreased expression of certain protease genes under water deficit during early developmental stages could be regarded as an indicator for drought resistance of winter wheat cultivars (Simova-Stoilova *et al*., 2010).

Plants respond to water stress generally by synthesis of ABA, inhibition of photosynthesis and respiration, accumulation of osmotically active compounds, synthesis of protective proteins, such as dehydrins and chaperones, by adjusting sink/source allocation and by speeding up senescence (Simova-Stoilova *et al*., 2010). Suppression of drought-induced leaf senescence in transgenic tobacco plants caused by the accumulation of CK due to IPT overexpression has been linked to an increase of dehydrins and heat shock proteins (Rivero *et al*., 2007). Production of CK also contributed to an enhanced drought tolerance in transgenic cassava and peanut (Qin *et al*., 2011). It has been illustrated that the application of exogenous ABA combined

with salinity stress provokes the over-expression of SAGs and the acceleration of senescence (Yang *et al*., 2003), suggesting the connection among leaf senescence, ABA and abiotic stress signaling (Podzimska-Sroka *et al*., 2015). Besides, drought-induced ABA was positively and significantly correlated with carbon remobilization from senescing leaves to grains in wheat plants subjected to drought stress (Yang *et al*., 2003). Potassium alterations also affect stress tolerance (Restrepo-Diaz *et al*., 2008). Drought stimulated signal transduction chains involving ROS and Ca^{2+} signaling lead to the induction of K^+ transporters and channels in roots and guard cells (Cheong *et* al., 2007). Barley genotypes with a higher K^+ nutritional status in the flag leaf showed superior drought tolerance by promoting ABA degradation and attenuation of starch catabolism, which delays flag leaf senescence (Hosseini *et al*., 2016).

Few studies give precise information about interactions between biotic stresses and leaf senescence and, within, those regarding pests are even less abundant than in the case of diseases. The effects and interactions of biotic stress and senescence may be interpreted in two ways: either the presence of a biotic factor promotes senescence after surpassing plant defenses through modification of common SAGs implied in primary metabolism; conversely, it could happen that senescence is already activated in the plant, naturally or induced by some abiotic factor, thus making the plant more prone for the establishment of pests and pathogens (Masclaux-Daubresse *et al*., 2010; Fagard *et al*., 2014; Ximénez-Embún *et al*., 2016). The first assumption would be the case, for instance, for an early senescence and premature cell death detected after down-regulation of *OsSAG12-1* in rice, after inoculation with *Xanthomonas oryzae* (Singh *et al*., 2013). Likewise, green peach aphid infestation in Arabidopsis accelerated senescence-like mechanisms as resembled in the elevated expression of several SAGs (Pegadaraju *et al*., 2005*;* Louis *et al*., 2010). The mechanisms that allow acclimation and adaptive responses to isolated biotic and abiotic stresses have been extensively characterized in a wide range of studies. Nevertheless, regarding combined biotic/abiotic stress responses, little information is still available. Deciphering the signaling pathways participating in the common crosstalk drawn by biotic and abiotic stresses will allow the identification of new targets for increasing environmental resilience in crops (Foyer *et al*., 2016). `The exposure to one type of stress confers a

general increase in resistance to a range of different stresses, a phenomenon called cross-tolerance´. This phenomenon relies on the synergistic co-activation of non-specific, stress-responsive pathways that cross biotic–abiotic stress boundaries, and which are usually related with altered redox and phytohormone signaling (Foyer *et al*., 2014). Cross-tolerance to different stresses triggered by an exposure to a single stress is widespread in plants (Munné-Bosch *et al*., 2013). Mechanisms of adjustment to water deficit may be associated to an enhanced cotton resistance to mites (Sadras *et al*., 1998). As aforementioned, ABA is very important during the response to drought episodes. Through stomatal closure, the ABA- induced signaling pathway intersects with both abiotic and biotic stress factors (Lee and Luan, 2012). In maize, differences at the proteome level were detected depending if two stresses, drought and the presence of the two-spotted spider mite, were applied individually or combined (Dworak *et al*., 2016).

1.3. REGULATION OF SENESCENCE AND STRESS: HORMONES, TRANSCRIPTION FACTORS AND REACTIVE OXYGEN SPECIES

Phytohormones are key regulators of leaf senescence (Li *et al*., 2012). The increasing knowledge on this regulatory field relies on several investigations about hormonal responses during abiotic and/or biotic episodes. The signaling processes involving plant hormones are modified under the presence of an external cue which triggers alterations in stress-responsive genes that eventually interfere with developmental senescence pathways (Zhang and Zhou, 2013). Several plant hormones, such as ethylene, ABA, SA and JA induce or accelerate senescence, as well as small molecules, such as oxygen. Conversely, CK, auxins, nitric oxide (NO) as well as molecules like Ca^{2+}, may retard leaf senescence (Li *et al*., 2012). Regarding developmental senescence, it appears that its onset is mainly regulated by CK levels, which seem to decrease along this event. Several groups induced the expression of this hormone through alterations in its biosynthetic route in order to delay the process (Podzimska-Sroka *et al*., 2015). Ethylene has a key role during fruit ripening and during the regulation of the onset of senescence, apart from being an important molecule tuning germination and seedling development (Podzimska-Sroka *et al*., 2015). Endogenous ABA levels increase during

senescence (van der Graaff *et al*., 2006), although this phytohormone is considered more an enhancer rather than a triggering factor. Furthermore, under drought, salinity or extreme temperature episodes, the content of ABA usually rises, which is consistent with its proved importance in the response towards abiotic stress (Guo and Gan, 2005). In plants defective in the SA pathway, developmental leaf senescence is delayed, but this is not observed during dark-induced conditions, which illustrates the relevance of this hormone specifically during natural senescence (Buchanan-Wollaston *et al*., 2005). Strigolactones are a class of plant hormones that fulfill several functions in plants, like the regulation of shoot branching and root architecture, as well as the mediation during leaf senescence and stress. The production of strigolactones is induced in response to nitrogen and phosphorous deficiency (Yamada and Umehara, 2015). Cystatins, widely known to be key regulators of CysProt activity in plants, are being successfully utilized in several biotechnological approaches towards stress tolerance. In a specific research in which the rice cystatin (oryzacystatin OCI) was ectopically expressed in soybean and Arabidopsis, plants presented an enhanced drought tolerance compared to WT presumably through effects on strigolactone pathways (Quain *et al*., 2014).

1.3.1. HORMONAL AND TRANSCRIPTION FACTORS CROSS-TALKING

Various TF families, such as NAC and WRKY, seem to be potentially involved in the regulation of senescence and stress response (Guo *et al*., 2004; Christiansen and Gregersen, 2014; Christiansen *et al*., 2016). Some of them were discovered during forward genetic screenings used to characterized Arabidopsis mutants with altered leaf senescence (Breeze *et al*., 2008). Some of these mutants presented alterations related to hormonal signaling (Woo *et al*., 2004; Kim, 2006). For instance, in a subtractive hybridization experiment, the *WRKY53* gene was identified and designated as a potential positive regulator of senescence, as illustrated knockout mutants with delayed senescence, whereas overexpression lines senesced prematurely (Miao *et al*., 2004). In addition, WRKY53 seemed to be possibly involved in the interaction of SA and JA signaling pathways along this process (Miao and Zentgraf, 2007).

The NAC family of TF is plant-specific, contains 117 members in Arabidopsis, 151 in rice, and 152 in both soybean and tobacco. Transcriptome analyses have associated approximately one third of Arabidopsis and many crop *NAC* genes with senescence, pointing them out as crucial regulators (Podzimska-Sroka *et al*., 2015). To date, ~50 *NAC* genes have been identified in barley (Christiansen *et al*., 2016). *HvNAC005* is associated with developmental senescence in this species, being significantly up-regulated after ABA treatment, but not during dark-induced senescence. Accordingly, RNA interference studies of wheat *Gpc-B1*, encoding the NAC protein NAM-B1, presented a significant reduction in grain zinc, iron, and protein content in parallel with a delayed leaf senescence phenotype (Uauy *et al*., 2006; Waters *et al*., 2009). In adittion, a positive regulator of senescence in rice, *OsNAP*, interfered during the grain-filling period affecting harvestable indexes (Liang *et al*., 2014). Another example implies the family or the drought-responsive element binding proteins (DREBs). *DREB2* and *DREB3* genes were overexpressed under the control of constitutive duplicated *CaMV35S* promoter and drought-inducible *ZmRAB17* promoter from maize. Transgenic barley plants carrying these constructs were more tolerant to drought without undesirable effects on plant growth and development (Mrízová *et al*., 2014). Besides, Je *et al*. (2014) demonstrated that DREB2 acts as transcriptional activator of the thermotolerance-related *cystatin 4* gene from Arabidopsis, with a concomitant and expected reduction in the associated protease activity.

In addition, the single stranded DNA binding protein (ssDNA) WHIRLY-1 seems to be a promising target in order to mitigate several stress signals. WHIRLY-1 was shown to bind to the promoter of the senescence-associated gene *HvS40*, a marker for leaf senescence in barley, which is induced both under natural and stress-induced senescence. WHIRLY-1 is expected to fulfill an important role during retrograde signaling between plastid and nucleus (Krupinska *et al*., 2014b; Foyer *et al*., 2014), which is in accordance with its first demonstrated function regarding nucleoid compaction inside the chloroplasts (Krupinska *et al*., 2013). This hypothesis was made over the fact that WHIRLY-1 is a thioredoxin target (Foyer *et al*., 2014), so it may act as a sensor of ROS alterations provoked by stress signals, which are first sensed in the chloroplasts. The WHIRLY-1 protein would change its structural conformation after

these signals, leading to a monomerization process. WHIRLY-1 monomers would reach the nucleus, where they may activate specific-stress and senescence-responsive genes, as those related with photosynthesis, possibly enabling NUE improvement in barley (Comadira *et al*., 2015).

1.3.2. REDOX REGULATORY NETWORKS

ROS conform one of the earliest responses of plant cells under abiotic and biotic stresses, and senescence. Oxygen free radicals interplay with sugars and nitrogen content, photoperiod signals, calcium cascades, and a big set of phytohormone signals acting synergistic or antagonistically to trigger and regulate leaf senescence. As chloroplasts are the main targets of ROS-linked damage during various environmental stresses and natural senescence, it is noteworthy that a well suited arsenal of antioxidant systems is contained inside this organelle (Khanna-Chopra, 2012). ROS behavior could be interpreted in two senses: as degrading harmful molecules when an excessive concentration is surpassed or as necessary signaling compounds. ROS signaling is controlled by production and scavenging, in contrast to Ca^{2+} signaling, which is based upon storage and release (Bieker *et al*., 2012). The primary targets of ROS signals are amino acids such as cysteine. ROS accumulation is also intrinsically linked to the production of NO. Redox gradient across the plasma membrane also deserves special attention regarding signaling in response to stress, since it seems to be directly involved in the regulation of specific protease-encoding genes that target proteins with carbonyl groups for degradation. Cellular redox state alterations imply a feed-forward re-amplification of the MAPK cascades, which function downstream of sensors/receptors to transform extracellular stimuli into intracellular responses, amplifying the transducing signal along the process (Munné-Bosch *et al*., 2013).

ROS are common elements in response to nutrient deficiencies. Interestingly, N deficiency promotes callose accumulation in vascular bundles and restricts the grain-filling rate in wheat coinciding with high ROS detection. A lower load of amino acids in the phloem after N deprivation increases the oxidation state in the cells promoting earlier senescence responses. As callose deposition at the neck region of

plasmodesmata determines its permeability during grain filling events, alterations in ROS metabolism will directly impact on macromolecular trafficking to the seed. The described situation is deleterious for seed quality and yield; however, it is necessary for the initiation of grain filling, since a minimum amount of ROS is required in order to activate senescence-associated remobilization of nutrients (Taylor *et al*., 2012). Drought is also considered to significantly increase the accumulation of ROS (Noctor *et al*., 2014). Likewise, ROS mediated in biotic stress responses, interacting with hormonal cascades (Kerchev *et al*., 2012). Interestingly, the progression of senescence along and throughout the plant architecture in tobacco plants also reflected ROS-related changes. Leaf interveinal and veinal regions senesced in a different manner in this species, due to a spatial heterogeneity in the accumulation of H_2O_2 and O_2^- which differentially regulated the expression of SDGs and SAGs. Authors speculated that integrity and compartmentation of the leaf are maintained until very late stages of senescence, enabling an efficient recycling (Niewiadomska *et al*., 2009).

1.4. SENESCENCE RELIES ON PROTEOLYSIS

1.4.1. GENERAL OVERVIEW OF DEGRADATION MECHANISMS IN PLANTS

Under stressful situations plants undergo changes related to their primary CH metabolism, using three main alternative substrates to obtain energy: proteins, chlorophylls and lipids (Hörtensteiner, 2013). Since plants are sessile organisms, recycling is especially important in order to face stress-induced senescence and avoid dramatic consequences over the offspring (Avila-Ospina *et al*., 2016). Plants use an escape strategy based upon a rapid degradation of source tissues and an accelerated development of sinks, therefore accelerating and ensuring seed production for next generation. Proteolysis, being the main hallmark during senescence and stress, fulfills the main goal of providing free peptides or amino acids to redistribute them within the plant. Amino acid catabolism will support the TCA cycle and will supply the mitochondrial electron transport chain (Araújo *et al*., 2011). Knowledge on which are the natural substrates for proteases is very limited due to the difficulty of its identification and the existence of functional redundancy (Avila-Ospina *et al*., 2014).

Several research teams are focusing their efforts in order to elucidate which are the key counterparts involved during senescence associated proteolysis, i.e., which are the targets for proteases (Kidrič *et al*., 2014; Tsiatsiani *et al*., 2012).

1.4.2. CHLOROPLAST DISMANTLING

Chloroplast proteins represent the main source for nutrient remobilization, especially nitrogen (Schiltz *et al*., 2004; Avila-Ospina *et al*., 2014). Most studies have been performed in this organelle rather than in the rest of cell compartments, although it was recently postulated that the mitochondria, the plasma membrane and the apoplast exert key signaling-related functions regarding nutrient remobilization during senescence (Martínez and Guiamet, 2014). Whilst chloroplast disorganizes, the photosynthetic capacity of the leaf progressively decreases (Sato *et al*., 2007; Chen *et al*., 2010). Plastid disorganization is reversible and the rest of cell organelles remain practically intact until the end of senescence (Taylor *et al*., 2012). As it is shown in Fig. 1.6, different environmental stresses may lead to different energetic cell status and this will determine the nature of degradation during senescence, presumably prevailing chloroplast, cytosolic or vacuolar proteases depending on the required mechanism (Carrión *et al*., 2014). Ubiquitin-related pathways could be only directed to specific cytosolic but not chloroplastic proteins during senescence. It was proposed that the ubiquitin-26S proteasome pathway mediates senescence-associated protein degradation through the ubiquitin N-end rule pathway, removing proteins which are mislocalized or unprocessed. At the onset of senescence, a negative regulator in the cytosol may be degraded through this system promoting a response which would trigger the dismantling of the chloroplast (Watanabe *et al*., 2009). In order to give precise information, an appropriate distinction between stromal (mainly participating in CO_2 fixation) and thylakoidal components (proteins, pigments and cofactors participating in light-harvesting processes within the photosystems and during the electron transport chain), should be established.

1.4.2.1. Stromal components

Rubisco and chloroplastic GS2 are the most studied stromal proteins recycled during senescence (Feller *et al*., 2008a; Ishida *et al*., 2008). Other enzymes, as those related to sulphur assimilation, have been poorly investigated. Most evidences established that degradation of these stromal proteins generally accounts earlier than chlorophyll and thylakoidal ones (proteins D1, LHCII of the PSII reaction center and PSII antenna; Krupinska *et al*., 2012).

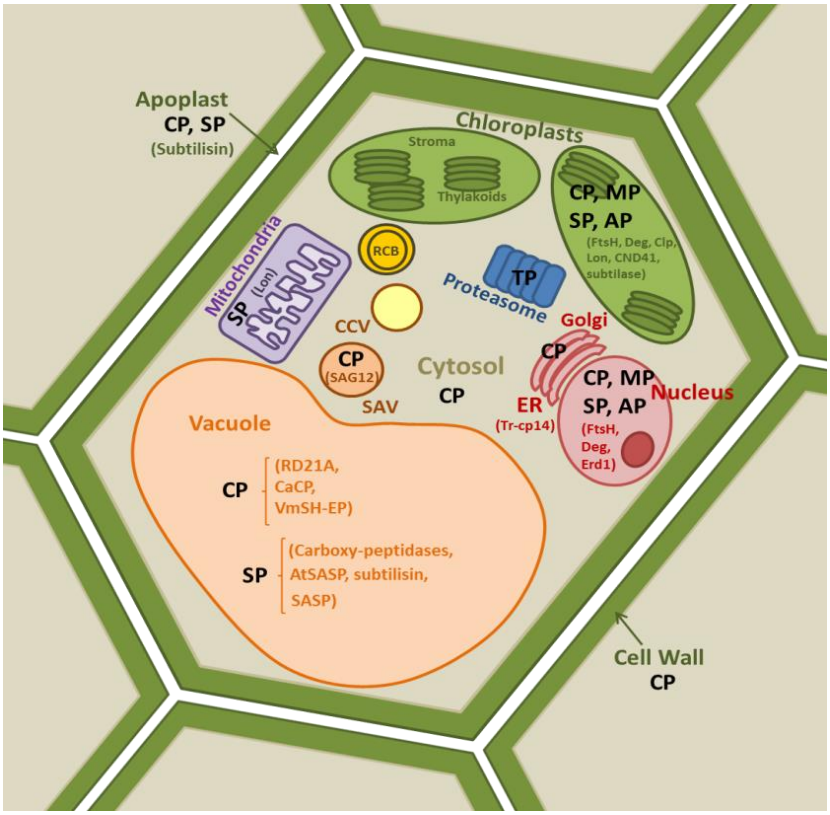


Fig. 1.6. Schematic representation of different cellular compartments: nucleus, Golgi apparatus, endoplasmic reticulum (ER), chloroplast, mitochondria, proteasome, cytosol, Rubisco containing bodies (RCB), Senescence Associated Vesicles (SAVs), Chloroplast Vesiculation-Containing Vesicles (CCVs), vacuole, apoplast and cell wall. The group of proteases identified in each compartment is shown: CysProt (CP), serine-proteases (SP), metallo-proteases (MP), threonine-proteases (TP) and aspartic-proteases (AP). From Diaz-Mendoza *et al*. (2016b).

Rubisco and Rubisco activase (the main regulator of Rubisco activity) appear to be the main targets for CysProt during foliar senescence (Prins *et al*., 2008), but Rubisco

activase is still poorly studied. In C4 plants, Rubisco, PEPC and pyruvate orthophosphate dikinase are the soluble proteins which, together, represent the same percentage as Rubisco alone in C3 plants (Feller *et al*., 2008b). For this reason, understanding the mechanisms of Rubisco degradation has become a key purpose for several research groups. Desimone *et al*. (1996) studied the nature of Rubisco degradation under oxidative stress in isolated chloroplasts of barley. Likewise, Irving and Robinson, (2006) investigated Rubisco degradation events in cereals. Through a proteomics approach, Schiltz *et al*. (2004) confirmed that Rubisco was the main source of nitrogen during seed filling in pea. Under different environmental conditions, the kinetics of Rubisco degradation may vary while comparing to other stromal and extraplastidial proteins (Feller *et al*., 2008a). Initiation of Rubisco degradation may account both near the C-terminus or the N-terminus (Thoenen *et al*., 2007). How proteolysis is promoted and initiated is being investigated. Several hypotheses stand that ROS might be involved, in terms of oxidizing certain cysteine residues, thus converting the protein in a more susceptible target for protease cleavage (Desimone *et al*., 1996), or "tagging" the protein for further degradation, provoking its denaturation through cross-linking of SH-groups (Garcia-Ferris and Moreno, 1994). It has been proposed that Rubisco fragments show an increased affinity to bind to the chloroplast external membranes (Irving and Robinson, 2006) facilitating its exit from this organelle. One of the most abundant amino acids found in the phloem of several cereal species undergoing leaf senescence is glutamine (Simpson and Dalling, 1981). The plastidial form (GS2) is quite susceptible to proteolysis as shown in isolated tobacco chloroplasts and is lost during early stages in cereal leaves, but the cytosolic (GS1), key enzyme for ammonia assimilation and *de novo* synthesis of amino acids from proteolyzed fragments, is maintained (Khanna-Chopra, 2012). Proteolysis of GS2 seems to be initiated through oxidative carbonylation of histidine residues (Ishida *et al*., 2008), although this ROS prompted degradation does not seem to be enough for complete degradation (Desimone *et al*., 1996). Some *in vitro* approaches showed that GS2 is degraded before other enzymes from carbon assimilation (e.g. Rubisco) (Thoenen *et al*., 2007). Besides Rubisco and GS2, Fischer and Feller, (1994) examined another set of plastidial enzymes by inmunoblotting in young winter leaves. Results proved that

proteolysis within the same organelle may be selectively regulated, since they observed different degradation rates.

1.4.2.2. Thylakoidal proteins

Degradation of proteins from PSII, which includes the reaction centers of photosynthesis and antenna systems, represents the second largest pool of remobilizable nitrogen from chloroplasts during leaf senescence, harboring 30% of the total chloroplast protein (Matile, 1992; Feller *et al*., 2008a). While disorganization of thylakoids proceeds and gerontoplasts develop, a proliferation of plastoglobuli is observed (Sorin *et al*., 2014). These formations contain rests of thylakoid galactolipids, probably hydrolyzed via the glyoxylate cycle, triggering signals for the rest of components ´degradation (He *et al*., 2002). Apart from membrane disassembling, the core photosynthetic proteins belonging both to PSI and PSII are also proteolyzed as observed in ultrastructural studies (Ghosh *et al*., 2001; Krupinska *et al*., 2012). The loss of cytochrome b6⁄f may precede degradation of photosystems I and II, and of ATP synthase. At early phases of senescence, the yield of PSII remains quite constant, meaning that this compartment seems to be dismantled later (Guiamet *et al*., 2002). Although consensus concerning that a certain degree of damage in thylakoids before chlorophyll degradation must exist, different theories regarding the sequence of degradation events over thylakoids have emerged. Some authors reported that PSII activity decreases earlier than that of PSI during senescence, whilst others sustained the opposite (Ghosh *et al*., 2001). A faster declined in PSII versus PSI was detected during heat-stress promoted leaf senescence in wheat (Hörtensteiner, 2013). Relative levels of some key components of PSI and PSII, such as D1 (PsbA) or Lhcb1 apoprotein, were analyzed in a high–yield variety of barley at late stages of senescence. Immunoblot and ultrastructural results illustrated a preferential degradation of grana over stromal proteins, resulting in an unexpected increase in the chlorophyll *a*/*b* ratio indicating that chlorophyll *b* is degraded faster (Krupinska *et al*., 2012). Authors speculated that this might be a means for avoiding the risk of photoinhibition by overexcitation of PSII, which is favorable to maintain ATP machinery in stroma thylakoids, to prevent overproduction of singlet oxygen and thus, to sustain higher

yields (Apel and Hirt, 2004). Although in most species grana thylakoids show higher stability compared to the stromal fraction, from these results it could be asserted that there is a wide range of variability among and within species, varieties and even different aged- chloroplasts.

A battery of studies has reported that the proteolytic machinery responsible for degradation of thylakoid proteins during senescence is within the chloroplast. It includes ATP-dependent proteases from Deg, Clp, FtsH and Lon families. FtsH proteases are the best studied metalloproteases in plants (Roberts *et al*., 2012). Leaf proteome analysis from genotypes with different NUE in oilseed rape reported that a plastidial FtsH plays a significant role in the breakdown of thylakoid proteins (Avice and Etienne, 2014). Nine members from the FtsH family in Arabidopsis contributed to plastid differentiation and repairing of the core protein of PSII, D1. As this status was also contemplated during senescence, it may be contributing to degrade this core protein (Carrión *et al*., 2014). Zelisko and Jackowski (2004) demonstrated that a similar metalloprotease was involved in the degradation of Lhcb3 in detached barley leaves induced to senesce under darkness.

1.4.2.3. Chlorophylls

The main hallmark of senescence, leaf yellowing, responds to the preferential degradation of chlorophyll over carotenoids (Matile, 1992). Chlorophyll degradation pathway owes its name to the first involved enzyme: PAO "pheophorbide *a* oxygenase". This important catalytic route is divided into two steps: formation of primary fluorescent chlorophyll-derived catabolites (pFCC), and modification followed by isomerization to render non-fluorescent catabolites (NCCs). The first phase occurs within the plastid and the second takes place in the cytosol and central vacuole (Hörtensteiner, 2013). Although chlorophylls contain much N, the 4 moles of N associated with each mole of chlorophyll are not exported from senescing leaves. It was demonstrated that colorless chlorophyll catabolites are not exported outside the cell but remains within the central vacuole in the form of linear tetrapyrrolic catabolites to further provide required by-products. A fine-tuned mechanism avoiding

accumulation of phototoxic pieces derived from chlorophyll catabolism was described within plant cells (Christ and Hörtensteiner, 2014). First studies on chlorophyll catabolism were performed with barley, and transformation of chlorophyll *b* into chlorophyll *a* before degradation was detected (Matile *et al*., 1992). Some experimental evidences have tried to correlate to some extent the involvement of proteases during chlorophyll degradation. Arabidopsis expressing *SPCP2*, encoding a sweet potato papain-like CysProt, showed that the decrease in chlorophyll in senescing leaves did not correlate with the induction of the protease, concluding that SPCP2 is not related to chlorophyll degradation initiation (Chen *et al*., 2010).

Few evidences support that SGR protein, lacking in stay-green mutants (*sgr*), has the key role of binding and destabilizing LHII during the initial steps of senescence (Thomas and Ougham, 2014). Various experimental approaches showed that SGR is acting just at the onset of senescence and it may act destabilizing the protein-chlorophyll complexes releasing apoproteins and pigments. It was postulated that SGR protein may be involved in the general process of N remobilization through the recruitment of specific proteases (Ghosh *et al*., 2001). After activation of proteins like SGR during senescence, partial destabilization of pigment-protein complexes occur inside the chloroplast. Then, some structural changes take place, which may allow the correspondent protease/s to access to their target apoprotein/s (Schelbert *et al*., 2009). In addition, Sato *et al*. (2007) suggested the implication of SGR in the activation of chlorophyll-degrading mechanisms in senescing tissues through the characterization of *sgr* mutants displaying higher stability of chlorophyll-protein complexes, but decreased levels of Rubisco than WT.

1.4.2.4. Traffic of proteolytic products

Observed mechanisms for chloroplast degradation along leaf senescence point out the existence of different proteolytic pathways, inside and outside the organelle, which may indicate the existence of redundant routes for photosynthetic protein degradation. Conversely, they may represent alternative ways of responding to different stresses and to meet variable needs, which in turn determines the

preferential degradation of one or another component in an spatial-temporal scale (Guiamet *et al*., 2002; Xie *et al.*, 2015).

Over the past years, biochemical, ultrastructural and molecular procedures have been developed in order to gain knowledge about the mechanisms for trafficking and degradation of compounds outside chloroplasts. Senescence Associated Vesicles (SAVs), Rubisco Containing Bodies (RCBs) linked to autophagy, and more recently, Chloroplast Vesiculation-Containing Vesicles (CCVs), have been proposed as the main mechanisms (Fig. 1.7). As it was demonstrated in Arabidopsis, soybean and tobacco plants, SAVs, small acidic organelles with a single membrane, only carried stromal proteins and contained most of the CysProt activity in senescing cells (Otegui *et al*., 2005; Prins *et al*., 2008; Martínez *et al*., 2008; Carrión *et al*., 2013). Thylakoid components were not detected, although a small portion of chlorophyll *a* appeared under certain conditions, which authors attributed to possible differences in pigment disassembly (Martínez *et al*., 2008). Besides, SAVs seemed independent of autophagy and they did not appear to reach the central vacuole, exhibiting an independent proteolytic activity (Xie *et al*., 2015). Data concerning SAVs formation and how the proteinaceous components enter and bind to the lumen still remain elusive. Likewise, there are few evidences regarding autophagy and RCBs formation. In this case, cytosolic components are engulfed by a specialized double-membrane structure (autophagosome) to finally attach to the vacuole and deliver carried components for recycling. Electron microscopy observations confirmed stromules protruding from chloroplasts and RCBs, which again only contained the stromal fraction, lacking visible proteolytic activity (Chiba *et al*., 2003; Ishida *et al*., 2008). Interestingly, authophagy-mediated processes are involved in the delivery of starch granules derived from the chloroplast to the vacuole during the night, a process not necessary linked to stress. Importantly, it has been postulated that autophagy mediates in nitrogen remobilization during grain filling in Arabidopsis, maize and rice. In barley, 24 autophagy-related (*ATG*) genes were identified from EST libraries (Avila-Ospina *et al*., 2016; Masclaux-Daubresse, 2016), and the expression of one of them, *HvATG5*, was much greater in the flag leaves than in seedlings, clearly indicating the key contribution of the flag leaf during nitrogen remobilization to the seeds.

Wang and Blumwald, (2014) demonstrated a third plastid degradation pathway, independent from SAVs and RCBs. The Chloroplast Vesiculation (CV) protein, firstly identified in rice (*Oryza sativa*) and with homologs in all sequenced plant species, was induced during senescence and after abiotic stress. Subsequent work on Arabidopsis showed how this protein induced the formation of CV-containing vesicles (CCVs) which contributed to plastidial degradation, both of stromal and thylakoid fractions. Authors also suggested that CCVs are formed from thylakoid membranes. Interestingly, knock-down lines for CV presented a retarded degradation of chloroplasts and an increased tolerance to stress (Wang and Blumwald, 2014). Apparently, CV seems to be unique and specific for chloroplast degradation, exhibiting more destructive effects than autophagy, which seems more related to general cellular degradation and recycling inside the cell (Xie *et al*., 2015).

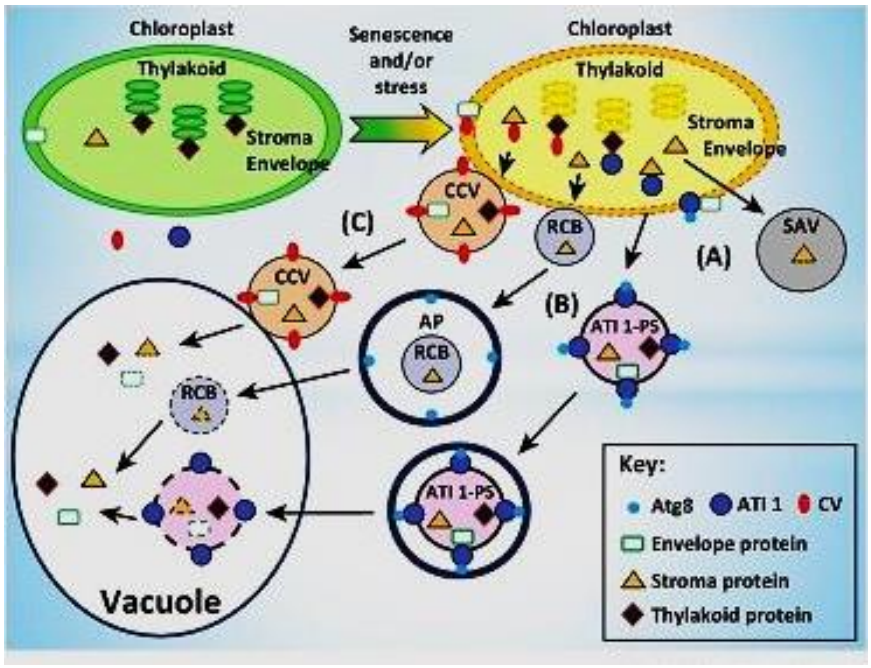


Fig. 1.7. Working model for the degradation pathways of chloroplast proteins displaying three different proteolytic routes. (A) SAVs; (B) RCBs; and (C) CCVs. What determines which degradation pathway will be activated, and whether these three pathways co-exist in the same cell at the same time still remain elusive. Dashed line indicate the breakdown of the chloroplast and the thylakoid membranes. AP, autophagosome. From Xie *et al*. (2015).

1.4.3. PLANT PROTEASES AND PROTEASE INHIBITORS

As previously stated, recycling of nutrients is undoubtedly the main goal during senescence. Thus, hydrolysis of a wide class of macromolecules from diverse organelles, most precisely protein degradation, would represent the most important process involved on it. Nearly 7% of around 2500 genes expressed in senescing leaves code for various types of hydrolases, including proteases (Gepstein, 2004). The first sort of classification for proteolytic enzymes was based on the catalytic mechanism regarding the location of the peptide bonds they cleave. Those enzymes that cleave peptide bonds within the polypeptide chain were named endopeptidases, whereas exopeptidases exert their cleavage mechanism at the termini positions. Within the last group, aminopeptidases and carboxypeptidases are further distinguished depending if they excise the peptide bonds at the N-end or at the C-end location, respectively (Kidrič *et al*., 2014). Additionally, based on the first classification proposed by Barrett, (1986), peptidases can be subdivided depending on the amino acid residue present in their catalytic site. Therefore, in accordance to the MEROPS peptidase database (http://merops.sanger.ac.uk/), current nomenclature is as follows: aspartic peptidases (A), cysteine peptidases (C), glutamic peptidases (G), metallo peptidases (M), serine peptidases (S), threonine peptidases (T) and asparagine lyases (N). The term 'peptidase' is usually applied for any proteolytic enzyme, although a few of them are not strictly hydrolases but instead lyases. In addition, those members of mixed catalytic type (P) and peptidases of unknown catalytic function (U) are also included into this classification. Finally, inhibitors for the peptidases are referred with the letter `I´ (Rawlings *et al*., 2016).

MEROPS database is a very useful resource continuously updated with information regarding proteolytic enzymes, inhibitors and, although less abundant, putative substrates. Here, the peptidases are subdivided into families and clans according to their evolutionary relationships. Families harbor homologous peptidases based on amino acid sequences similarities. A clan conforms a set of families, for which there is evidence of a common ancestry (Rawlings *et al*., 2016).

1.4.3.1. Cysteine Proteases. C1A and C13 families

According to Van der Hoorn, (2008), serine proteases represent the most abundant class in plants, followed by aspartic members. Regarding senescence, almost nearly all groups of proteases seem to intervene (Roberts *et al*., 2012), but the most frequently detected class among protease SAGs corresponds to the cysteine group (CysProt; Guo *et al*., 2004; Díaz-Mendoza *et al*., 2014; Velasco-Arroyo *et al*., 2016). Among about 800 proteases encoded by plant genomes, approximately 140 correspond to CysProt encompassing 15 families belonging to five clans. The papain-like (C1) from clan CA is the most abundant and most reported plant CysProt family. Members of the papain-like subfamily C1A are the most widely studied among plant CysProt, conforming the main group that participates along leaf senescence (Díaz-Mendoza *et al*., 2014, Velasco-Arroyo *et al*., 2016). Besides, legumains (family C13), metacaspases (family C14), calpains (family C2) and proteases related to ubiquitin-dependent pathways (families C12, C19 and C85) have also been identified as proteolytic enzymes with putative roles during this process (Martinez and Diaz, 2008; Julián *et al*., 2013).

C1A proteases were classified based on their homology to cathepsins in mammals (Martínez and Diaz, 2008; Martinez *et al*., 2012). They were grouped as cathepsins L-, B-, H- and F-like according to their gene structures and phylogenetic relationship. C1A proteases participate in protein degradation during senescence and abscission processes, programmed cell death, and accumulation and mobilization of storage proteins in seeds and tubers (van der Hoorn, 2008; Martinez *et al*., 2009; Díaz-Mendoza *et al*., 2014; Díaz-Mendoza *et al*., 2016b). Aside, C1A CysProt corresponding genes are strongly expressed in response to multiple stresses, such as darkness, drought, nutrient starvation, extreme temperatures, salt, or pest and pathogen attacks (Parrott *et al*., 2010; Guo and Gan, 2012; Diaz and Martinez, 2013; Kempema *et al*., 2015; Velasco-Arroyo *et al*., 2016). Members of this family share a highly conserved catalytic mechanism including the three amino acids Cys, His and Asn in the catalytic triad, and a Gln residue which seems to be essential for maintaining an active enzyme conformation (Fig. 1.8). Furthermore, they usually contain three disulfide bonds and their chain is folded to form a globular protein with two interacting domains delimiting

a cleft at the surface where substrates can be bound (Kidrič *et al*., 2014). This tertiary structure is similar between C1A peptidases from animal and plant origin. Pre-proteins comprise an N-terminal signal peptide with a few amino acids which acts as a tag to determine the precise location for the protein into the secretory pathway; a propeptide sequence of 130–150 amino acids; and the mature protein, which is about 200–300 residues long. In order to ensure an efficient proteolysis, both in temporal and spatial scales, CysProt are synthesized as inactive precursors. To become active, C1A CysProt need to be self-processed or hydrolyzed by other enzymes (Wiederanders *et al*., 2003; Cambra *et al*., 2012b). Propeptides contain the non-contiguous ERFNIN signature in cathepsins L- and H-like or the ERFNAQ variant in cathepsin F-like, whereas cathepsin B-like proteases lack this motif. The propeptide blocks substrate access to the active site of the enzyme, and binds in reverse orientation compared to substrate binding.

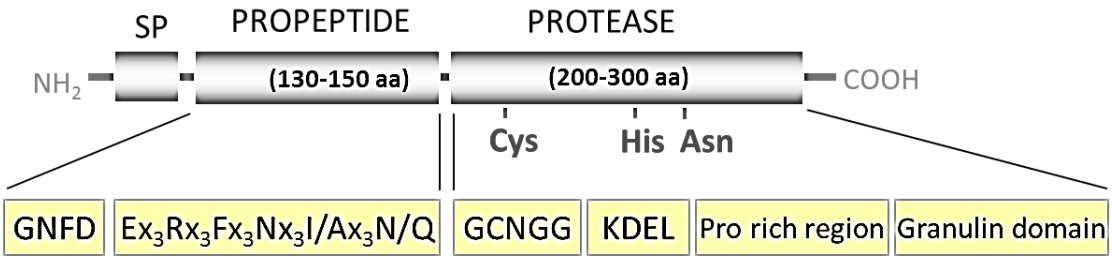


Fig. 1.8. General schematic representation for C1A CysProt. SP, Signal peptide region. In the propeptide region, C1A CysProt contain a consensus motif GxNxFxD, apparently essential for the correct processing of the peptidase precursors, and the non-contiguous ERFNIN signature found in L- and H-like cathepsins, or the ERFNAQ variant in F-like cathepsin, both of unknown function. This signature lacks in B-like cathepsins. In the mature part of the protease, the active site residues Cys, His and Asn are crucial for the catalytic mechanism. The GCNGG like-motif, common to all CysProts, and several cysteine residues, presumably involved in the formation of disulphide bridges to maintain the three-dimensional structure of the protein, are also identified. KDEL indicates the ER retention signal found in some C1A members. In some cases, a Pro-rich domain and a granulin domain are identified (Cambra *et al*., 2012a,b).

Some C1A members are synthesized with the C-terminal ER-retention signal KDEL that target these proteases to specialized lytic vesicles (Fig. 1.8). Moreover, a subclass of proteases from the papain family presents a C-terminal extension with a Pro-rich region and a granulin domain with homology to animal proteases of the

epithelin/granulin family. This extension participates in the regulation of protease solubility and in its self-activation (Yamada *et al*., 2001) .

C1A protease activity is regulated at the transcriptional and protein levels. Protease expression is mainly controlled by TF. Importantly, protein activity is regulated by binding to specific inhibitors (cystatins and propeptides), cofactors, and by means of the previously described mechanism of zymogen activation, in which pH is determinant. Complexity is additionally increased through post-transcriptional alternative splicing and differential polyadenylation (Simova-Stoilova *et al*., 2010). Lately, the C1A protease activity seems to be regulated at the post-translational level by legumains, Asn-specific CysProt involved in polypeptide processing and protein breakdown (Martínez *et al*., 2012)**.**

Legumains are CysProt belonging to the C13 family and clan CD, with increasingly recognized physiological significance in plants. Legumains are also named asparaginyl endopeptidases or vacuolar processing enzymes (VPEs), since they are vacuolar CysProt that intervene in the breakdown of peptidic bonds after asparagine or aspartic amino acid residues (Álvarez-Fernández *et al*., 1999). These proteases use a cysteine and a histidine to catalyze the enzymatic reaction. VPEs were shown to mediate in cell death in response to a variety of stress inducers and during development of different organs (Hara-Nishimura *et al*., 2005; Julián *et al*., 2013). In plants, the activity of legumains is regulated by the members of the cystatin family of peptidase inhibitors with an extension in the C-terminal part of the protein (Martinez *et al*., 2007; Cambra *et al*., 2010).

1.4.3.2. Phytocystatins

Protease inhibitors are proteins, usually small, in a range from 2-20 kDa. They can be classified according to their reaction mechanism (competitive, non-competitive, uncompetitive, suicide protease inhibitors), or to their specificity, i.e., depending if they are able to inhibit one or several classes of proteases, one specific family or even solely one protease type (Kidrič *et al*., 2014). As with their counterparts, the MEROPS

database offers a detailed and appropriate classification and is organized in a similar way to that for proteases. Currently there are 81 protease inhibitors families in the MEROPS database (release 10.0), including 22 of plant origin. Of the latter, 10 families include those isolated exclusively from plants. One of the largest families of CysProt inhibitors in plants corresponds to the plant cystatin or phytocystatin (PhyCys) family (I25). Cystatins are tightly bound and reversible inhibitors of C1A CysProt and, some members, also inhibit the C13 legumain family. Their inhibitory mechanism is characterized by a tight and reversible interaction with their target, which involves a tripartite wedge formed by the partially flexible N-terminus containing a Gly residue and two hairpin loops carrying, respectively, a conserved Gln-X-Val-X-Gly motif (QXVXG, where X is any amino acid) and a Trp residue (Martínez and Díaz, 2008; Martínez *et al*., 2009). Some PhyCys have a molecular mass around 23 kDa, due to the C-terminal extension with a conserved Ser-Asn-Ser-Leu motif (SNSL), responsible for their capability for inhibiting the legumain family. The asparagine of the SNSL motif is essential in this inhibition (Martínez *et al*., 2007). Additionally, PhyCys of 85-87 kDa, designed as multicystatins, which contain several cystatin domains, have also been characterized (Nissen *et al*., 2009). Besides, most PhyCys have a signal peptide suggesting a non-cytosolic location (Martinez and Diaz, 2008).

Like their CysProt targets, endogenous PhyCys are regulated by internal and environmental cues. PhyCys have been repeatedly implicated in the control of plant development and defense due to their action over endogenous and heterologous CysProt (Neuteboom *et al*., 2009). They have an important role by tuning endogenous proteolytic activities during seed maturation and germination (Hwang *et al*., 2010; Cambra *et al*., 2012b; Diaz-Mendoza *et al*., 2016a), PCD events (Belenghi *et al*., 2003), fruit ripening (Neuteboom *et al*., 2009), and they also regulate plant defense by controlling the activities of CysProt from pests and pathogens (Martinez *et al*., 2009; Martínez *et al*., 2012, 2016). Lately, PhyCys involvement regarding abiotic stress tolerance and/or crop quality amelioration represent research topics with a rising attention (Je *et al*., 2014; Quain *et al*., 2014).

1.4.3.3. C1A papain-like cysteine proteases and their phytocystatins counterparts in senescence

Among all plant biological functions in which C1A papain-like CysProt and PhyCys participate, those related to senescence, nutrient recycling and stress tolerance towards abiotic stresses perhaps belong to the less studied area. Nonetheless, in the last decades an increasing number of research groups are currently dealing with these topics. Proteases are highly abundant during senescence, both under natural or induced conditions. Roberts *et al*. (2012) proposed a classification based on the expression profile along the time-course for leaf senescence. The authors drew a subdivision taking into account the putative and variable biological roles: Class 1 of senescence-associated proteases comprises those expressed both in green (non-senescent) and senescent tissues, with barely invariable levels. They seem to be involved in housekeeping functions in order to maintain cell viability. Class 2 includes proteases which show low basal levels in green tissues and are rapidly induced soon after senescence, probably required for bulk proteolysis. Class 3 clusters members which are predicted to fulfill significant roles in the late stages of senescence and cell death, being specifically synthesized *de novo* and exclusively in senescent tissues. Class 4 comprises proteases which seem to be involved in the early breakdown of chloroplastic proteins, and which are transiently expressed during senescence. Finally, proteases which are inversely associated with senescence can be grouped inside Class 5 (Fig. 1.9).

During natural and induced-senescence, CysProt members consistently represent the most up-regulated group of proteolytic enzymes (Bhalerao *et al*., 2003, Guo *et al*., 2004). For instance, Drake *et al*. (1996) already isolated and analyzed two cDNAs encoding tomato CysProt (SENU2 and SENU3) expressed during the later stages of visible leaf senescence, as well as during seed germination, indicating a possible role in protein turnover. The sweet potato (*Ipomoea batatas*) senescence-associated papain-like CysProt SPCP2 and SPCP3 presented an enhanced accumulation both under natural and induced senescence, as demonstrated when ectopically expressed in Arabidopsis, causing increased sensitivity to drought and salt stresses (Chen *et al*.,

2013). Virus-induced gene-silencing of *CaCP* from pepper (*Capsicum annuum* L.) provoked an enhanced tolerance to salt- and osmotic-induced stress, apart from its participation during developmental senescence (Xiao *et al.*, 2014). In contrast, in tobacco NtCP1 seemed to be related to senescence, but NtCP2 was only relevant in young leaves, with a possible role more linked to PCD, as it was proposed for similar KDEL-tailed CysProt (Beyene *et al*., 2006). Another CysProt expressed in Arabidopsis leaves under water-stress or/and senescence was RD21, which contains a C-terminal granuline extension. After its accumulation in the vacuoles in an aggregated form, it slowly renders a soluble protease by removing the granuline domain during leaf senescence (Yamada *et al*., 2001).

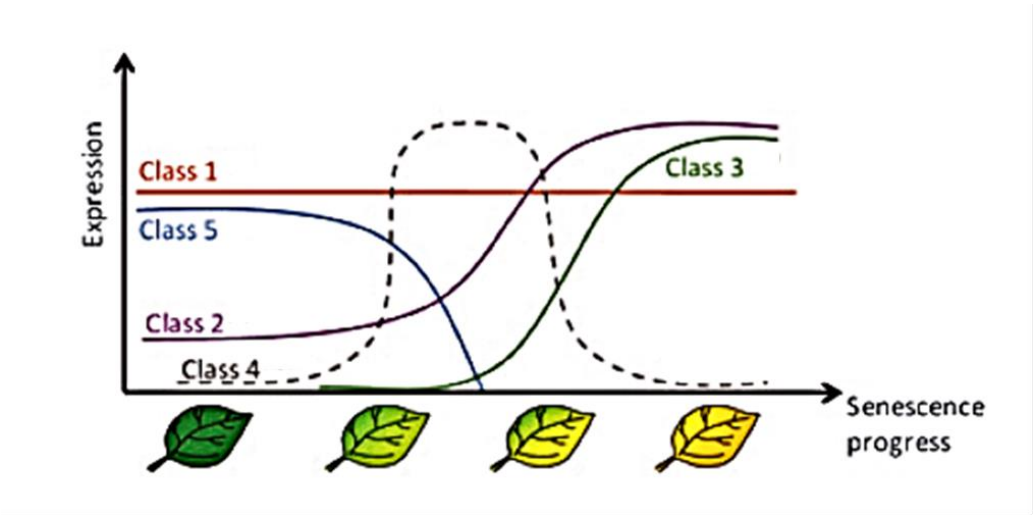


Fig. 1.9. Schematic representation of the protease classes participating along leaf senescence. Subdivision takes into account the putative and variable biological roles and the time-course. Adapted from Roberts *et al*. (2012).

In wheat, the flag leaf starts to senesce during post-anthesis, when the grain filling has already begun and nutrient recycling is required from source leaves (Krupinska and Humbeck, 2004). D.E. Martinez *et al*. (2007) showed that vacuolar CysProt of wheat were common in attached flag leaves senescing naturally during post-anthesis and also after stress. Thoenen *et al*. (2007) demonstrated that a wheat cysteine endopeptidase was involved in the degradation of the large subunit of Rubisco although the results indicated that depending on the senescence-inducing conditions, different proteolytic enzymes may be involved. Changes on leaf protein

content and Rubisco turnover due to the expression of the rice cystatin OC-I in tobacco were translated in decreased CysProt associated activities and higher Rubisco contents, clearly involving CysProt in its degradation in leaves under optimal and stress conditions (Prins *et al*., 2008). Besides, papain-like CysProt may regulate related partners along senescence, as observed in the Arabidopsis Cathepsin B group. Triple mutants for the three *AtCATHB* isoforms resulted in retarded senescence and a seven-fold decrease in theaccumulation of the senescence marker gene *SAG12*, which seemed to be downstream of AtCATHB members during this process (McLellan *et al*., 2009). Knowledge about senescence-associated CysProt has also been applied looking at postharvest amelioration of leafy vegetables. Thus, using the senescence-specific SAG12 promoter from Arabidopsis, the expression of the *ipt* gene was monitored during development and postharvest in lettuce, resulting in a delayed senescence in mature heads concomitant with higher concentrations of CK and hexoses in the older leaves (McCabe *et al*., 2001). Similarly, this construct was used to transform the miniature potted rose (*Rosa hybrida* cv. Linda), where it resulted in a reduced sensitivity to ethylene and a delayed senescence phenotype (Zakizadeh *et al*., 2013). Members of the legumain family have also been reported to mediate in the activation of downstream proteases involved in amino acids recycling during senescence (Rojo *et al*., 2003). Likewise, papain-like CysProt are processed by legumains in order to degrade reserve proteins in *Vigna mungo* and *O. sativa* (Kato *et al*., 2005).

A wealth of evidence also suggests that serine and aspartic proteases play key roles during senescence and N remobilization. Serine proteases were detected as the predominant type in wheat during monocarpic senescence both during heat stress and under natural conditions (Chauhan *et al*., 2009). In addition, silencing of the chloroplast aspartic protease CND41 from tobacco leaves resulted in delayed senescence and accumulation of Rubisco in the oldest leaves, suggesting a failure in N remobilization. Conversely, its overexpression led to accelerated senescence and increased Rubisco degradation (Kato *et al*., 2005).

Biotechnological approaches based on PhyCys have been mostly directed to hamper insect and acari feeding (Atkinson *et al*., 2004; Carrillo *et al*., 2011a) and to

achieve improved antiphatogenic effects (Carrillo *et al*. 2011a,b; Martinez *et al*., 2016). Besides, PhyCys-based strategies have been designed to reach better yields of bio-engineered proteins such as vaccines and metabolic enzymes (Pillay *et al*., 2012). Apart from the well-known defense function, the other key role of PhyCys as plant regulators of endogenous protein turnover has also been documented for diverse plant physiological processes (Belenghi *et al*., 2003; Benchabane *et al*., 2010; Diaz-Mendoza *et al*., 2016a,b). In contrast, studies regarding acquisition of resistance or tolerance against some particular negative abiotic stimulus, as well as research linked to seed quality or yield, are more scarce (Quain *et al*., 2014).

In order to ameliorate resilience and quality in the face of climate change, it is paramount to identify specific PhyCys members differentially expressed under various abiotic stresses. In addition, it is crucial to understand how PhyCys and their targets CysProt maintain an equilibrium which allows a recovery after a given stress, instead of an excessive proteolysis which would result in irreversible PCD (Fig. 1.10; Kunert *et al*., 2015).

Overexpression of several PhyCys genes has been the strategy to investigate the mechanisms behind the increasing tolerance to cold, drought, oxidation, high salinity, wounding or heat stress in several species (Van der Vyver *et al*., 2003; Hwang *et al*., 2010). The up-regulation of transcript levels for several CysProt in response to high temperatures led to hypothesize that PhyCys and CysProt may be counterparts in the specific response to heat stress (Huang and Xu, 2008). To highlight, the study performed by Je *et al*. (2014) demonstrated that the *cystatin 4* gene from Arabidopsis mediated in thermo-tolerance processes under the control of the DREB2C cascade. Transformed tobacco plants expressing OCI showed more resistance towards the negative impacts of chilling episodes (Van der Vyver *et al*., 2003). Its ectopic expression in tobacco, soybean and Arabidopsis presumed a protective role towards the photosynthetic machinery during both natural and stress-induced senescence (Prins *et al*., 2008; Quain *et al*., 2014). Eason *et al*. (2014) characterized the role of a protease inhibitor (BoCPI-1) in broccoli (*Brassica oleracea* var. italic) during the regulation of protease activity along postharvest senescence. In wheat, varying effects on the

transcript levels for several plant inhibitors and some CysProt were observed when comparing roots and leaves of water deprived plants with different tolerance to drought (Vaseva *et al*., 2014). All reported effects seem to be settled on the precise inhibition mechanisms of the PhyCys against their specific targets, the CysProt.

From previously remarked data and based on formerly information (Martinez and Diaz, 2008), it cannot be elusive that an important coevolution of plant proteases, specifically C1A CysProt, and PhyCys, appears critical for many plant physiological events. Despite current evidences indicating that upon abiotic stresses plant cells perform dramatic restructuring with parallel and striking alterations on CysProt and PhyCys levels, the precise interactions and regulatory networks underpinning these crucial modifications are still poorly understood.

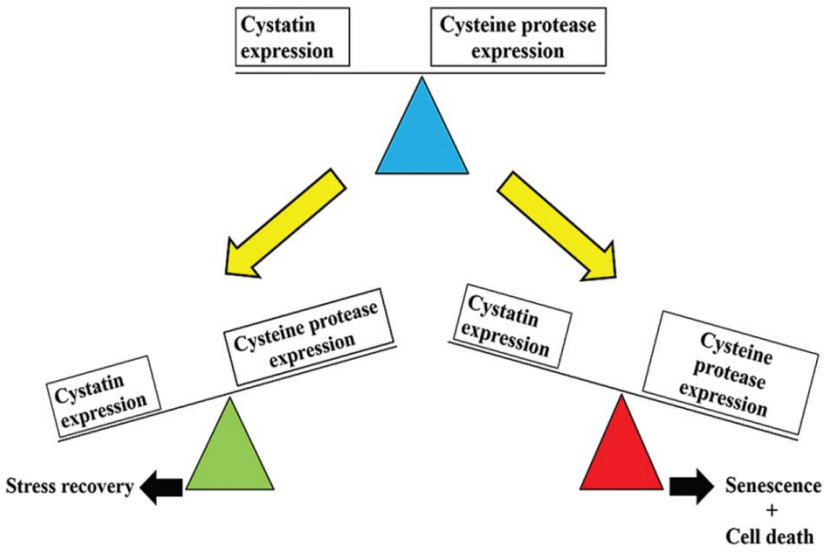


Fig. 1.10. Outline representing a balance which indicates the prevalence of PhyCys or CysProt expression after stress perception, leading to recovery if these inhibitors are restricting proteolysis or, conversely, inducing senescence and ultimately cell death if characteristic degradation and recycling are activated. From Kunert *et al.* (2015).

The Protein Data Bank (PDB, rcsb.org; Berman *et al*., 2000) contains only a few examples from these interactions due to the difficulties of isolating and solving complex structures. The structure of the tarocystatin–papain complex from the tropical species taro (*Colocasia esculenta)* was resolved by Chu *et al*. (2011). Results

indicated a similar binding mode to inhibit CysProt activity as that observed for the human cystatins. Conversely, many individual structures for both proteases and cystatins have been released (Diaz and Martinez, 2013). Since 1968, date in which the three-dimensional structure of papain was determined by X-ray diffraction at 2.8 ˚A resolution (Drenth *et al*., 1986), the crystal structure of several plant C1A CysProt has been resolved (e.g. barley EPB2; Bethune *et al*., 2006). In the case of cystatins, the nuclear magnetic resonance (NMR) structure of OC-I was the first available (Nagata *et al*., 2000), and more recently, a crystal structure from potato multicystatin 2 (PMC2) was resolved (Nissen *et al*., 2009). As these physical *in vitro* interactions are usually difficult to achieve, *in silico* data may facilitate the elucidation of putative functions underlying proteolysis-related members. Homology modeling has been extensively used as the approach for predicting protein complex structures. For instance, the interaction between the cathepsin B (HvPap-19) and the cystatin 6 (HvCPI-6) from barley was suggested through this docking technique. Besides, this protein–protein interaction was further evidenced *in vivo* through a Bimolecular Fluorescence Complementation (BiFC) assay (Martinez *et al*., 2009). The formation of a CysProt-cystatin complex was reported in senescent spinach leaves using several molecular and biochemical assays (Tajima *et al*., 2011). More recently, Vorster *et al*. (2013) reviewed the involvement of the CysProt-cystatin system in another key process, Rhizobia-mediated N_2 fixation, during nodule development and senescence in legumes.

1.5. BARLEY AS A MODEL SPECIES FOR THE POACEAE FAMILY

1.5.1. BARLEY AS AN ECONOMIC, GENETIC AND CLIMATE-CHANGE ADAPTABLE RESOURCE

Domesticated barley (*Hordeum vulgare* L.) belongs to the monocotyledonous family of Poaceae which embraces a great number of agriculturally important species such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.). It derives from its wild antecessor *H. vulgare* spp *spontaneum*, being among the world's earliest domesticated crop plants. Archaeological evidence indicates that barley and wheat were domesticated 10,000 years ago in the Fertile Crescent (Schulte *et al*., 2009;

Mrízová *et al*., 2014). Cultivated barley represents the fourth most important cereal grain in both area and tonnage harvested (FAO statistics; http://faostat.fao.org, 2015), ranking after maize, rice and wheat. Barley is an inbreeding crop showing a wide genetic variability which has rendered cultivars particularly tolerant to cold, salinity, drought and alkaline soils. Hence, it represents an economically important species that can be cultivated in diverse environments ranging from boreal to equatorial regions, and evenly in artificially irrigated fields in Sub-Saharan Africa (Mrízová *et al*., 2014). Due to its early maturation stage feature and its paramount importance for the malting and brewing industry, the barley grain is one of the best-studied systems within cereal crops and it can be regarded as a general model for Poaceae seed development and germination processes (Sreenivasulu *et al*., 2008; Schulte *et al*., 2009). Approximately three-quarters of the barley global production are destined to animal forage, around 20% is malted for use in alcoholic and non-alcoholic beverages, and the remaining 5% is used for human feed. Although the human diet is not a primary use, barley grain is particularly enriched in soluble dietary fiber, offering a wealth of potential health benefits, and still representing the major calorie source in several parts of the world (Schulte *et al*., 2009). In addition, barley grain has been successfully used as a bioreactor for the production of therapeutic proteins for the past few years (Mrízová *et al*., 2014). Finally, it should be considered the potential exploitation of barley straw for the production of second and third generation biofuels, especially bioethanol, due to its cell-wall polysaccharides enrichment (Li *et al*., 2011).

As aforementioned, barley is an excellent model species for genetic research within the Poaceae family. Golden Promise is a two-rowed spring barley malting cultivar that is routinely used in biotechnological applications as it is amenable to transformation, currently representing the most responsive genotype in tissue culture (Møller *et al*., 2011). Improvements in transformation efficiency, reduction of time required for preparation of stable transgenic lines, extensive barley germplasm and mutant collections and, most importantly, availability of its sequenced genome (International Barley Genome Sequencing Consortium, 2012), support this relevance. Barley is a diploid species with a large haploid genome of 5.1 gigabases (Gb) distributed within 7 chromosomes (2n = 2x = 14; Mayer *et al*., 2012). Knowledge of the

full genome sequence is essential for understanding natural genetic variation and development of modern strategies for breeding programs. Although its functional annotation is still incomplete, the gene space provides a new molecular and cellular insight into the biology of the species, providing a platform to advance gene discovery and genome-assisted crop improvement in association with comparative sequence and transcriptome data (Mayer *et al*., 2012). A systematic synteny analysis with model species from the Poaceae family with already annotated genomes (rice, maize, sorghum and Brachypodium) confirmed the existence of over 30,000 barley genes, mainly located to specific chromosomal loci. It is assumed that full annotation of the barley genome will appear soon, as more than 90% of predicted gene structures are currently available on public databases (http:// webblast.ipk-gatersleben.de/barley/index.php). Since 2003, Affymetrix Inc. provides a GeneChip Barley Genome Array containing more than 22,000 unique probes designed on the basis of EST libraries (Close *et al*., 2004). The technology so far allowed over sixty whole-transcriptome comparative studies focused on malting properties, pest and disease control, abiotic stress tolerance, nutritional characteristics, and reproductive development (http://www.plexdb.org). Abundant alternative splicing, premature termination codons and novel transcriptionally active regions suggest that post-transcriptional processing represents an important regulatory layer (Mayer *et al*., 2012).

Given the mentioned importance of chloroplast organelles in cereals, the existence of detailed information concerning its genome would be very informative. An important study carried out by Zhelyazkova *et al*. (2012) released key data about primary transcriptome of the barley chloroplast genome, exhibiting many more promoters than genes and unveiling numerous noncoding RNA candidates. Very interestingly, a barley whole exome capture was also performed (Mascher *et al*., 2013). Authors proposed that targeted sequencing of the mRNA-coding exome reduces barley genomic complexity more than 50-fold. They reported the implementation and evaluation of a whole exome capture assay for cultivated barley, demonstrating its applicability to genome-wide variant discovery in the genus *Hordeum* and beyond.

Barley is an excellent model for understanding agricultural responses to climate change (Dawson *et al*., 2015). For example, under elevated CO_2, the carbon and energy supplies are usually higher, which could facilitate the energetically expensive salt tolerance mechanisms present in some crops. In this context, *H. vulgare* seedlings adapt to hypersalinity in soil by actively increasing solute concentration and cell wall elasticity. Both processes improved plant water uptake and leaf turgor maintenance leading barley species to succeed in salinized areas in which growth is not currently possible (Pérez-López *et al*., 2010)**.** In addition, elevated CO_2 also reduced negative drought effects on nitrogen metabolism in barley plants, as stated during drought and recovery experiments (Robredo *et al*., 2011). In this case, high levels of CO_2 would enhance nitrate reduction, and this enhancement would be mediated by the stimulation of both synthesis and activity of Nitrate Reductase (NR). Likewise, GS activity raised in the study, leading to an increase in the amount of ammonia assimilated and in the protein content. In conclusion, elevated CO_2 seems to palliate drought effects on nitrogen metabolism by improvement of photosynthesis and mitigation of water deficit, although other factors could be involved.

Large collections of barley germplasm containing geographically diverse elite varieties, landraces and wild accessions contain alleles that could ameliorate the effects of climate change (Mayer *et al*., 2012). In a review assessing barley's resilience as a crop, Newton *et al*. (2011) described many of the genes that may be involved in responding to important abiotic and biotic stresses in a climate change framework. Recently initiated international projects such as WHEALBI (www.whealbi.eu; 2014) are using exome capture tools to characterize carefully chosen panels of barley and wheat seeking for climate-related adaptive traits. In the transnational project CLIMBAR (http://plen.ku.dk; 2014), additional questions linking climate change and epigenetic memory are addressed. It is known, for example, that epigenetic modifications are responsive to drought and act in barley seeds (Kapazoglou *et al*., 2013). The particular goal for this project consists on determining if epigenetic information modulated within precise climate scenarios for different parts of Europe by 2070 may be transmitted to the next generation in barley plants.

1.5.2. PROTEASES AND CYSTATINS INVOLVED IN BARLEY GERMINATION, SENESCENCE AND STRESS

In barley, 41 papain-like CysProt (C1A), 8 legumain-like CysProt (C13) and 13 cystatins have been described (Martinez and Diaz, 2008; Julián *et al*., 2013; Díaz-Mendoza *et al*., 2014). The functional characterization of an important battery of these members has been performed in order to expand knowledge on different physiological processes (Martinez *et al*., 2009; Cambra *et al*., 2012; Julián *et al*., 2013; Díaz-Mendoza *et al*., 2014; Diaz-Mendoza *et al*., 2016a; Velasco-Arroyo *et al*., 2016). The classification for the whole C1A CysProt family in this species, based on an evolutionary comparative genomic analysis, rendered four groups according to gene structures and phylogenetic relationships: 34 cathepsin L-, 3 cathepsin B-, 3 cathepsin F- and 1 cathepsin H- like C1A proteases. The largest group, composed by cathepsin L-like members, is further subdivided into five subgroups (Martinez and Diaz, 2008). On the other hand, the entire cystatin gene family from barley, which contains 13 non redundant genes, was identified and characterized along with their target enzymes, the barley cathepsin L-like proteases (Martinez *et al*., 2009). The structural and functional diversity within the cystatin family in barley was likewise previously studied in depth by Abraham *et al*. (2006).

Cereal grains, crucial on human and livestock nutrition, largely accumulate starch, proteins and lipids. These compounds are used during the germination process, a period that is crucial for the survival of the seedling until photosynthesis is fully established. During grain development and maturation, proteins involved in germination are stored in the endosperm together with starch and lipids. Storage, structural, metabolic and protective proteins are present in the grains. Storage proteins represent nearly 80% of these components and fall into three different fractions: albumins, globulins and prolamins, these last being the most abundant, and named hordeins in barley (Shewry and Halford, 2002). There are three groups of hordeins: B (sulfur-rich), C (sulfur-poor), and D (high Mr), with several subgroups within the B-group (Shewry *et al*., 1995). Hordeins are coordinately expressed during endosperm development and their expression is tightly regulated (Diaz *et al*., 2005;

Rubio-Somoza *et al*., 2006; Moreno-Risueño *et al*., 2007). Hydrolysis of these reserves is consequently crucial, being accomplished by several enzymes, such as amylases and proteases, which are either stored during grain maturation or newly synthesized during early germination. The products of this degradation are absorbed by the scutellum to nurture the developing embryo and favour seedling establishment. Limited proteolysis mediated by peptidases is essential for initiation of storage protein breakdown (Müntz, 1996). C1A CysProt conform one of the most abundant group of proteases participating along these processes. Their role during germination has been reported in a wide range of both monocot and dicot species (Grudkowska and Zagdanska, 2004; Tan-Wilson and Wilson, 2012). Some of the barley C1A proteases expressed in grain tissues have been characterized. Jacobsen *et al*. (1970) performed the initial research relating C1A CysProt and germination processes in barley. Zhang and Jones, (1995) reported that 27 C1A CysProt are among the 42 proteases involved in the germination of barley grain. In this species, several cathepsin L-like proteases of the scutellar epithelium and the aleurone layer secreted to the endosperm upon germination in response to GA were identified (Mikkonen *et al*., 1996; Martinez *et al*., 2009). A cathepsin H-like protease isolated from GA-induced aleurone cells was targeted to vacuoles, and a cathepsin B-like protein was expressed in this tissue also after treatment with GA (Holwerda and Rogers, 1992; Martínez *et al*., 2003). Interestingly, Sreenivasulu *et al*. (2008) developed a detailed transcriptomic analysis regarding barley grain maturation, desiccation and germination, in two distinct tissue fractions (starchy endosperm/aleurone and embryo/scutellum). In this study, several C1A CysProt were highly expressed. Among them, a protein (probe Contig2402_s_at) belonging to the cathepsin F-like group, which had not been previously characterized in the barley grain, and that corresponded to the HvPap-1 protein identified in barley by Martinez and Diaz, (2008). This F-like peptidase was modulated by its own propeptide and its inhibitors, the cystatins, was able to *in vitro* degrade different substrates, including barley endosperm proteins (hordeins, albumins, and globulins), was localized in protein bodies and vesicles of the embryo, and was induced by GA in aleurone cells (Cambra *et al*., 2012a,b). Based on these results and considering recent data (Diaz-Mendoza *et al*., 2016a), it can be cocluded that this protease fulfills a key role in the mobilization of storage proteins, mainly hordeins, during germination,

probably together with other cysteine and serine proteases. Functional interactions between cystatins and cathepsin L-like proteases were inferred from their common implications as counterparts during mobilization of storage proteins upon barley seed germination (Martinez *et al*., 2009; Cambra *et al*., 2012b) Cystatins have been extensively related to the regulation of physiological processes in seeds (Benchabane *et al*., 2010). The barley cystatin HvCPI-2 encoded by the *Icy-2* gene was a good inhibitor of different barley cathepsin L- and F-like CysProt. It was strongly expressed in the germinating embryo and repressed by GA in aleurone layers, suggesting a key role for HvCPI-2 in the regulation of the CysProt activity in the barley grain (Martinez *et al*., 2009; Cambra *et al*., 2012b).

Related to legumains, an in-depth molecular and functional characterization of the whole family from barley was performed by Julián *et al*. (2013). The study revealed a multifunctional role within the group, since there was a wide variability in expression depending upon the biotic/abiotic stimulus applied. Strikingly, results evidenced that these proteases presented caspase-like activity, and that one specific member, HvLeg-2, was able to degrade storaged seed globulins. Besides, the ability of the cystatin HvCPI-4, the only member with a long C-terminal extension conferring additional capacity to inhibit both CysProt from C1A and C13 families, was tested. Results indicated that HvCPI-4 modulated the proteolytic action of the HvLeg-2 *in vitro*.

Regarding senescence and barley, several investigations have shed light about particular features on this topic, some of them through multiple–omics technologies. In 2005, Parrott *et al*. analyzed the activities and transcript levels for several proteolytic enzymes from putative plastidial, cytosolic and vacuolar origin, detecting a clear induction after the exposure to elevated carbon levels in stem girdled leaves. In 2010, the same group performed a similar experiment inducing high leaf C/N, which is known to accelerate senescence, in order to analyze the specific behavior of the family C1A, resulting in a clear overexpression of several members in the treated samples. Other reports contain information about individual members. This is the case for the papain-like HvCP3, which was highly induced in green leaves of barley, but it seemed not to be involved during recycling as it drastically decreased along natural senescence

(Watanabe *et al*., 2009). Authors postulated a possible maintainer role in cytosol for this protease.

Senescence of barley leaves has been studied under field or altered conditions, and many experiments have been developed using the flag leaf, known as a key source of nutrients during proteolysis leading to seed filling. A cDNA library screening obtained from naturally and dark-induced senescent barley leaves, permitted the isolation of three cDNA clones. *HvSF6* and *HvSF42* transcripts accumulated in both experimental settings, while the transcripts of *HvSF2* did it exclusively during natural senescence (Scharrenberg *et al*., 2003). The sequence of the cDNA clone *HvSF42* corresponds to the papain-like CysProt HvPap-1 (Martinez and Diaz, 2008). The transcriptomes of barley flag leaves collected from field plots supplied with two N regimes (standard and high), were analyzed with the purpose of identifying genes specifically associated with nitrogen remobilization during leaf senescence (Hollmann *et al*., 2014). The strongly induced levels of expression observed for a NAC TF, a serine protease and various autophagy-related genes in those samples collected from standard N regimes, led to hypothesize that the corresponding proteins fulfill important roles during nitrogen remobilization under natural conditions. In comparison, those genes upregulated in both regimes would possibly be implicated in general senescence processes associated with late leaf development. Among them, the papain-like CysProt HvPAP-14 and HvPAP-20 were detected, as well as the serine protease SCPL51. qRT-PCR confirmed a higher upregulation of HvPAP-20 under high nitrogen supply, in contrast to the pattern observed for SCPL51. The serine protease was previously shown to be upregulated in germinating barley seeds (Druka *et al*., 2006; Sreenivasulu *et al*., 2008), and after anthesis in leaves of a high-grain-protein variety compared with the leaves of a low- grain-protein variety (Jukanti *et al*., 2008). Results are in accordance with the idea that SCPL51 is involved in nitrogen remobilization, an event likewise essential for two intimately related processes, senescence and germination (Schaller, 2004). In addition, HvPAP-20 encodes a cathepsin B-like CysProt (Martínez and Diaz, 2008), also known to be upregulated during barley germination (Druka *et al*., 2006; Sreenivasulu *et al*., 2008).

Nitrogen remobilization during senescence, especially in cereals like barley and wheat, has captured the attention of numerous multidisciplinary groups (Gregersen *et al*., 2008; Distelfeld *et al*., 2014). Apart from those reports researching over specific genes, such as those encoding proteases and cystatins, other papers use a wide omics approach to identify abiotic stress-responsive genes (reviewed in Gürel *et al*., 2016). As an example, the responses of barley root and shoot proteomes to long-term nitrogen deficiency and short-term nitrogen and ammonium starvation were investigated to provide insights into mechanisms of N uptake and assimilation (Møller *et al*., 2011). A report giving the first characterization of 24 *HvATG* genes from barley was recently released, providing molecular data to further understand regulation of the autophagy machinery during natural and dark-induced leaf senescence and in response to nitrogen deficiency (Avila-Ospina *et al*., 2016). Interestingly, the expression level for one specific *HvATG* gene was much more perceptible in the flag leaf than in the seedlings, highlighting again the importance of this organ during recycling and grain filling along senescence. An important set of studies are likewise focused on transcription factors related to senescence and stress events in barley. WHIRLY1 (Foyer *et al*., 2014) functions in the control of responses to nitrogen deficiency but not to aphid (*Myzus persicae*) infestation (Comadira *et al*., 2015). This protein is known to bind to the promoter of the senescence-associated gene *HvS40*, marker for natural and stress-induced senescence (Krupinska *et al*., 2014a). Furthermore, in a recent study focused on the barley transcription factor HvNAC005, Christiansen *et al*. (2016) demonstrated that it is associated with developmental but not with dark-induced senescence, as do another barley NAC members and a papain-like CysProt. HvNAC005 is significantly up-regulated after ABA treatment, as supported by ABA-responsive elements in its promoter. Data provided support that HvNAC005 represents a strong positive regulator of senescence in barley, working in the complex crosstalk with hormonal and specific signaling pathways and representing another possible target for optimizing nutrient remobilization in cereals.

There exists a wide diversity of cultivars among cereals species, as a consequence of the great effort made though classical breeding in order to obtain varieties with enhanced yields. According with this statement, several research groups

have developed comparative studies focused on structural properties of the photosynthetic machineries, i.e., chloroplasts, through detailed microscopy observations. Very interestingly, in the high-yield barley variety Lomerit, a preferential degradation of grana thylakoids was detected (Krupinska *et al*., 2012), in contrast to the usual pattern observed in other cultivars (Scharrenberg *et al*., 2003). This is a favorable alternative strategy of chloroplast dismantling which would be indeed favorable for the plant's photosynthetic performance to maintain stroma thylakoids with the ATP synthesis machinery, leading to increased and stable harvest indexes (Krupinska *et al*., 2012).

There is an increasing prevalence of studies focused on drought/senescence effects using this model cereal, mainly relying on metabolomics and proteomics data. Drought episodes during juvenile stages of crop development led to premature leaf senescence, negatively impacting on biomass production. Thus, the expression profiling of genes involved in drought and leaf senescence in juvenile barley was investigated in a genome wide association study through an expression quantitative trait loci (eQTL) approach for an important set of barley cultivars (Wehner *et al*., 2016). Significant correlations were detected within the group of genes involved in drought stress and those intervening in leaf senescence. Released data may help to develop markers for future barley breeding programs in order to ameliorate tolerance to drought in this and related crops. In other research study, proteomic and metabolomic changes in leaves and roots of two barley genotypes, with contrasting drought tolerance, were evaluated. Several of the identified proteins and metabolites whose accumulation levels increased under drought in the susceptible variety presented an elevated constitutive accumulation in the drought-resistant line (Chmielewska *et al*., 2016). A whole transcriptome response, both for aerial and root tissues, was deeply analyzed under severe drought and subsequent re-watering in WT barley plants during stem elongation (Kokáš *et al*., 2016). In the companion paper (Vojta *et al*., 2016), information was expanded and interesting details arose. Through an RNA-seq study authors inspected all processes related with CK metabolism. The introduction of one CK-degrading enzyme into the barley genome under the control of a mild promoter resulted in a CK-insensitive phenotype, which enabled plants to regenerate better after

a water deficit episode. Leaf revitalization was accompanied by up-regulation of photosynthetic genes, mainly those encoded by the chloroplast genome, translated into a greater regeneration of transgenic plants and higher biomass accumulation. Interestingly, up-regulation of four aquaporin genes was likewise detected in all transgenic genotypes, perhaps allowing the faster recovery in comparison to WT plants (Vojta *et al*., 2016).

Suming up, the broad number of genetic tools and the great amount of knowledge gathered on barley physiology make this crop a model for dissecting the role of proteases and their inhibitors under natural and stress conditions in order to design biotechnological strategies leading to improve stress tolerance in crops.

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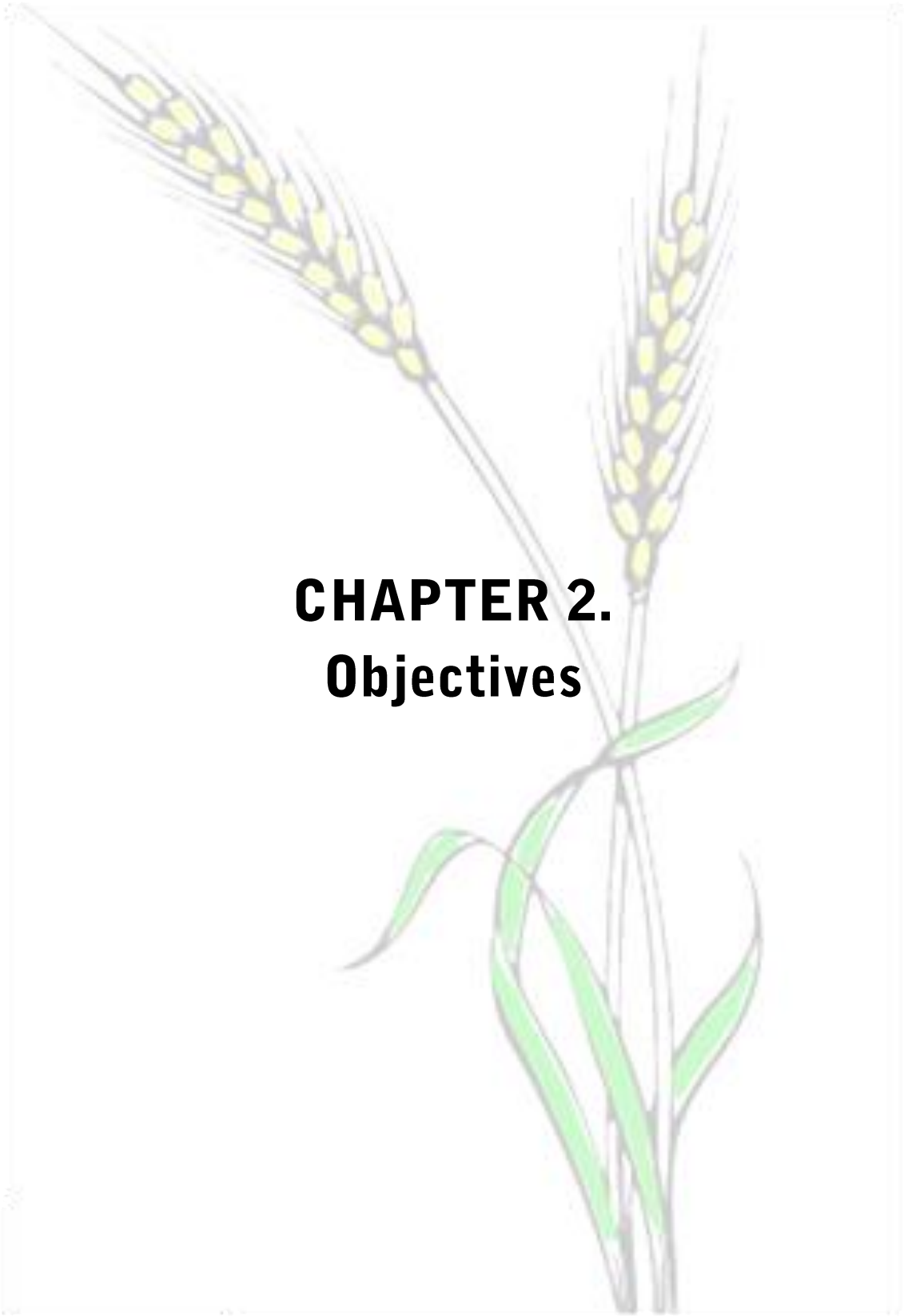


CHAPTER 2.
Objectives

Reports on proteases, cystatins and leaf senescence, especially those related with abiotic stresses, still leave many uncovered gaps. Thereupon, the current thesis has tried to perform, on one side, an exhaustive research about the state of the art. Besides, the current PhD dissertation tries to expand knowdlege through experimental evidences, using barley as a model species, and trying to undertake the following specific objectives:

2.1. To examine the response of the whole barley C1A cysteine protease and cystatin families at the transcriptional level, after the induction of leaf senescence by a specific abiotic stress, i.e., darkness and drought. These studies aimed to select relevant members with significant expression to further investigate their possible functional roles during abiotic-induced senescence.

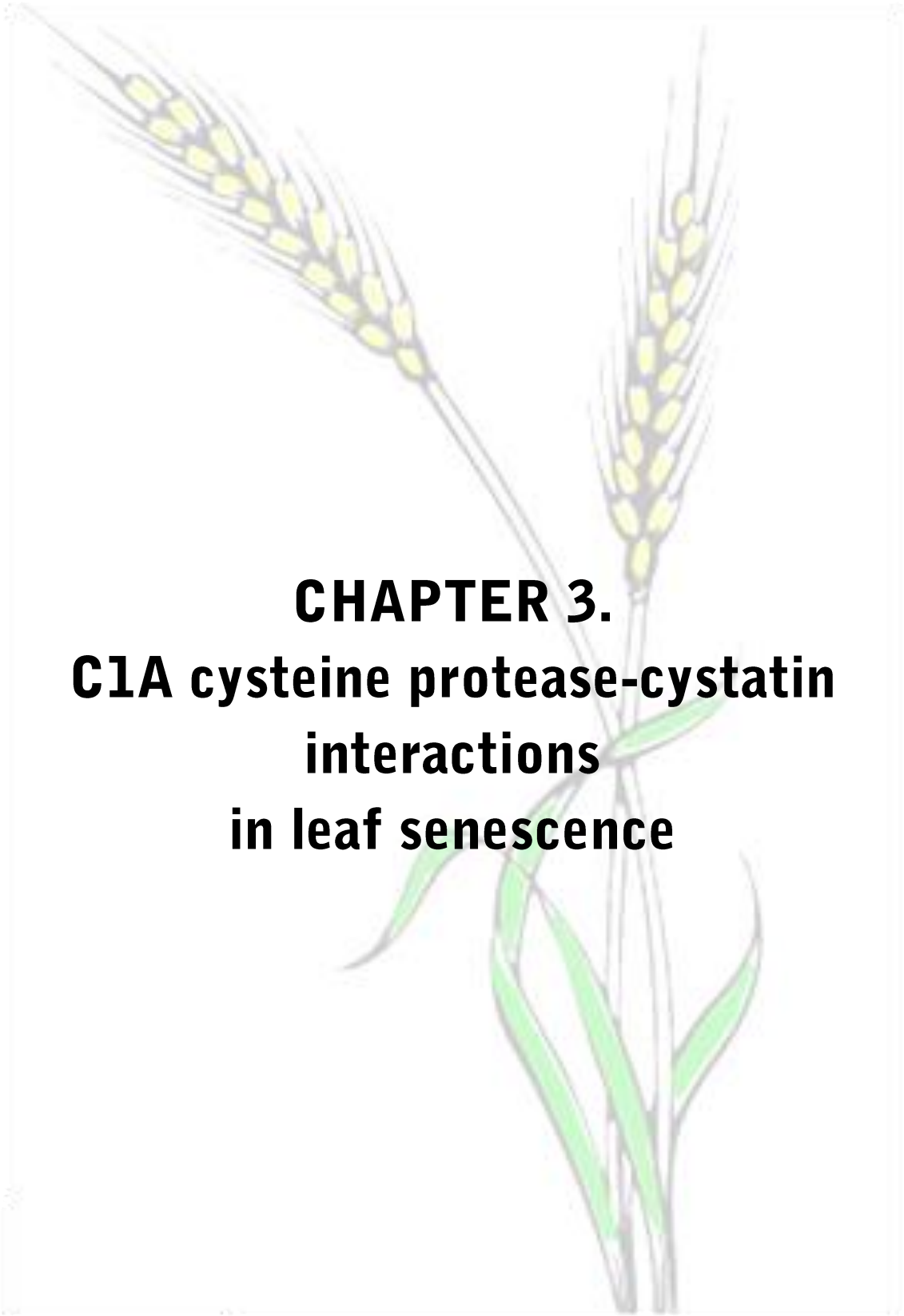
2.2. To characterize previously generated homozygous transgenic barley plants with altered expression for those individual members, either corresponding to C1A proteases (*HvPap-1*, overexpressing and silencing lines), or cystatins (*Icy-2* and *Icy-4*, silencing lines).

2.3. To examine how alterations in the levels of an individual member, HvPap-1, could affect leaf senescence progression. Accordingly, senescence was induced by darkness and several proteolysis-related parameters were in-depth studied. The aim was to conclude if this particular protease may significantly contribute to protein degradation and mobilization associated to leaf senescence.

2.4. To examine and compare how alterations in the levels of HvPap-1, together with alterations in the levels of a cystatin, HvCPI-2, could affect the hydrolysis of storage proteins upon barley grain germination. The aim was to *in vivo* corroborate formerly information about the key role of the protease. Furthermore, analyses with the cystatin lines could provide relevant information about the interplay and balance between cysteine proteases and cystatins along germination.

2.5. To dissect the role of two protease inhibitors, the cystatins HvCPI-2 and HvCPI-4, during drought-induced senescence, through molecular, biochemical, physiological and phenotypical analyses. These studies aimed to provide new information on how cystatins could be related with tolerance or sensitivity towards particular environmental cues, through alterations in the plant´s lifespan.

2.6. To integrate all information and try to correlate these intimately linked processes, leaf senescence and germination, with the aim to provide a theoretical and empirical basis for future designing biotechnological strategies by manipulation of plant senescence.



CHAPTER 3.
C1A cysteine protease-cystatin interactions in leaf senescence

3.1. LEAF SENESCENCE AND PROTEIN BREAKDOWN

Leaf senescence is a physiological process that leads to a massive degradation of macromolecules to mobilize nutrients from leaves to sink tissues in order to sustain further growth and development. Senescence is controlled by intrinsic and environmental factors that trigger a coordinated sequence of events such as loss of chlorophyll with the subsequent reduction of photosynthesis, degradation of macromolecules, relocation of nutrients, dismantling of cellular components and finally, cell death (Krupinska, 2007; Lim *et al.,* 2007; Jing and Nam, 2012; Krupinska *et al.,* 2012). These chemical, structural and metabolic changes involve modification in the expression of thousands of genes, down- or up-regulated during the senescence time course. Each senescence-promoting factor up-regulates a subset of Senescence-Associated Genes, known as SAGs, sequentially involved in perception, signal transduction pathways and final responses, with all of them subjected to complex regulatory crosstalk (Breeze *et al*., 2011; Guo, 2013).

Protein breakdown is one of the most important hydrolytic processes in the senescent leaf with a crucial role in nutrient recycling, especially nitrogen. Many of the SAGs encoding proteases are synthesized *de novo* or induced during senescence. Among the 800 proteases encoded by plant genomes, serine- and cysteine-proteases (CysProt) are the most abundant enzymes associated with leaf senescence described in different plant species (Bhalerao *et al.,* 2003; Roberts *et al.,* 2012; Diaz and Martinez, 2013). Members of aspartic- and threonine-proteases have also been described as participants in leaf senescence whereas few reports have shown a role for metallo-proteases in this process (Graham *et al.,* 1991; Roberts *et al.,* 2012). Expression studies have evidenced changes in the temporal patterns of proteases along senescence, which is consistent with increases in proteolytic activities and reductions, mainly of chloroplastic proteins (Breeze *et al.,* 2011; Roberts *et al.,* 2012). Particularly, Rubisco (D-Ribulose-1,5-bisphosphate carboxylase/oxygenase) which represents the major nitrogen investment in crops and the first source of transportable nitrogen is the main target of proteases (Guo *et al.*, 2004; Masclaux-Daubresse *et al.,* 2007; 2010; Feller *et al*., 2008).

The degradation of most of the chloroplastic proteins is probably initiated within the organelle, mediated by its own proteases, and it is followed outside through the action of other proteases. Intra-plastid proteolysis involves multiple isomeric forms of FtsH-, Clp- and Lon-like ATP-dependent proteases and DegP ATP-independent proteases, both derived from bacterial ancestors (Olinares *et al.,* 2011). However, the targets and the functional significance of many of these proteases are still not characterised. It is known that members of the DegP and Clp serine-protease and FtsH metallo-protease classes are up-regulated in senescing leaves and participate in the degradation of plastid proteins such as those of photosystem II (Roberts *et al.,* 2012). Moreover, Kato *et al.* (2004) described the proteolytic action of the chloroplast CND41 aspartic protease on Rubisco break down during senescence as well as its implication in nitrogen translocation. The overexpression of CND41 reduced Rubisco in senescing tobacco leaves whereas CND41 silenced lines delayed senescence and maintained higher levels of Rubisco in older leaves (Kato *et al.,* 2004; 2005). In addition, the non-chloroplastic proteolysis is produced by the action of vacuolar proteases and by the independent Senescent-Associated Vacuoles (SAVs) enriched in CysProt activities (Otegui *et al.,* 2005, Martinez *et al.,* 2008; Carrion *et al*., 2013). The lytic central vacuoles also contain C1A CysProt among other enzymes (Thoenen *et al.,* 2007; Ishida *et al.,* 2008; van Doorn *et al.,* 2011). This extraplastidial pathway of degradation is dependent on *ATG* genes and implies a complex trafficking of proteins from the chloroplast to the central vacuole, not yet studied in depth. The detection of Rubisco-containing bodies (RCBs) carrying stromal proteins to the central vacuole in senescing leaves of several plant species corroborate this dynamic protein traffic (Chiba *et al.,* 2003; Ishida *et al.,* 2008; Prins *et al.,* 2008; Carrion *et al*., 2013). Interestingly, Prins *et al.* (2008) found larger increases in the amount of immunogold-labelled Rubisco in RCBs as well as in the chloroplast of tobacco plants overexpressing the rice CysProt inhibitor, cystatin OC-1, in comparison to the non-transformed controls. Besides, OC-1 protein was immuno-located in the cytosol, vacuole and chloroplasts of these transformed plants. It was even detected the presence of cytosolic inclusion bodies containing crystalline structures. These bodies strongly suggested interactions of the OC-1 cystatin with CysProt based on their similarity to inclusions bodies found in tomato transgenic plants over-expressing wound- and jasmonate-inducible tomato

cystatin (Madureira *et al*., 2006). The subcellular location of these proteins evidence the link between the protein degradation machinery in the chloroplasts, cytosol and vacuoles.

All these data confirm the importance of C1A CysProt as the main proteases located in different cell compartments during the leaf senescence process, as well as the important regulatory effect of cystatins in the leaf senescence physiology. However, there are still many gaps to be clarified indicating that more studies have to be done.

3.2. C1A CYSTEINE PROTEASES IN LEAF SENESCENCE

The MEROPS database is dedicated to the analyses of proteolytic enzymes (Rawlings *et al.,* 2013). In this database proteases are classified into clans based on structural similarity or sequence features, and clans are divided into families based on common ancestry. Among the approximately 140 CysProt that belong to 15 families in 5 clans, papain-like family C1A CysProt (family C1, clan CA) is one of the most abundant groups of plant proteases responsible for protein degradation in many physiological processes, including leaf senescence (Martinez *et al.,* 2012; Roberts *et al*., 2012; Diaz and Martinez, 2013). We focus our attention in this complex C1A peptidase family although other CysProt such as legumains or vacuolar processing enzymes (family C13), metacaspases (family C14), calpains (family C2) and proteases related to ubiquitin-dependent pathways (family C12, C19 and C85), have been also identified as enzymes with putative roles in plant senescence (Diaz and Martinez, 2013; Rawlings *et al.,* 2013). As all CysProt, C1A proteases have a nucleophilic cysteine thiol in their catalytic triad (Cys, His and Asn) and are synthesized as inactive or weakly active precursors to prevent unspecific proteolysis and to guarantee that the mature enzyme is formed in the right place and/or at the right time. To become active, the C1A peptidases need to be processed either by self-processing or with the aid of processing enzymes. Protease precursor activation depends on the pH, the action of other proteases and protease inhibitors, and the cellular or extra-cellular environment (Diaz and Martinez, 2013). Additionally, some cathepsin L-like members also contain a C-

terminal extension sequence, which includes a Pro-rich region and a granulin domain with a high homology to animal proteases of the epithelin/granulin family (Yamada *et al.,* 2001).

Based on their similarity to cathepsins, a family of lysosomal proteolytic enzymes of mammalian cells, all the C1A proteases from plants can be grouped as L-, B-, H-, or F-like cathepsins (Martinez and Diaz, 2008). L-like cathepsins are distributed in five independent groups (A to E) whereas H-, F-, and B-like cathepsins are not sub-classified. Transcriptomic and proteomic data have consistently assigned a major role to members of the four cathepsin L-, B-, H- and F-like classes in leaf senescence in different plant species (Guo *et al.,* 2004; Gregersen *et al*., 2008; Ruuska *et al.,* 2008; Martinez *et al.,* 2012). Figure 3.1 represents a phylogenetic tree constructed according to Martinez and Diaz (2008). The tree includes plant C1A proteases previously described as up-regulated enzymes during leaf senescence (Table S 3.1), as well as barley C1A CysProt (see below the section on barley C1A CysProt in leaf senescence). Examples of C1A proteases involved in leaf senescence are described in the next subsections.

3.2.1. CATHEPSIN L-LIKE CYSPROT

SAG12, originally identified in *Arabidopsis*, is probably the best studied senescence-induced cathepsin L-like CysProt. It shows a strictly senescence-associated pattern of expression in leaves and is currently used as a senescence marker (Hensel *et al.,* 1993; Lohman *et al*., 1994). Otegui *et al.* (2005) demonstrated that the AtSAG12 fused to the green fluorescent protein driven by the *AtSAG12* promoter colocalized together with Rubisco and other stromal proteins in SAVs in Arabidopsis. More recently, the same research group has shown in a very elegant way that active CysProt are responsible for the degradation of some plastid proteins in SAVs during dark-induced senescence of tobacco leaves (Carrion *et al*., 2013). Studies with potential orthologs of *Arabidopsis SAG12* gene in other dicot species, such as *BnSAG12-1* and *BnSAG12-2* in *Brassica napus* (Noh and Amasino, 1999; Desclos *et al.,* 2008; Gombert *et al.,* 2006)*, SPG31* in

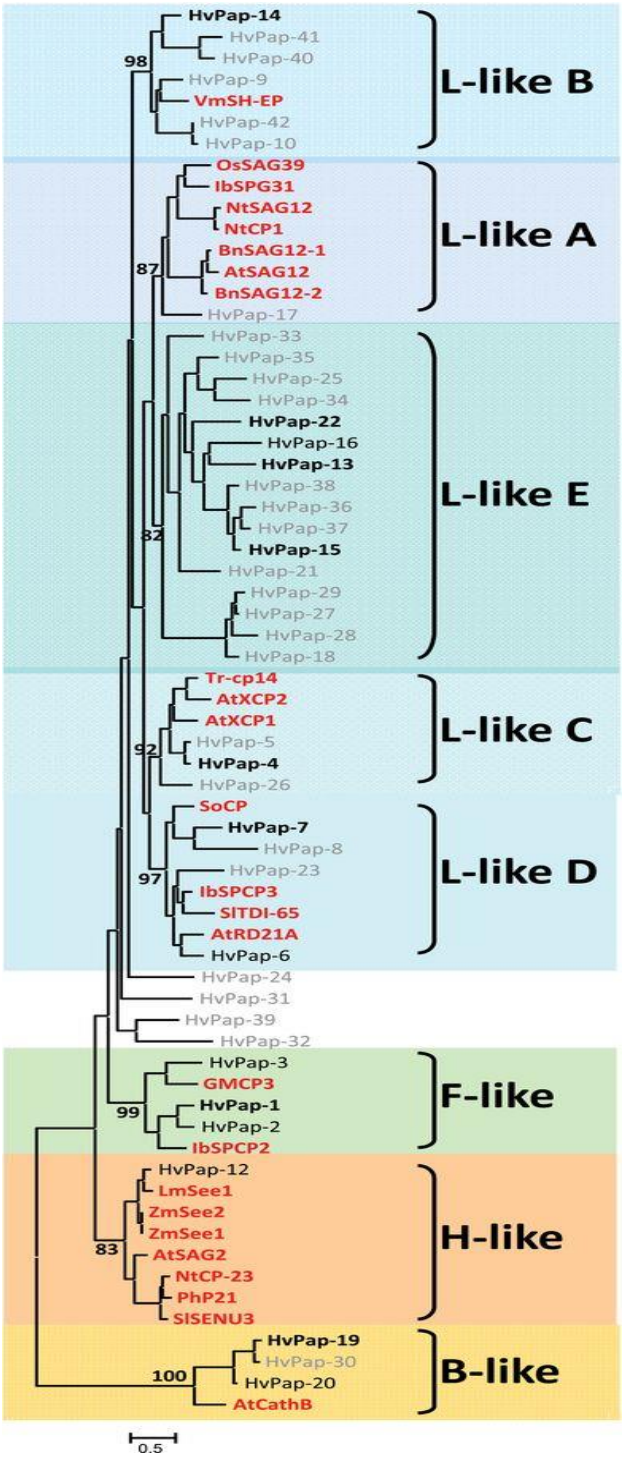


Figure 3.1. Phylogram of the C1A CysProt from barley and the senescence associated C1A cysteine proteases from other plant species (in red). The amino acid sequences were aligned by MUSCLE and analyzed with the Maximum Likelihood method. aLRT values from the main phylogenetic clades are indicated. The senescence induced barley CysProt are highlighted in bold. The barley CysProt that are not expressed in leaves are marked in grey.

sweet potato (Chen *et al*., 2002) and *NtCP1* and *NtSAG12* in tobacco (Beyene *et al*., 2006; Carrion *et al.,* 2013) corroborated this role of SAG12. In rice, the *SAG39* gene expressed at late senescence stages is the putative ortholog of *Arabidopsis SAG12*. When the isopentenyltransferase gene (*ipt*), that is involved in cytokinin synthesis, was expressed under the *SAG39* promoter in rice plants, the chlorophyll level of the flag leaf used to monitor senescence confirmed the stay-green phenotype *vs* wild-type plants. Changes in the cytokinin content led to early flowering and greater number of emerged panicles in the transgenic rice lines. Besides, measurements of sugar and nitrogen contents in flag leaves demonstrated a transition in the source-sink relationship in transgenic plants triggered at the onset of leaf senescence with the nitrogen content decreasing more slowly. In contrast, sugars were removed more rapidly than in wild-type plants (Liu *et al*., 2010). XCP1 and XCP2 are other cathepsin L-like CysProt involved in *Arabidopsis* senescence. XCP1 is xylem-specific and its ectopic expression produces early leaf senescence and a rapid loss of chlorophyll (Funk *et al*., 2002). Recently, the Tr-cp14 protease, with high homology to XCP1 and XCP2 has been identified in white clover (*Trifolium repens*). This protease is localized in the endoplasmic reticulum and was associated with the development of tracheary elements in senescent leaves (Mulisch *et al.,* 2013). Immunocytochemical analysis showed that chloroplasts of senescing French bean leaves were translocated into the central vacuole where VmSH-EP protease is located, suggesting a possible involvement of this protease in the degradation of Rubisco (Minamikawa *et al.,* 2001).

The granulin domain C1A CysProt has also been associated with degradation in senescence in different plant species. An example is RD21A, which causes a major dominant protease activity in *Arabidopsis* leaf extracts and it is responsible for induced proteome degradation in the vacuoles of senescing leaves (Yamada *et al.,* 2001; Gu *et al.,* 2012). RD21A is synthesized as a 57-kDa precursor and then is slowly processed to the 33-kDa mature protein via a 38-kDa intermediate. These intermediates are accumulated in the vacuoles as aggregates and after maturation a soluble protease is formed by removing the granulin domain during leaf senescence (Yamada *et al.,* 2001). Phylogenetic analysis showed that RD21A displayed a close relationship with SPCP3, a granulin CysProt from sweet potato, significantly induced upon natural and dark-

treatment senescence (Chen *et al*., 2006). Similarly, the 41-kDa CysProt with the granulin domain SoCP is induced in senescent leaves of *Spinacia oleraceae* (Tajima *et al*., 2011). Another example of granulin domain CysProt is the tomato 65-kDa protease TDI-65, related to drought-induced senescence as well as programed cell death (Harrak *et al.,* 2001).

3.2.2. CATHEPSIN H-LIKE CYSPROT

AtSAG2 is a cathepsin H-like CysProt that participates in leaf senescence in *Arabidopsis* (Hensel *et al.,* 1993). NtCP-23 from tobacco shows also significant similarities in its amino acid sequence to plant senescence-associated AtSAG2. Its expression is induced by amino acid remobilization in naturally senescing leaves (Ueda *et al*., 2000). *SENU2* and *SENU3* genes from tomato, with a significant homology to cathepsin H-like CysProt, are highly expressed in advanced stages of senescent leaves. Transgenic lines deficient in ethylene biosynthesis, in which leaf senescence was delayed, showed that the accumulation of SENU2 and SENU3 was slowed down but not prevented (Drake *et al*., 1996). Other examples of senescence-associated cathepsin H-like CysProt are: PhP21, first identified in callus of petunia grown under low cytokinin concentrations, that showed the highest expression levels in senescing petunia leaves (Tournaire *et al.,* 1996); LmSee1, identified in leaves of ryegrass (*Lolium multiflorum)* that is also induced during senescence (Li *et al*., 2000; 2004); and two maize senescence related genes *See1* and *See2* that are highly expressed in leaves from earlier senescent maize varieties than in stay-green varieties (He *et al.,* 2005).

3.2.3. CATHEPSIN B-LIKE CYSPROT

As far as we know, the only report of cathepsin B-like CysProt associated with leaf senescence is the case of *AtCathB* genes of Arabidopsis described by McLellan *et al.* (2009). Three *CathB* homologues (*AtCathB1, AtCathB2* and *AtCathb3*) were identified in Arabidopsis and showed significant increases in expression after 2 days of dark-induced senescence. This increased gene expression was most marked for the *AtCathB3* gene. Single, double and triple knockout mutants presented a senescence-

related phenotype but only the *Atcathb* triple mutant evidenced a significant delay in dark-induced senescence compared to the wild-type. A RT-qPCR analysis revealed a significant decrease of the *SAG12* marker accumulation in the triple mutants at 4 days post dark-treatment compared with the wild type *SAG12* expression levels. This was also accompanied by significant greater chlorophyll content in the triple mutant than in the control (McLellan *et al.,* 2009).

3.2.4. CATHEPSIN F-LIKE CYSPROT

Following the previous classification, SPCP2 is a cathepsin F-like CysProt isolated from senescing leaves of sweet potato, which is enhanced in natural and induced senescent leaves and weakly detected in mature green tissues (Chen *et al.,* 2010). Its overexpression caused altered developmental characteristics and stress responses in transgenic *Arabidopsis* plants. GMCP3 of *Glycine max* is another cathepsin F-like CysProt induced by senescence and diverse stresses in non-seed tissues (Esteban-Garcia *et al.,* 2010).

Besides the CysProt from different classes commented above, there are other studies regarding the role of CysProt in leaf senescence. However, many of them correspond to partial gene/protein sequences or there is no information about which CysProt class they are. These CysProt have been found in Solanaceae, legume, cereal and woody species (Khanna-Chopra *et al.,* 1999; Xu and Chye, 1999; Bhalerao *et al.,* 2003; Sillanpää *et al*., 2005; Thoenen *et al.,* 2007). The results from these reports suggest that differences in protease classes, activity timing, compartmentalization and regulatory factors depend on the plant species and the senescence- inducing conditions (Buchanan-Wollaston *et al*., 2003; 2005; Thoenen *et al.,* 2007).

3.3. BARLEY C1A CYSTEINE PROTEASES IN LEAF SENESCENCE

Most studies performed so far on CysProt and leaf senescence involved individual members from multiple plant species. Determining the functions of all gene family members within a unique species and identifying their targets would be very

informative. Barley could be assumed as a model for leaf senescence in monocots according to the numerous research analyses focused on this cereal species. Barley natural leaf senescence has been studied under field conditions to identify up-regulated genes (Scharrenberg *et al.*, 2003). A cDNA library prepared from mRNA of senescent flag leaves was differentially screened and three cDNA clones were isolated. Their expression patterns were studied either in leaves at different developmental stages or in darkness-induced senescence. cDNA clone *HvSF42 (HvPap-1)* was found to be accumulated during senescence of flag leaves as well as during dark-induced senescence of attached primary foliage leaves (Scharrenberg *et al*., 2003).

High levels of carbohydrates were previously shown to promote the onset of senescence (Parrott *et al.,* 2007). Carbohydrate accumulation in barley plants can be experimentally induced by steam-girdling at the leaf base by occluding the phloem. Gene regulation under these conditions was investigated using the 22K Affymetrix Barley GeneChip array and quantitative real-time reverse transcriptase PCR (RT-qPCR) (Parrott *et al.,* 2007). Several C1A CysProt, among other senescence-specific genes, were up-regulated under these conditions, including the cathepsin L-like sequences Contig10941_at (renamed *HvSAG12* or *HvPap-13*), Contig9006_at (*HvPap-8*) and Contig5626_s_at (*HvPap-14*) and the cathepsin B-like sequences Contig2680_at (*HvPap-19*) and Contig2683_s_at (*HvPap-20*). Analysis of barley leaf senescence and protease gene expression showed that whereas *HvPap-13* is only specifically induced under conditions of high carbohydrate content, *HvPap-8* is up-regulated under high carbohydrate levels and low to moderate nitrogen levels. This implies that *HvPap-8* most likely participates in bulk protein degradation during barley leaf senescence (Parrott *et al*., 2010).

Massive analyses of gene expression have facilitated the unveiling of the molecular events and pathways associated to leaf senescence. Particularly, microarray barley experiments highlight the importance of transcriptomic approaches to find out the members of a particular protein family involved in a specific process (Parrott *et al*., 2007; Gregersen *et al.,* 2008). However, the results of these microarray analyses should be validated by RT-qPCR to establish the accuracy of the analyses and to avoid

the putative problem of gene specificity. The genome of barley has been recently published and annotated (International Barley genome Sequencing Consortium, 2012) and can be accessed to determine the extent of the C1A family in this species. Searches in the annotation of the barley genome (webblast.ipk-gatersleben.de/barley) using BlastP as well as the nucleotide sequence using tBlastN, together with searches in the Gene Index expressed sequences collection (compbio.dfci.harward.edu/tgi), has allowed us to identify the whole C1A family in barley (Table S 3.2). It is made up by 41 members (*HvPap-1* to *HvPap-42*, being *HvPap-10* and *HvPap-11* different allelic variants of the same gene).

The participation of all members of the C1A peptidase family in barley leaf senescence was assessed by RT-qPCR analysis (see Supplementary data S3). C1A protease gene expressions were compared in control *vs* senescing barley leaves (Figure 3.2). Ten out of the 34 barley L-like cathepsins were expressed in leaves in control and/or senescing conditions. Seven of them (*HvPap-4, -7, -8, -13, -14, -15* and *-22*) were induced when plants were grown under darkness, especially for the *HvPap-4* and *HvPap-22* genes (Figure 3.2A). The expression of B-, F- and H-like cathepsins was higher than L-like cathepsins in control leaves (Figure 3.2B). Cathepsin H-like (*HvPap-12*), two out of the three cathepsins F-like (*HvPap-1* and *HvPap-2*) and two out of the three cathepsins B-like (*HvPap-19* and *HvPap-20*) were expressed. The most expressed members of these C1A sub-families (*HvPap-1, 12* and *19*) were induced in response to darkness, with higher relative expression levels than that of L-like enhanced cathepsins. These results suggest a wide representation of members of the barley C1A family, including representatives of all the main groups of C1A CysProt. Barley genes from all the groups represented in the phylogenetic tree (Figure 3.1) were induced by leaf senescence, with the exception of group L-like A, in which *HvPap-17* was expressed in leaves but was not induced after darkness treatment. C1A proteases from other plants were strongly represented in the cathepsins H-like group and absent in the group L-like E. Surprisingly, *AtSAG12,* the widely used CysProt marker for senescence, belongs to the same group (L-like A) that the non-induced *HvPap-17* gene and its previously described orthologous *HvPap-13* is found in a different subgroup (L-

like E). The relatively short distance between these two sub-groups suggests that their members could share similar physiological function.

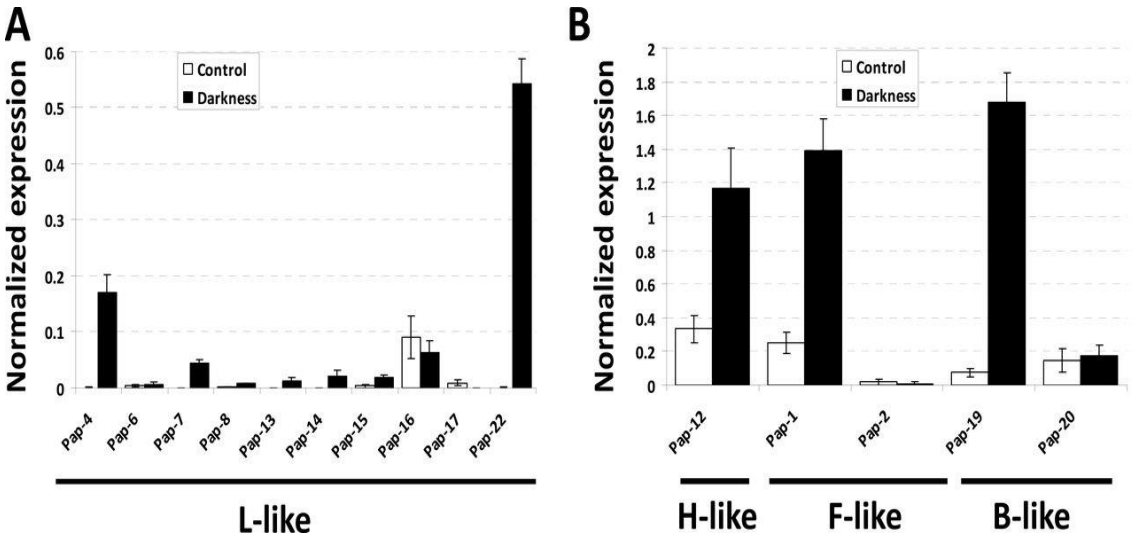


Figure 3.2. Expression of barley C1A CysProt genes in leaves after seven days of senescence treatment (darkness) or seven days of 16:8 h light/dark photoperiod (control), as determined by real time quantitative PCR. Values are expressed as relative mRNA levels of C1A CysProt genes normalized to barley c*yclophilin* mRNA content. A. Expression of L-like cathepsins. B. Expression of H-, F-, and B-like cathepsins.

3.4. CYSTEINE PROTEASE-CYSTATIN INTERACTION IN LEAF SENESCENCE

Plant cystatins (phytocystatins) are natural specific inhibitors of cysteine proteases of the papain C1A family, although some of them with a carboxy-terminal extension are also able to inhibit CysProt of the legumain C13 family (Martinez *et al.,* 2007). The structure of the tarocystatin-papain complex has been recently resolved showing that the phytocystatin inhibitory mechanism is produced by a tight and reversible interaction with its target C1A enzymes (Chu *et al.,* 2011). This interaction involves a tripartite wedge formed by the partially flexible N-terminus containing a glycine residue and two hairpin loops carrying a conserved Gln-Xaa-Val-Xaa-Gly motif in the central region of the polypeptide (where Xaa is any amino acid) and a Pro-Trp (or Leu-Trp) in the C-terminal region. Indirect inhibitory protease assays using commercial protease-degradable fluorescence substrates and measuring the capacity of inhibition of cystatins on proteases, support this physical interaction (Martinez *et al.,* 2009).

Likewise, interplay between C1A CysProt and their inhibitors have been tested by experiments using plant tissues. Plant protease-cystatin complexes have been purified by immunoaffinity or hydrophobic chromatography from leaves of maize and spinach (Yamada *et al.,* 1998; Tajima *et al.,* 2011). Cystatins and cathepsin L-like proteases, tagged to fluorescence proteins, have been localized to the endomembrane system in plant cells and interaction between them has been detected by bimolecular fluorescence complementation assays (Martinez *et al.,* 2009).

Phytocystatins have a dual function, as defense proteins and as endogenous regulators of protein turn-over. They participate in physiological processes, such as plant growth and development, programmed cell death, accumulation and mobilization of storage protein in seeds and tubers (Solomon *et al.,* 1999; Martinez *et al.,* 2009; Weeda *et al.,* 2009; Cambra *et al.,* 2012), plant defense (Carrillo *et al.,* 2011; Santamaria *et al*., 2012) and senescence (Kleber-Janke and Krupinska, 1997; Sugawara *et al.,* 2002; Prins *et al.,* 2008; Neuteboom *et al.,* 2009, Tajima *et al*., 2011). Sugawara *et al.* (2002) described the Dc-CP1 CysProt inhibitor Dc-CPIn involved in the regulation of petal wilting in senescing carnation (*Dianthus caryophyllus*) flowers. The recombinant Dc-CPIn protein completely inhibits the activity of Dc-CP1 extracted from carnation petals. Northern blot analysis showed that the mRNA for *Dc-CP1* accumulated in large amounts, whereas that for *Dc-CPIn* disappeared, corresponding to the onset of petal wilting in flowers undergoing natural senescence and exogenous ethylene-induced senescence. Based on these findings, a role of Dc-CPIn in the regulation of petal wilting was suggested, acting as a suppressor of petal wilting inhibiting Dc-CP1 CysProt.

Neuteboom *et al.* (2009) characterized AcCYS1, a pineapple cystatin, with a 63 residues AE-rich N-terminal trunk (NTT) that enhances inhibition (>95%) of fruit bromelain CysProt and is post-translationally removed during fruit ripening. *AcCYS1* mRNA was present in roots and leaves but was most abundant in fruit. Ripe fruit extracts proteolytically removed the NTT of 27-kDa AcCYS1 *in vitro* to produce a 15-kDa species that poorly inhibits bromelain, which implies an increase in tissue proteolysis, softening, and degradation during fruit ripening (Neuteboom *et al.,* 2009).

The most detailed analysis of the participation of a cystatin in leaf senescence is that of the biochemical and molecular characterization of a senescence-related cysteine protease-cystatin complex from spinach leaf (Tajima *et al.,* 2011). This complex was composed of the 41-kDa CysProt SoCP and a 14-kDa cystatin CPI. Purified recombinant CPI had a strong inhibitory activity against SoCP and the release of the cystatin from the SoCP-CPI complexes implied a concomitant activation of the enzyme activity. The coordinated expression of the mRNAs of *CPI* and *SoCP* in senescent leaves suggested that this protease was involved in leaf senescence. A second analysis that supports a role of phytocystatins in leaf plant senescence was reported by Prins *et al.* (2008) on the effect of transgenic tobacco plants expressing the rice cystatin OC-I (oryzacystatin I) on leaf protein accumulation. These plants grew more slowly than the controls and showed changes in leaf protein content with an increased abundance, among others, of two Rubisco activase isoforms, together with delayed leaf senescence. Western-blot analysis of 14-weeks old plants revealed considerable higher levels in the amount of Rubisco protein in the overexpressing OC-I leaves compared to controls. These results demonstrated that C1A CysProt and phytocystatins were involved in Rubisco turnover in leaves undergoing senescence, and confirmed the importance of the protease-inhibitor interaction in leaf senescence. In barley, the isolation of a cDNA clone that includes a complete open reading frame with homology to the sequence of a cystatin in leaves during dark-induced senescence as well as during natural senescence under field conditions, suggests a role of this protease inhibitor in leaf senescence (Kleber-Janke and Krupinska, 1997). The whole family of barley cystatins was identified and biochemically and molecularly characterized by Martinez *et al*. (2009). Thirteen proteins were described (HvCPI-1 to -13), which were assigned to three different phylogenetic clades (Group A, HvCPI-1 to -4; Group B, HvCPI-5 and -9; Group C, HvCPI-6 to -8 and HvCPI-10 to -13). Blast searches in the barley genome have not found any additional barley cystatins. Their expression patterns in different tissues, sub-cellular location, as well as inhibitory capacity against barley CysProt were analyzed. The participation of all barley cystatin family members in leaf senescence was assessed by RT-qPCR (Supplemental data S3). Figure 3.3 shows that six barley cystatins (genes *Icy3*- to *-6*, *-8* and *-9;* encoding HvCPI-3 to -6, -8 and -9 proteins) were induced by darkness treatment, one cystatin expressed in leaves was

not induced (gene *Icy-2*; HvCPI-2 protein), and the rest of cystatins are not expressed in leaves under control or senescence conditions. The co-induction of C1A CysProt and cystatins by darkness senescence treatment supports a tight regulation of this physiological process, in which protease inhibitors have a role modulating the degradative activity of endogenous induced proteases. Therefore, the balance of CysProt and cystatins accumulation levels is crucial for the regulation of the senescence process induced by darkness. This co-regulation could be extended to the senescence induced by some other stimuli, and to the physiologically different natural senescence.

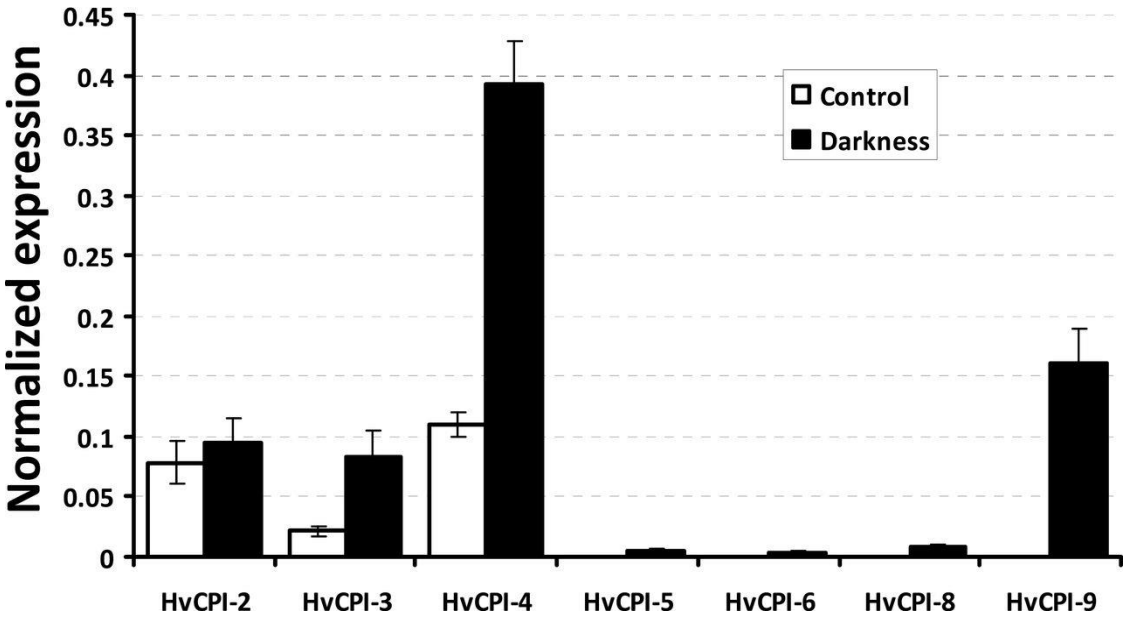


Figure 3.3. Expression of barley cystatin genes in leaves after seven days of senescence treatment (darkness) or seven days of 16:8 h light/dark photoperiod (control), as determined by real time quantitative PCR. Values are expressed as relative mRNA levels of cystatin genes encoding HvCPI proteins, normalized to barley c*yclophilin* mRNA content.

3.5. CONCLUSIONS

The senescence-associated proteolysis includes different subcellular compartments, several types of proteases and regulators and a complex trafficking of proteins that leads to a massive protein turn-over with a crucial role in nutrient recycling. CysProt and cystatins have appeared as active partners on the proteolytic events during leaf senescence. The current knowledge of this process allows the creation of a

hypothetical model of degradation pathways involving the chloroplast and extraplastidial compartments where CysProt and cystatins develop their actions (Figure 3.4). The degradation of proteins induced by senescence treatments might take place within the plastid itself mediated by CysProt, among other proteases. Rubisco, other stromal proteins and thylakoid proteins are probably bound to the chloroplast envelope membrane to promote association between chloroplasts and other organelles (Prins *et al*., 2008). Outside the chloroplast, plastid proteins including Rubisco, occur in the vesicular transport system mediated by SAVs where proteolysis may continue due to the presence of active CysProt (Carrion *et al*., 2013). Besides, RCBs derived from chloroplast and carrying stromal proteins or their hydrolytic products, are redirected to the central vacuole. Thereafter, protein degradation products appear in the vacuoles either to be broken down in smaller molecules or to be transiently storage as amino acids until they are re-localize to other plant tissues. Cystatins, localized in cytosol, chloroplasts and vacuoles may participate regulating the CysProt activities (Prins *et al.,* 2008; Martinez *et al.,* 2009), as is indicated in Figure 3.4. These CysProt inhibitors must be subjected to a complex regulatory crosstalk in response to specific factors operating during senescence. Further studies on senescent leaves are needed to clarify the unknown steps on protein transport and degradation as part of the dismantling of cellular components during leaf senescence.

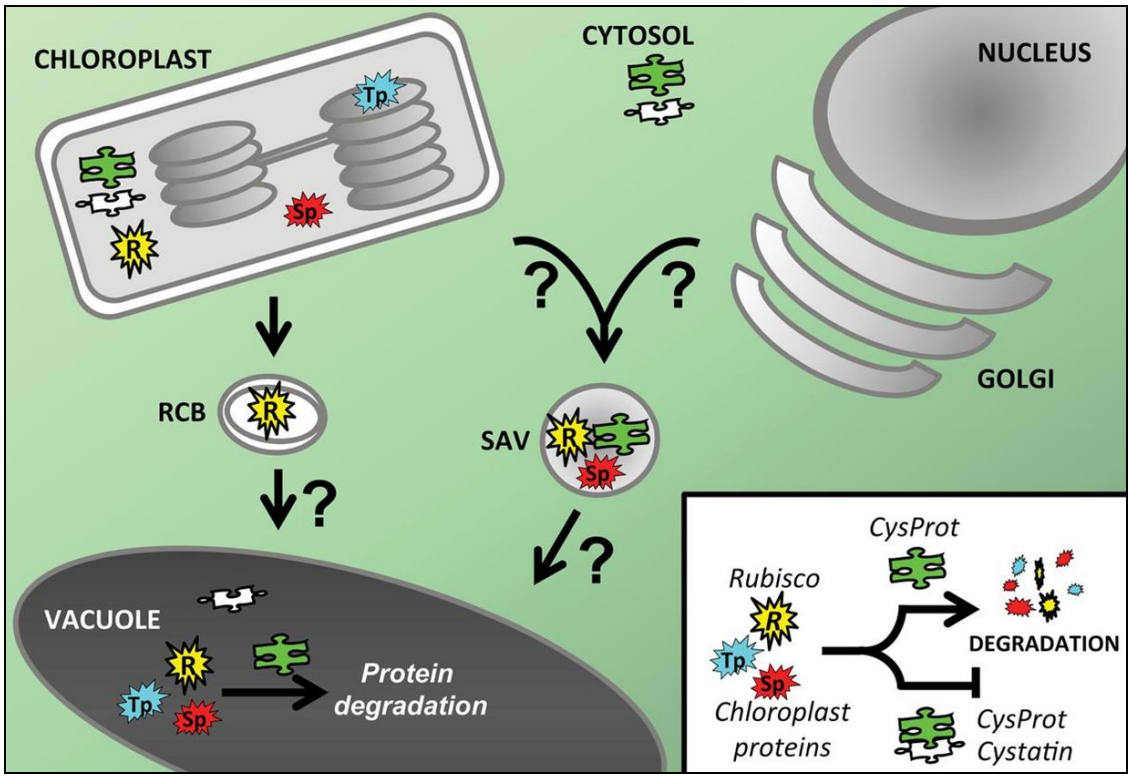


Figure 3.4. Scheme of potential pathways of degradation for chloroplast proteins during leaf senescence, mediated by CysProt among other proteases, and regulated by cystatins. SAV: Senescecne Associated Vacuole; RCB: Rubisco-Containing Body; R: Rubisco (yellow colour); SP: stromal protein (red colour); Tp: thylakoid protein (blue colour). Insert: scheme of CysProt (green colour) and cystatin (white colour) interaction regulating the protein degradation process.

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3.7. SUPPLEMENTAL DATA

Supplemental File S 3.1. RNA extraction, cDNA synthesis and Real-time quantitative PCR analyses.

For real-time quantitative PCR (qRT-PCR) studies, seven day-old plants of barley (*Hordeum vulgare*) cv. Bomi grown in a 16/8 h light/dark photoperiod were incubated either in darkness or kept them in the same photoperiod during seven additional days. Leaf samples from these plants were collected, frozen into liquid N_2 and stored at -80 ºC until being used for RNA/protein extraction.

Total RNA was extracted from frozen samples by the phenol/chloroform method, followed by precipitation with 3 M LiCl and digestion with DNAse. cDNAs were synthesized from 1 µg RNA using the High Reverse Transcription kit (Applied Biosystems) following manufacturer's instructions. qRT-PCR analyses were performed for duplicated samples by means of a CFX96 Real-time system (BioRad) using a SYBR Green detection system. Quantification was normalized to barley *cyclophilin* mRNA levels. The primers used for PCR amplification are described in Table S 3.2.

Table S 3.1. C1A CysProt genes induced in leaf senescence in different plant species.

CysProt Gene	Accession number[a]	Species	Reference
AtSAG12	At5G45890	*Arabidopsis thaliana*	Lohman *et al*. 1994 Otegui *et al*., 2005
NtSAG12	ADV41672.1	*Nicotiana tabacum*	Carrion *et al*., 2013
BnSAG12-1	AAD53011.1	*Brassica napus*	Noh and Amasino, 1999
BnSAG12-2	AAD53012.1	*Brassica napus*	Noh and Amasino, 1999
IbSPG31	AAL14199.1	*Ipomoea batatas*	Chen *et al*., 2002
NtCP1	AY881011.2	*Nicotiana tabacum*	Beyene *et al*., 2006
OsSAG39	CAD40026.2	*Oryza sativa*	Liu *et al*., 2010
AtXCP1	At4g35350	*Arabidopsis thaliana*	Funk *et al*., 2002
AtXCP2	At1g20850	*Arabidopsis thaliana*	Funk *et al*., 2002
Tr-cp14	AAP32192.1	*Trifolium repens*	Mulisch *et al*., 2013
VmSH-EP	P12412.1	*Vigna mugo*	Minamikawa *et al*., 2001
AtRD21A	At1g47128	*Arabidopsis thaliana*	Yamada *et al*., 2001 Gu *et al*., 2012
IbSPCP3	AAK48495.1	*Ipomoea batatas*	Chen *et al*., 2006
SoCP	AB377534.1	*Spinacia oleracea*	Tajima *et al*., 2011
SlTDI-65	AAD48496.1	*Solanum lycopersicum*	Harrak *et al*., 2001
AtSAG2	At5g60360	*Arabidopsis thaliana*	Hensel *et al*., 1993
NtCP-23	BAA96501.1	*Nicotiana tabacum*	Ueda *et al*., 2000
SlSENU3	CAA88629.1	*Solanum lycopersicum*	Drake *et al*., 1996
PhP21	AAC49361.1	*Petunia x hybrida*	Tournaire *et al*., 1996
LmSee1	CAB71032.1	*Lolium multiflorum*	Li *et al*., 2000
ZmSee1	X99936.1	*Zea mays*	Li *et al*., 2004 He *et al*., 2005
ZmSee2	NP001105479.1	*Zea mays*	He *et al*., 2005
AtCathB	At4g01620	*Arabidopsis thaliana*	Guo *et al*., 2004 Parrott *et al*., 2007 McLellan *et al*., 2009
IbSPCP2	AAK27969.1	*Ipomoea batatas*	Chen *et al*., 2010
GMCP3	NP001236888.1	*Glycine max*	Esteban-Garcia *et al*., 2010

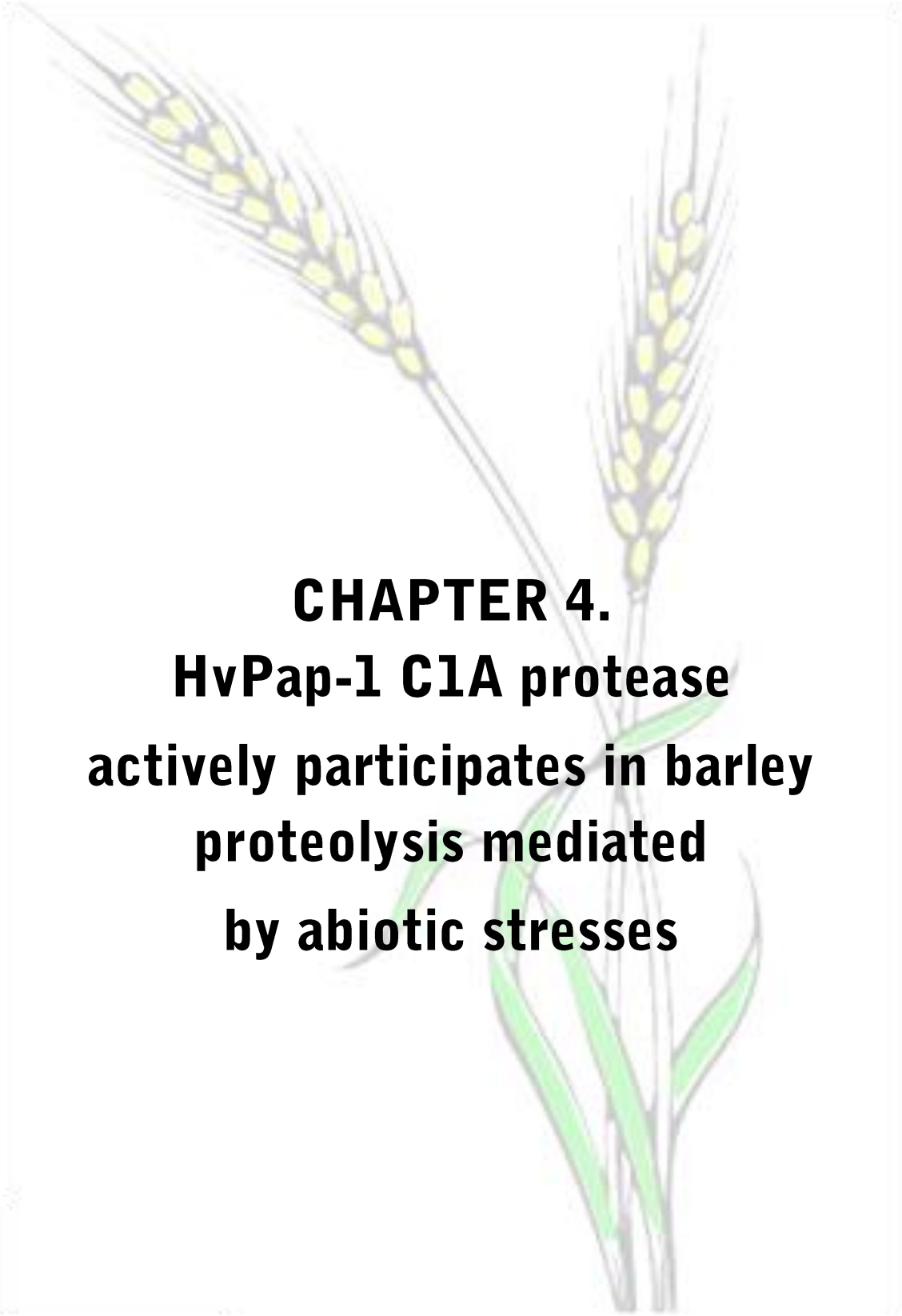
[a] NCBI or TAIR (for *Arabidopsis thaliana* genes) accession numbers

Table S 3.2. Gene specific primer pairs and accession numbers for barley C1A CysProt genes.

Barley C1A CysProt Gene	Barley Genome Accession Number[a]	Oligonucleotide sequences (5' to 3')
HvPap-1	MLOC_79498.2	HvPap-1F TCCTGGAGTCGATCTTTGGTTTC
		HvPap-1R CAAGCATACTGTTGCGGCTTC
HvPap-2	MLOC_67471.3	HvPap-2F ATGGCTCGCCTCCGCCTCCGC
		HvPap-2R CTATTCCTTCTTAGAGGTATG
HvPap-3	MLOC_61862.1	HvPap-3F ATGGCCGCCCGGCTCGTCTCC
		HvPap-3R TCACGGGGCGACGGCGACGG
HvPap-4	MLOC_66677.2	HvPap-4F CCTTGAGAGTCCTTGTTCCCGA
		HvPap-4R CCATGTTCGTCGTTTTAACCGA
HvPap-5	MLOC_65684.2	HvPap-5F TGCTGTTCTTCTCCTCTGTGTC
		HvPap-5R AGCCACTTCTCAAACAGCTC
HvPap-6	MLOC_81876.1	HvPap-6F TGCAATTGACGGCAAGAAGA
		HvPap-6R TGGATCACCAGGTGATCATTTG
HvPap-7	AK371876	HvPap-7F AAAAACGCGAAGGTGGTGAG
		HvPap-7R TTCTGCAGCGATTTCTCGTC
HvPap-8	AK358908	HvPap-8F AGTTTGCTGCAGCGATAACC
		HvPap-8R GACCTTGGAGAAAGCTTGCTTC
HvPap-9	MLOC_73076.2	HvPap-9F ACTGCGACAACGTCAACAAC
		HvPap-9R TCTTCTGGATGAACTGGAAGGC
HvPap-10	MLOC_76470.1	HvPap-10F TCGATCCATGTGCTTATCCGA
		HvPap-10R AACACACGCCTAATCCTTGGC
HvPap-11	MLOC_76470.1	HvPap-11F TCGATCCATGTGCTTATCCGA
		HvPap-11R AACACACGCCTAATCCTTGGC
HvPap-12	MLOC_34809.1	HvPap-12F ATGTGCGCTATTGCTACCTGC
		HvPap-12R CACCTTATTCATGTCTGGCGAA
HvPap-13	AK365745	HvPap-13F AGTTGGACCATGGTTGCAAC
		HvPap-13R TCACAAGTGTCGTCCTTTGC
HvPap-14	AK368788	HvPap-14F TCGCACTTCCAGTTCTACTCG
		HvPap-14R CCCCATGAGTTTTTCACGATCC
HvPap-15	MLOC_74627.1	HvPap-15F TGATGAACGCTGTGGCAAAC
		HvPap-15R TACATGGCCCGTTGTAGATTCC
HvPap-16	MLOC_23006.1	HvPap-16F CTGGATCGGTAAGAACTCGTGG
		HvPap-16R TGATGGAGGTGCCATCATATGA
HvPap-17	AK364080	HvPap-17F AGCTGCGTGTGCATTTATCATG
		HvPap-17R GCGGTGAAATATGCAACCCA
HvPap-18	AK251286.1	HvPap-18F TGCGGTTCCAAGTCTTCAAG
		HvPap-18R TCGCCGAACCTGTTCATACTC
HvPap-19	AK364590	HvPap-19F TCGATGAAGAACATCGCCG
		HvPap-19R TGCCCGCTTAATTTGACAGG
HvPap-20	AK368127	HvPap-20F GGAGGTCACGCTGTCAAGTT
		HvPap-20R GTATCCGTCATCACCCCATC
HvPap-21	MLOC_47161.1	HvPap-21F AAGAACTCGTTTGGCCAGTC
		HvPap-21R TACGAGTTGATGCTGCACAG
HvPap-22	AK354649	HvPap-22F AGAACGAGTTCACCGACCTTAC
		HvPap-22R ATCTCCGGCCAAAGTAGTGATG
HvPap-23	BI948364[b]	HvPap-23F TGGAGCTGTTAGTCGAGTCAAG
		HvPap-23R TTCCACTGCTGCAATTGCTG
HvPap-24	MLOC_70665.1	HvPap-24F ACAGCAACGAAAGGGCTTTG
		HvPap-24R TGTGGGTGGTGGTTTTGTTG
HvPap-25	CAJW010136629[c]	HvPap-25F AGTACTGGCTGGTGAAGAACTC
		HvPap-25R TTTCGCCATCTTCGGGTAGAC
HvPap-26	MLOC_4941.2	HvPap-26F TTCTTGCTGCTAGGCTTTGC
		HvPap-26R ACCAGGAGCTGAAGAGATCATG
HvPap-27	MLOC_3692.1	HvPap-27F TTCACCGGGTTCAACAACAC
		HvPap-27R TGAAACTTGATGCCGGTGAC
HvPap-28	MLOC_25346.1	HvPap-28F TCACAGGTTCCAACAACACC
		HvPap-28R ACAAGCAAGTGCCCTGAAAC

HvPap-29	MLOC_68042.1	HvPap-29F ACAAGCTGTTGTTCGACCAC
		HvPap-29R TAGAACTGGAACTCCCACGTG
HvPap-30	AK368614	HvPap-30F GCTGCTCGTCGTCCTCTC
		HvPap-30R AGTTTGCGAGGTATGGGTTG
HvPap-31	AK371580	HvPap-31F AAACAACAACGCCGTTACCC
		HvPap-31R ATGGCGTGGAAACCTTCAAC
HvPap-32	AK370699	HvPap-32F ATGCCGGCTTCAACAAGAAC
		HvPap-32R TTTTTGACACCGCTGACAGC
HvPap-33	MLOC_67087.1	HvPap-33F AGTCAAGCACCAAGGTCACTG
		HvPap-33R CTGCTGCTCTGACATGGAAATG
HvPap-34	MLOC_54413.1	HvPap-34F AATCCACGGGTGACAAGTACTG
		HvPap-34R AAGGATTTTGCGCTGCATGC
HvPap-35	MLOC_70133.1	HvPap-35F TACTTCATCAAGACGGGGAAGC
		HvPap-35R TCTCCATGATCCACTGGAAAGC
HvPap-36	MLOC_25316.1	HvPap-36F AAGGGAAATGCGGATGTTGC
		HvPap-36R TTCTTCCACCATTGCAACCG
HvPap-37	MLOC_11887.1	HvPap-37F AAGGTCAGTGCAAAGGGAAC
		HvPap-37R ACACTTCGCTGTTGCTCTTG
HvPap-38	MLOC_55561.1	HvPap-38F TTTCGATCGCGTCAAACAGC
		HvPap-38R TGCATGTGTCAGCTTAGTGG
HvPap-39	AK371477	HvPap-39F AACCGGCAAACTCGTTTCAC
		HvPap-39R TTAAACCACCGTTGCAACCC
HvPap-40	MLOC_54873.1	HvPap-40F ATCCTTGTGGGCGTTGTATG
		HvPap-40R TGCGTGCATTCTCCTTGAAC
HvPap-41	MLOC_69737.1	HvPap-41F ACAAGCGTGAAGACACAAGG
		HvPap-41R ATTGTTGCGCAGACAACGTC
HvPap-42	AK248416.1	HvPap-42F TTCGCCGGCTCCCGCGTCAA
		HvPap-42R TGGCGCCAGTCCACGGACCG

[a] Barley genome, annotated genes (http://www.public.iastate.edu/~imagefpc/IBSC Webpage/IBSC Template-seq resources.html)
[b] Barley Gene Index reference number (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=barley)
[c] Morex genomic reference number (http://www.public.iastate.edu/~imagefpc/IBSC Webpage/IBSC Template-seq resources.html)



CHAPTER 4.
HvPap-1 C1A protease actively participates in barley proteolysis mediated by abiotic stresses

4.1. INTRODUCTION

Plants respond to different environmental stresses by reprogramming the expression of subsets of genes depending on each stress-promoting feature. Once these molecular events are initiated, a complex physiological network is activated, triggering metabolic pathways that finally impact on the plant physiology (Suzuki *et al.,* 2014; Zmienko *et al.,* 2015). Generally, to overcome abiotic/biotic stresses, plants develop alternative strategies by inducing premature senescence and early flowering. Plant proteolysis associated with these physiological processes is essential for plant survival by promoting recycling of nutrients from stressed tissues to growing or sink tissues. As an outcome, a massive degradation of macromolecules, dismantling of cellular structures, mainly chloroplasts, and the subsequent mobilization of mineral nutrients and N-containing molecules to sustain further growth and development are observed (Hörtensteiner, 2007; Diaz-Mendoza *et al*., 2014). Up-regulation of proteases, from plastidial and nuclear genomes, are needed for protein breakdown throughout stress responses, implying a complex traffic of proteins, peptides and amino acids among subcellular compartments (Roberts *et al.,* 2012; Carrion *et al.,* 2013; Diaz-Mendoza *et al.,* 2014). In particular, Cysteine Proteases (CysProt) of the C1A papain family are the predominantly up-regulated plant proteases (Roberts *et al.,* 2012; Diaz and Martinez, 2013; Diaz-Mendoza *et al.,* 2014). C1A CysProt genes are strongly expressed in response to multiple stresses, such as darkness, drought, nutrient starvation, extreme temperatures, salt and pest and pathogen attack (Rabbani *et al.,* 2003; Parrott *et al*., 2010; Guo and Gan, 2012; Diaz-Mendoza *et al.,* 2014; Kempema *et al.,* 2015). However, changes in protease gene expression do not necessarily lead to changes in protease activity, probably due to parallel alterations in the expression of genes encoding protease inhibitors (Diaz-Mendoza *et al.,* 2014; Kidric *et al.,* 2014).

The role of C1A proteases in response to abiotic stresses is clearly shown in Arabidopsis plants constitutively expressing CysProt genes from wheat (*TaCP*) and sweet potato (*SPCP2*). Over-expressing plants showed enhanced tolerance to drought and/or salt stress and higher enzymatic activity than the wild-type (WT) plants (Chen *et al.,* 2010; Zang *et al.,* 2010). In contrast, the sweet potato *SPCP3* CysProt ectopically

expressed in Arabidopsis plants caused altered phenotypic traits and increased sensitivity to drought stress (Chen *et al.,* 2013). Furthermore, suppression of the *CaCP* protease in pepper plants retarded salt- and osmotic-induced leaf stress responses (Xiao *et al.,* 2014), and expression of an antisense construct of the cathepsin H-like protease gene *BoCP5* delayed floret senescence in broccoli (Eason *et al.,* 2005). Altogether, these data suggest that different CysProt may play opposite roles in the same or similar physiological processes. In this context, the interplay between CysProt and their specific inhibitors, cystatins, is very relevant and remains subjected to a complex regulatory crosstalk dependent on the specific treatment to induce plant stress. In the last decades, transgenic expression of phytocystatins has been used to improve plant behaviour under biotic and abiotic stresses although little has been published about the pathways involved (Martinez *et al*., 2009; 2012). Recently, transgenic soybean and Arabidopsis plants over-expressing a rice cystatin displayed enhanced drought tolerance by altering strigolactone pathways (Quain *et al.,* 2014).

Most abiotic stresses are closely related to plant senescence and activate the down-regulation of genes involved in the photosynthetic process in parallel to the up-regulation of genes responsible for chlorophyll breakdown in chloroplasts (Gregersen *et al.,* 2008; Krupinska *et al*., 2012). Chloroplasts regulate the onset of plant senescence by generating reactive oxygen species (ROS) and modifying the oxidative state of electron transporters (Keech *et al*., 2007; Queval and Foyer, 2012, Baxter *et al.,* 2014). In addition, genes associated with mitochondrial electron transport, β-oxydation of fatty acids, glutamine and asparagine synthesis and nucleic acid fragmentation are also up-regulated in senescent leaves (Buchanan-Wollaston, 1997; Yang and Ohlrogge, 2009; Avila-Ospina *et al.,* 2014; Christiansen and Gregersen, 2014; Orsel *et al.,* 2014; Sakamoto and Takami, 2014).

In cereals, abiotic stresses induce degradation of leaf nitrogen-containing macromolecules (mainly proteins) to free amino acids (glutamate, glutamine and asparagine, among others) prior to be phloem loaded and transported to developing grains. This process provides most of the nitrogen needed for grain filling. Particularly, chloroplast proteins including Rubisco (D-Ribulose-1,5-bisphosphate

carboxylase/oxygenase), Calvin cycle enzymes and proteins involved in photosynthetic light reactions are the main targets for proteases and represent the first source of transportable nitrogen (Masclaux-Daubresse *et al*., 2007; 2010; Feller *et al.,* 2008). Variations in the stress type and timing may disturb the active photosynthetic period leading to alterations in efficient N and micronutrient mobilization and, therefore, affecting crop quality and yield (Gregersen *et al.,* 2013; Distelfeld *et al.,* 2014). Small-grain cereals such as barley, wheat and rice, mobilize up to 90% of the nitrogen from vegetative tissues to the grains (Gregersen *et al.,* 2008). Understanding these proteolytic pathways, in particular chloroplastic protein degradation, may extend the photosynthetic period, as occurs in "stay green" phenotypes, and modify the total nitrogen content of the grain. Thus, the manipulation of key factors involved in protein remobilization such as C1A proteases, seems a promising strategy to improve the outcome of senescence.

Barley is an excellent model for studying proteolysis induced by stress within monocots according to the numerous research analyses focused on this species and the comprehensive knowledge of the whole C1A CysProt family (Martinez *et al*., 2009; Diaz-Mendoza *et al*., 2014). Besides, barley has been recently considered a translational model for adaptation to climate change (Perez-Lopez *et al*., 2012; Dawson *et al.,* 2015).

In this work, we demonstrate the contribution of barley C1A proteases in the proteolytic processes induced in leaves by darkness and nitrogen starvation abiotic treatments. We have generated barley transgenic plants over-expressing and silencing the stress-induced HvPap-1 cathepsin F-like protease to characterize the stress-response features triggered by the biotechnological modification of proteolytic pathways. A senescence-delayed phenotype observed in *HvPap-1* silenced lines during natural and induced-senescence entails the big potential to this genetically engineered plants.

4.2. MATERIALS AND METHODS

4.2.1. PLANT MATERIAL AND GROWTH CONDITIONS

Grains of barley (*Hordeum vulgare* L. cv "Golden Promise") were germinated in trays with vermiculite (to induce severe stress conditions) or soil (to induce moderate stress conditions), daily watered and incubated at 22ºC under 16 h light/8 h dark photoperiod for 7 d in Sanyo MLR-350-H chambers. 7 d-old plants were incubated in continuous darkness at 22ºC or with 16 h light/8 h dark photoperiod (control plants). In parallel, 7 d-old plants were grown in pots filled with vermiculite and daily watered with complete Hoagland nutrient solution (Hoagland, 1920) for control plants, or with Hoagland nutrient solution without any N source to induce severe senescence, at 22ºC and 16 h/8 h light/dark photoperiod. Whole plant leaves were harvested after 3 and 7 d of treatment (darkness, nitrogen starvation and control), frozen into liquid nitrogen and stored at -80ºC for further analysis.

Barley transgenic lines over-expressing or silencing the barley *HvPap-1* gene (OE Pap1 and KD Pap1, respectively) were generated in collaboration with the IPK-Gatersleben, Plant Reproductive Biology Group and molecularly analyzed as described by Diaz-Mendoza *et al.* 2016. Transgenic plants were grown in soil under the same temperature and photoperiod conditions described above. Moderate stress mediated by darkness was induced in 7 d-old transgenic and control plants grown in soil as described above. Whole plant leaves were harvested after 7, 14 and 21 d of treatment (darkness/control), frozen into liquid nitrogen and stored at -80ºC for further analysis.

4.2.2. PHOTOSYNTHETIC PIGMENT MEASUREMENTS

Chlorophyll *a* and *b*, total chorophyll and carotenoids (xanthophylls and carotenes) were quantified in WT and transgenic lines incubated under abiotic stresses and control conditions. 100 mg of leaves were ground in a mortar with liquid nitrogen and suspended in 15 ml of 80% (v/v) acetone in photo-protected tubes. After centrifugation at 13 000 *g* for 2 min, the absorbance of 1 ml of the supernatant was

measured at 470, 663 and 646 nm, for carotenoids, chlorophyll *a* and chlorophyll *b*, respectively, using a UV-vis spectrophotometer (UltroSpec™ 3300pro, Amersham Bioscience). Pigment content was calculated using the extinction coefficients and equations determined by Lichtenthaler (1987).

4.2.3. PROTEIN QUANTIFICATION AND PROTEASE ACTIVITIES

Total soluble proteins were extracted from treated and control leaves by grinding plant tissues into liquid nitrogen before the addition of 500 µl of extraction buffer (150 mM NaCl, 50 mM sodium phosphate, pH 6 and 2 mM EDTA). After centrifugation at 16 300 *g* for 10 min at 4ºC, the supernatant was used for protein quantification according to the method of Bradford (1976), with bovine serum albumin as standard.

Protease activities were assayed by measuring the hydrolysis of substrates containing the AMC (7-amino-4-methyl coumarin) fluorophore carried out in microtiter plate format. The standard assay volume was 100 µl, using 5 µg of barley protein extract and the corresponding substrate added to a final concentration of 0.25 mM. Cathepsin B-like and L-/F-like activities were assayed using Z-RR-AMC (*N*-carbobenzoxy-Arg-Arg-AMC) and Z-FR-AMC (*N*-carbobenzoxy-Phe-Arg-AMC) substrates, respectively. For these CysProt a buffer containing 0.1 M of sodium phosphate pH 6.5, and 10 mM cysteine, 10 mM EDTA and 0.1% (v/v) Brij 35 was used. Legumain-like activity was determined using the substrate Z-AAN-AMC (*N*-carbobenzoxyloxy-Ala-Ala-Asn-7-AMC) at 100 µM concentration, in 50 mM HEPES buffer (pH 7.5) containing 2.5 mMdithiothreitol (DTT). Trypsin-like activity was analyzed using Z-R-AMC (*N*-carbobenzoxy-Arg-AMC) and elastase-like activity using MeOSAAPV-AMC (MeOSuc-Ala-Ala-Pro-Val-AMC) in buffer Tris-HCl 0.1 M, pH 7.5. The reaction was incubated at 30ºC for 1 h and emitted fluorescence was measured with a 365 nm excitation wavelength filter and a 465 nm emission wavelength filter. All assays were carried out in triplicate. Blanks were used to account for spontaneous breakdown of substrates and results were expressed as nmol of hydrolysed substrate per mg of protein per min (nmol mg^{-1} min^{-1}). The system was calibrated with known amounts of AMC in a standard reaction mixture.

4.2.4. REAL-TIME RT-QPCR ANALYSIS

Total RNA was extracted from frozen barley leaves by the phenol/chloroform method, followed by precipitation with 8 M LiCl (Oñate-Sanchez and Carbajosa, 2008) and digestion with DNase. cDNAs were synthesized from 2 µg of RNA using High Reverse Transcription kit (Applied Biosystems) following the manufacturer's instructions. RT-qPCR analyses were performed by triplicate samples by means of a CFX96 Real-time system (BioRad) using the SYBR Green detection system. Quantification was standardized to barley cyclophilin (*HvCycl*) mRNA levels following Diaz-Mendoza *et al.* (2014). The primers used are shown in Supporting Information Table S 4.1.

4.2.5. IMMUNOBLOT ANALYSIS

Protein extracts were prepared from frozen leaf samples as indicated above. After separation on SDS-polyacrylamide gels (12%, w/v) according to Laemmli (1970), proteins were electro-transferred onto nitrocellulose membrane (GE Healthcare) and blocked in PBS (phosphate buffered saline) antisera buffer containing 5% (w/v) powdered skim milk, for 3 h. Immunoblotting was performed with anti-peptide polyclonal antibodies specifically selected against each protease. Supporting Information Table S 4.2 shows the peptide sequences used to get the antibodies. All protease antibodies were produced in rabbits by Pineda Antibody Services. Polyclonal antibody against the Large Subunit of Rubisco (anti-LSR) was supplied by Agrisera. Optimal dilutions of primary antibodies were adapted to each protease. Peroxidase-conjugated anti-rabbit IgG (Sigma) diluted at 1:10,000 (v/v) was used as secondary antibody for detection with ECL Plus (GE Healthcare).

4.2.6. SPECIMEN PROCESSING FOR MICROSCOPY

Thin strips of leaves cut from control and senescence-induced barley plants were fixed in a freshly prepared solution of 4% (w/v) formaldehyde in PBS first at room temperature under vacuum until the specimens sank, then overnight at 4ºC. Samples were washed in PBS and dehydrated in a series of increasing concentrations of

methanol in water as follows: methanol 30%, 50% and 70% (v/v) for 30 min each and methanol 100% for 90 min (with 3 changes) at 4ºC. Specimens were progressively infiltrated in LRwhite resin (Agar Scientific) in series of mixtures methanol:LRwhite (v/v) with increasing concentrations of the resin (2:1; 1:1; 1:2) for 1 h each at 4ºC, then let in pure resin with 0.5% (v/v) benzoin- methyl- ether as a catalyst, overnight at 4ºC. Polymerisation in capsules was performed under UV light at -20ºC for 2 days and at 22ºC for 1 day. 1-2 µm thin sections were cut from the polymerised blocks in a Leica EM UC6 ultramicrotome.

4.2.7. STRUCTURAL ANALYSIS AND IMMUNOFLUORESCENCE DETECTION OF HVPAP-1, HVPAP-16 AND HVPAP-19

Thin sections of 1-2 µm were carefully collected on water drops on 10-well Teflon-printed slides (Fisher Scientific Inc.), let dry down and stored at room temperature until further use. To assess any possible structural rearrangements at the subcellular level in the stressed samples *vs* the controls, the sections were stained with 0.05% (w/v) toluidine blue O (TBO, Panreac), rinsed in distilled water, mounted and observed on a Zeiss Axiophot microscope under bright field. Photographs were taken with a Leica DFC300 FX CCD camera using the Leica Application Suite 2.8.1 build 1554 acquisition software.

For immunofluorescence the sections on the 10-well slides were hydrated with PBS for 5 min and unspecific binding sites were blocked by 10 min incubation with 5% (w/v) BSA (bovine serum albumin) in PBS. Then, they were incubated with 20 µl drops/well of either a rabbit-raised antibody to the CysProt (HvPap-1, HvPap-16, HvPap-19) applied 1:50 (v/v) in PBS, for 1 h at room temperature in a humid chamber. After two washes of 15 min in PBS, an Alexa Fluor 488 anti-rabbit antibody (Molecular Probes) was applied in a 1:25 solution in 2.5% (w/v) BSA in PBS, for 45 min at room temperature in a humid chamber and darkness. Subsequent to another two washes of PBS for 15 min each, the slides were mounted in a 50:50 (v/v) solution of glycerol/PBS.

4.2.8. CONFOCAL IMAGING OF HVPAP-1, HVPAP-19 AND HVPAP-16

Serial sections were collected on a Leica SP8 confocal microscope using the laser excitation lines of 488 nm (to detect the proteases) and 633 nm (to detect the red auto-fluorescence from the chlorophyll). All series were captured under the same conditions (pinhole size, gain, offset, magnification). The management of the series was performed with either the LAS-AF-Lite 3.1.0_8587 or Fiji software. To composite the corresponding figure in Adobe Photoshop CS3, the maximum projections of the green and red channels were overlaid for each treatment shown. Only the automatic levels were adjusted.

4.2.9. STARCH QUANTIFICATION

Thirty mg of fresh leaves from transgenic and WT barley lines darkness-treated were used for total starch quantification with STA20 Kit (Sigma) following the manufacturer´s recommendations. Dilutions were carried out as necessary to fit into linearity of the glucose standard curve. Measurements were performed six times for each sample. After calculations, starch content was expressed as grams of transformed starch per 100 grams of initial fresh weight.

4.2.10. DATA ANALYSIS

Statistical differences among treatments and/or lines were analyzed by one-way ANOVA followed by Tukey´s (HSD) multiple comparison test performed using the soft R Project (v.3.1.2) package.

4.3. RESULTS

4.3.1. STRUCTURAL AND PHYSIOLOGICAL CHANGES IN LEAVES UNDER SEVERE STRESSES

For comparison purposes two stress treatments were firstly used to analyze barley leaf responses. 7 d-old plants were grown in vermiculite under continuous darkness or in

hydroponic cultures without any N source, for 3 and 7 d. Chlorophyll, carotenoids and protein contents were analyzed as standard parameters of abiotic stress. A significant decrease in the chlorophyll level was observed in darkness- and N-depleted leaves, at both time points. This effect was particularly striking in plants grown under darkness conditions in comparison to controls (Fig. 4.1A,B). Similarly, a significant reduction in the chlorophyll/carotenoids ratio was observed after 7 d of treatment, which was particularly conspicuous in leaves grown under N starvation. The level of total soluble proteins was also reduced in the treated plants *vs* controls (Fig. 4.1A,B). An early yellowing phenotype found in darkness- and N-starved leaves was mainly observed in the leaf apex (Supplementary Fig. S 4.1), paralleled strong subcellular rearrangements in the mesophyll, as seen on toluidine blue O (TBO)-stained sections (Fig. 4.1C). After 7 d under darkness, the chloroplasts looked smaller and round-shaped losing their typical peripheral location within the cell and forming aggregates. Under N starvation, small chloroplasts kept their peripheral position although assumed a rather spherical shape with spaces among them (Fig. 4.1C). All these phenotypic, cell structure and physiological parameters confirm strong alterations associated with severe senescence occurring under darkness and N starvation treatments.

4.3.2. C1A PROTEASES AND PROTEOLYTIC PATTERNS ARE MODIFIED IN BARLEY LEAVES UNDER INDUCTION OF SEVERE SENESCENCE

The activity of two main protease groups, cysteine- and serine-proteases, was analyzed using specific substrates for cathepsin L-/F- and B-like and legumain (CysProt) or trypsin and elastase (serine-proteases), respectively. Members of both protease groups, in particular trypsin, cathepsin L-/F- and B- like participated in the degradation of leaf proteins. Only cathepsin L-/F- and B- like activities were significantly increased in senescent leaves (up to 2.5- and 3.4-fold under 7 d of darkness and N starvation treatment, respectively) compared to the non-treated controls (Fig. 4.2).

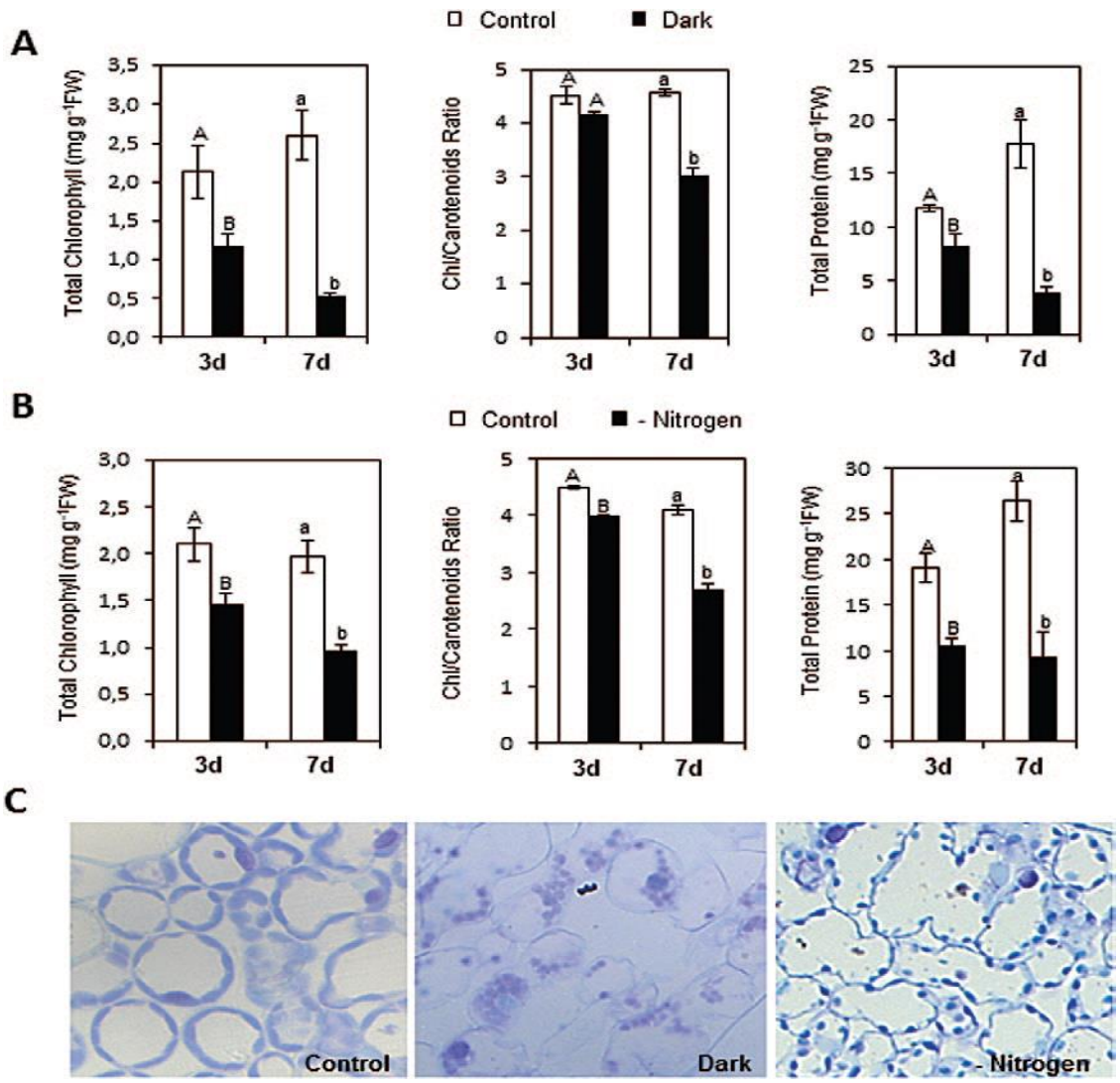


Figure 4.1. Biochemical and cell structural changes in barley leaves after severe stress treatments. (A) Chlorophyll content, chlorophyll/carotenoids ratio and total protein content of barley leaves grown in vermiculite under continuous darkness or with 16 h/8 h photoperiod for 3 and 7 days. Data are means ± standard error of six measurements. (B) Chlorophyll content, chlorophyll/carotenoids ratio and total protein levels of barley leaves grown in Hoagland nutrient solution with or without N source for 3 and 7 days. Data are means ± standard error of six measurements. Significant differences between control and treated plants are indicated with capital letters (3 d) and small letters (7 d) ($P < 0.05$, HSD). (C) Structural cell changes of barley leaves grown under continuous darkness, N starvation or control conditions for 7 d. Leaves were stained with 0.05% (w/v) toluidine blue O and observed on a Zeiss Axiophot microscope under bright field.

The expression profile of the 41 C1A CysProt barley genes and the 13 genes encoding cystatins in 7 d of darkness-induced leaf senescence was previously assessed by quantitative Real Time-PCR (RT-qPCR) by our group (Diaz-Mendoza *et al.*, 2014).

Based on these data, three cathepsin L-like (*HvPap-4, -6* and *-16* genes), one cathepsin F-like (*HvPap-1* gene), one cathepsin H-like (*HvPap-12* gene) and one cathepsin B-like (*HvPap-19* gene) were selected to study their expression under stresses induced by continuous darkness and by the absence of N at 3 and 7 d. The results, expressed as mRNA levels normalized to the constitutively active barley *cyclophilin* gene, revealed that the cathepsin F-, B- and H-like genes were highly induced in stressed leaves *vs* controls. This induction was dependent on the treatment with some differences on the expression time course. Small alterations on the expression of *HvPap-4*, *HvPap-6* and *HvPap-16* cathepsin L-like were also detected after darkness and N deprivation (Fig.4.3A,B).

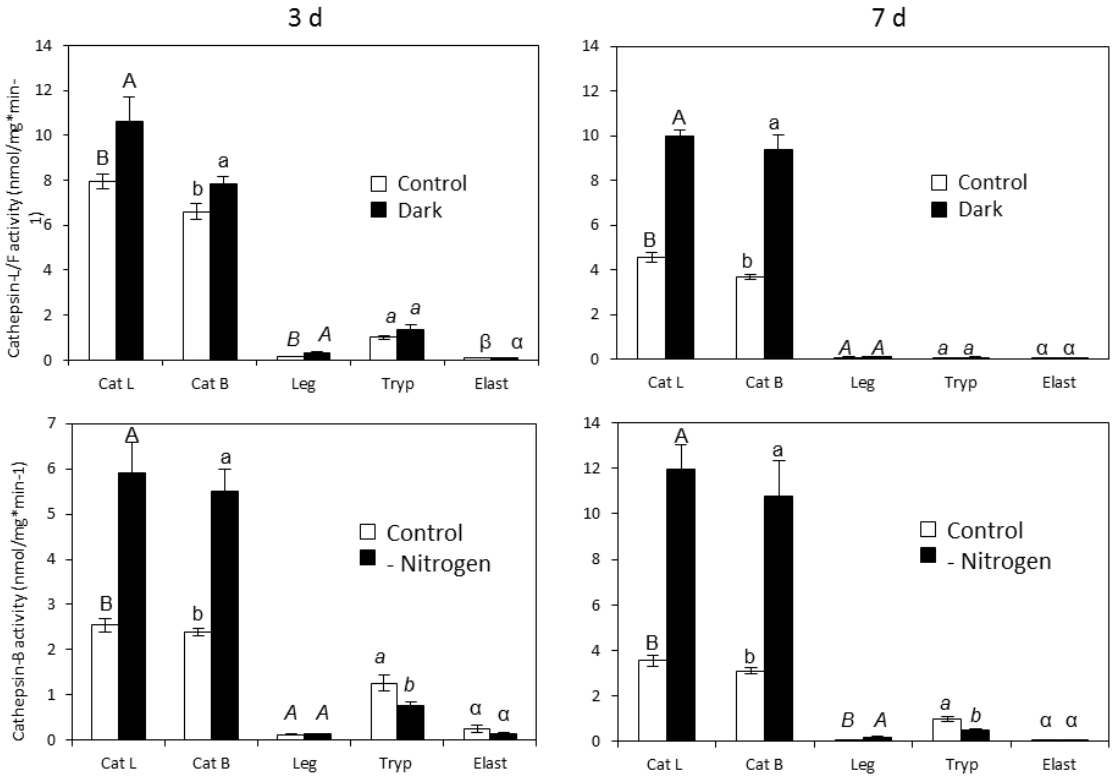


Figure 4.2. Proteolytic patterns of barley leaf after stress treatments. Proteolytic activities of barley leaves grown in vermiculite under continuous darkness or with 16 h/8 h photoperiod, or grown in Hoagland nutrient solution with or without N source for 3 and 7 d. Specific substrates to be degraded by cathepsin L-/F-like (Cat L/F) and B-like (Cat B), legumain (Leg), trypsin (Tryp) and elastase (Elast) were used. Data are means ± standard error of six measurements. Significant differences between control and treated plants are indicated with different letters ($P < 0.05$, HSD).

Immunoblot assays using antibodies against peptides of the HvPap-1, HvPap-16 and HvPap-19 CysProt were performed after checking peptide specificity to avoid cross-reactivity (Diaz-Mendoza *et al.,* 2016). Results from immunoblots pointed out a link between transcript and protein accumulation patterns in most of the samples (Fig. 4.3). As previously reported by Cambra *et al.* (2012), the protein profile of HvPap-1 showed two bands of different size corresponding to the immature protein (40 kDa), the same size of the inactive recombinant protein expressed in *Escherichia coli,* and the mature processed form (26 kDa). Both bands increased their signal in senescing leaf samples at 7 d of treatment. A similar induction pattern was observed for the active form of the HvPap-19 cathepsin B-like protein while no differences were detected for HvPap-16. The two treatments (darkness and N starvation) also altered Rubisco levels in barley leaves. A clear depletion of the Rubisco enzyme was found by a specific antibody against its large subunit (LS), more strikingly in leaves grown under 7 d of continuous darkness (Fig. 4.3C).

To analyze the subcellular localization of these C1A CysProt in stressed leaves, immunofluorescence was performed with the same specific antibodies used for immunoblot experiments. The maximum projection of confocal Z series is shown in Fig. 4.4. Green fluorescence signal from the CysProt HvPap-1, HvPap-19 and HvPap-16 was mainly localized to the epidermis in control leaves (Fig. 4.4A-C). After 7 d without any nitrogen source the labelling of HvPap-1 and HvPap-19 was observed in a high number of small vesicles within mesophyll cells (Fig. 4.4D,E). As seen on TBO-stained samples (Fig. 4.1C), the chloroplasts looked more spherical and smaller than those from the controls. Besides, their distribution was different, with large spaces between them at the periphery of the cell. A more striking phenotype was observed after 7 d in darkness. HvPap-1 and HvPap-19 CysProt localized to larger patches and the chloroplasts clustered together (Fig. 4.4G,H). The red auto-fluorescence from the chlorophyll was weaker in the senescence-induced specimens than in the controls. The localization pattern of HvPap-16 was restricted to epidermal layers in both treatments (Fig. 4.4F,I), as in the controls.

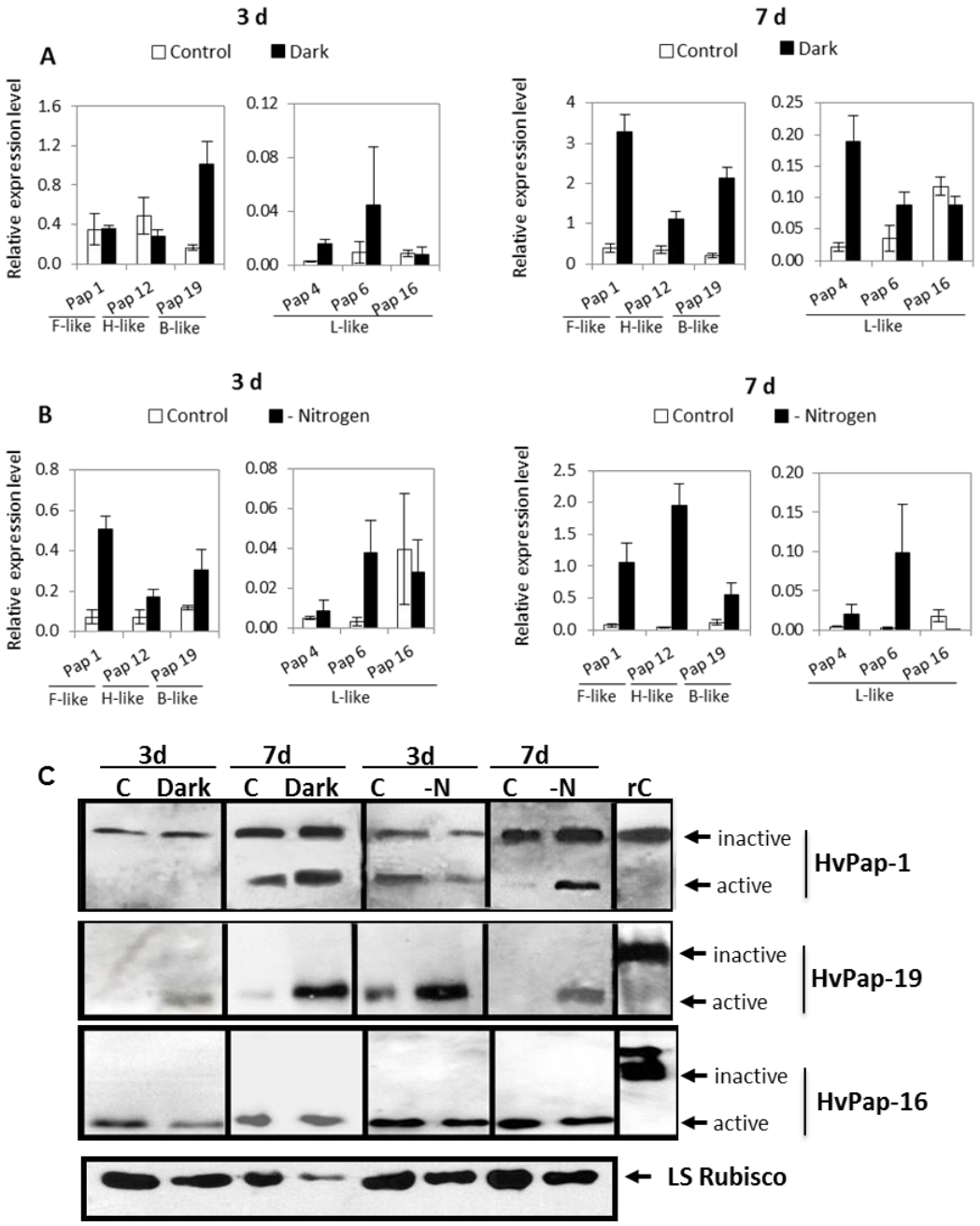


Figure 4.3. Transcripts and protein levels of C1A CysProt (cathepsin F-, H-, B- and L-like) in barley leaves after severe stress treatments. (A) Expression of *CysProt* genes (*HvPap-1, -4, -6, -12, -16* and *-19*) in leaves grown in vermiculite under continuous darkness or with 16 h/8 h photoperiod, for 3 and 7 d. (B) Expression of *CysProt* genes (*HvPap-1, -4, -6, -12, -16* and *-19*) in leaves grown in Hoagland nutrient solution with or without N source, for 3 and 7 d. Data were determined by RT-qPCR and expressed as relative mRNA levels of *C1A CysProt* genes normalized to barley *cyclophilin* mRNA content. (C) Protein accumulation pattern of CysProt in barley leaves after severe stress treatments using immunoblot assays. Recombinant CysProt proteins purified from *E. coli* cultures (rC) were used as control size. Molecular bands corresponding to inactive and active forms of CysProt are indicated by arrows. Rubisco protein pattern was analyzed using a specific antibody against its Large Subunit (LS Rubisco).

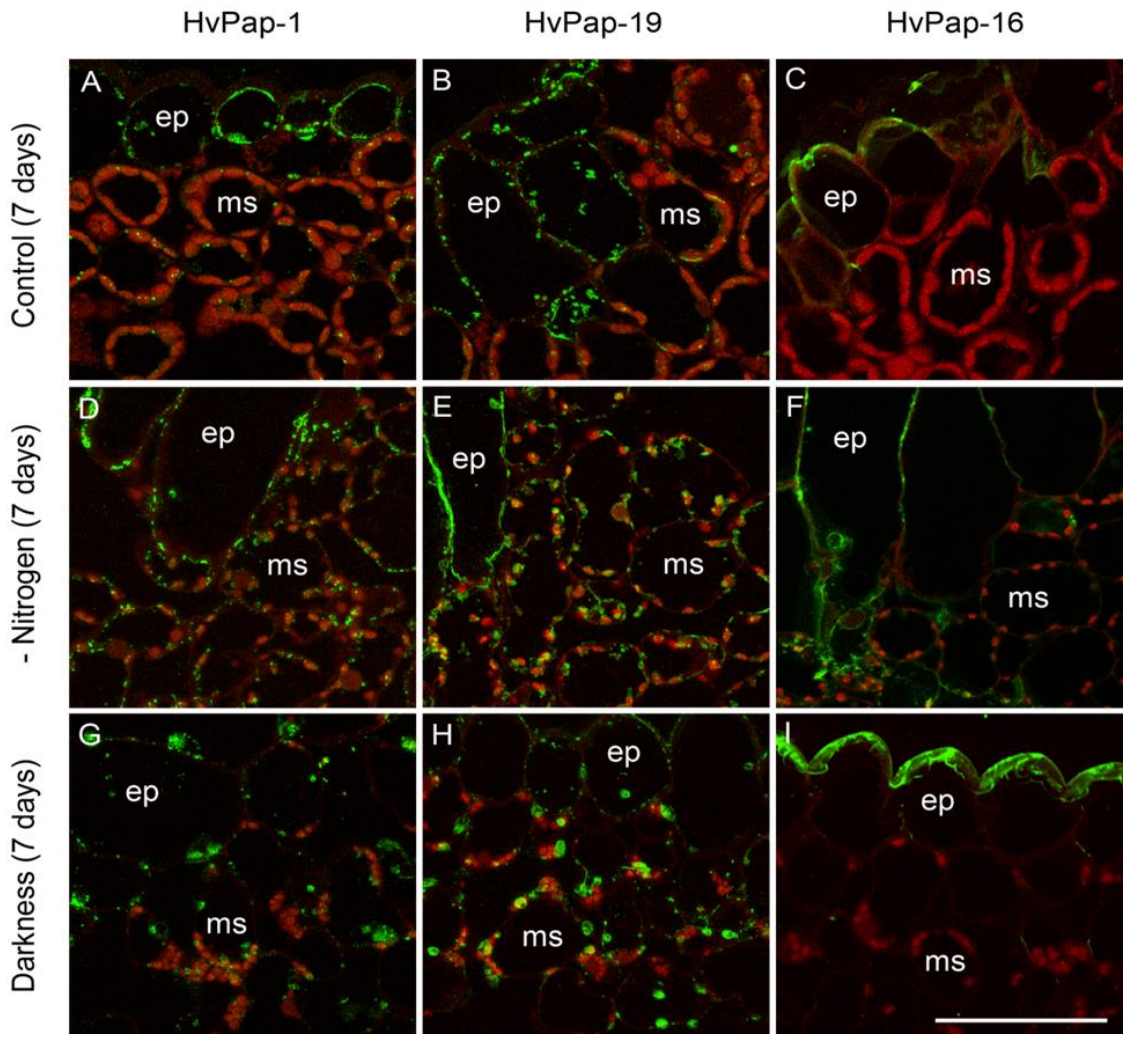


Figure 4.4. Overlay of maximum projections of confocal series during the immunofluorescence localization of CysProt (green) and the detection of chlorophyll auto-fluorescence (red) in old barley leaves after 7 d of darkness, N starvation or control treatments. CysProt HvPap-1 (A, D, G), HvPap-19 (B, E, H) and HvPap-16 (C, F, I). Epidermal cells (ep); mesophyll cells (ms). Bar: 50 µm.

4.3.3. TRANSGENIC BARLEY LINES OVER-EXPRESSING OR SILENCING THE HVPAP-1 CYSPROT

To investigate the *in vivo* behaviour of C1A CysProt, the *HvPap-1* gene was selected based on its high expression levels induced by the described stress treatments and because it is a member of the barley cathepsin F-like family, which has been thus far poorly studied in plants. For that, transgenic plants overexpressing (OE Pap1 lines) or silencing (KD Pap1 lines) the *HvPap-1* gene previously generated were analyzed (Diaz-

Mendoza *et al.,* 2016). The phenotypes of the selected transgenic lines revealed differences throughout the plant growth cycle in comparison to WT plants since the first stages of development (Supplementary Fig. S 4.2). OE Pap1 lines presented early yellowish symptoms in the apex of the oldest leaves at 3 wk of growth while evidences of stress were not observed either in KD Pap1 lines or in the WT plants at this time point (Supplementary Fig. S 4.2). After 5 wk, OE Pap1 lines showed lower numbers of green leaves than the silencing or WT ones and this feature became more prominent in the following wk. At 9 wk, when the spikes had appeared, a clear delay in natural senescence in the KD Pap1 lines respect to the over-expressing and non-transformed plants was observed. This delayed-senescence phenotype of *HvPap-1* silenced lines was remarkable at 10-11 wk, when KD Pap1 1175 and 1130 lines still maintained green leaves whereas OE Pap1 lines and WT plants turned yellow and completely dried-out (Fig. 4.5).

4.3.4. TRANSGENIC BARLEY HVPAP-1 LINES SHOW ALTERATIONS ASSOCIATED WITH STRESS MEDIATED BY DARKNESS

The implication of the studied CysProt in response to darkness was analyzed by comparing transgenic and non-transgenic lines. To mimic natural conditions, experimental plant growth parameters were slightly modified with respect to the severe treatment previously used. 7 d-old plants were grown in soil and then subjected to continuous darkness for 3, 7, 14 and 21 d. As expected, leaves from dark treated plants were shorter than those grown under photoperiod conditions. After 21 d of darkness, the leaves were much smaller than in the controls and presented severe damages (Supplementary Fig. S 4.3). These phenotypic observations and the determination of standard parameters associated with stress (chlorophyll, carotenoids and proteins) led us to select the 14 d-time point for further molecular, biochemical

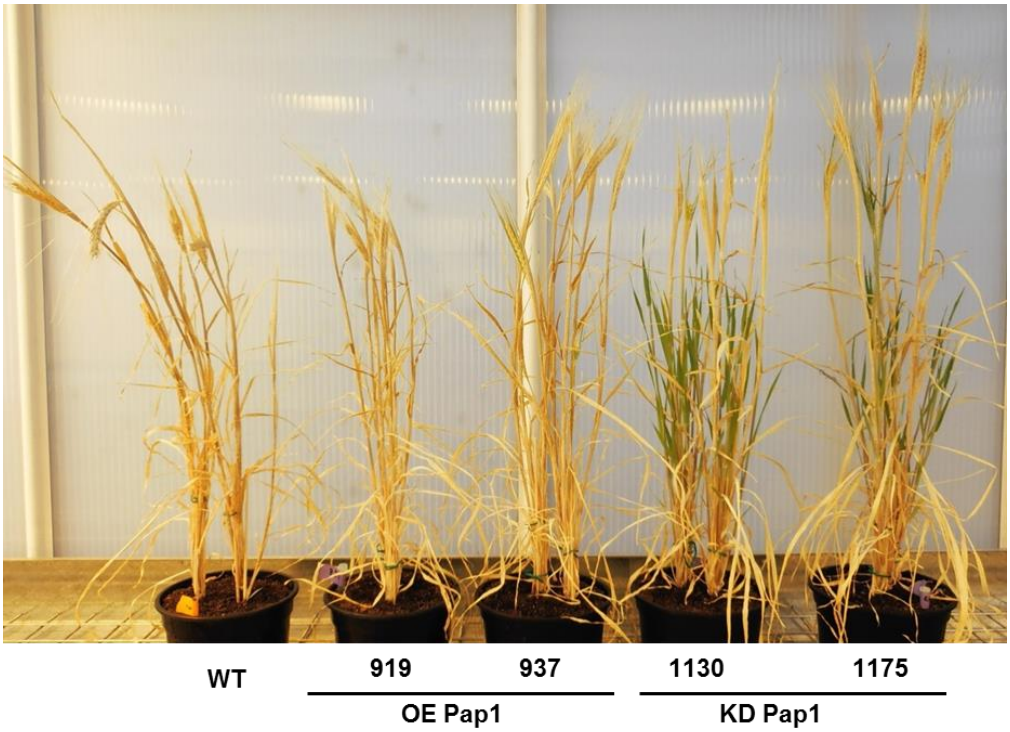


Figure 4.5. Natural senescence phenotypes of 10 wk-old *HvPap-1* over-expressing (OE Pap1: 919 and 937 lines), silencing (KD Pap1: 1130 and 1175 lines) and wild-type (WT) barley plants.

and physiological studies. According to Keech *et al.* (2007) and Zmienko *et al*. (2015), long term treatment of continuous darkness (2 wk) are suited to study protease impact on chloroplast degradation.

The phenotype of transgenic and control lines after 14 d of continuous darkness presented slower growth and shorter size than the same genotypes grown under dark/light photoperiod (Fig. 4.6). However, the most interesting observation was the absence of brownish symptoms in the oldest leaf of whole darkened KD Pap1 lines. In contrast, strong stressed phenotypes were detected in the oldest leaf apex of OE Pap1 lines as well as in the WT plants after 14 d of darkness (Fig. 4.6). The expression patterns of some CysProt in darkness-treated and control leaves from selected over-expressing and silencing lines were investigated by RT-qPCR assays. As shown in Supporting Information Fig. S 4.4, *HvPap-1* transcripts increased in plants grown under darkness independently of the transgene insertion, although messenger levels were lower in knock down lines than in over-expressing or WT lines. The mRNA profile for

other genes encoding CysProt was also analyzed in these transgenic plants. The expression of the *HvPap-19* gene was up-regulated in darkness-treated OE Pap1 and KD Pap1 lines. *HvPap-6* and *HvPap-12* genes presented similar expression patterns under dark and control conditions in most over-expressing and silenced *HvPap-1* lines. The *HvPap-16* gene was strongly repressed in response to darkness in all transgenic lines and WT (Supplementary Fig. S 4.4).

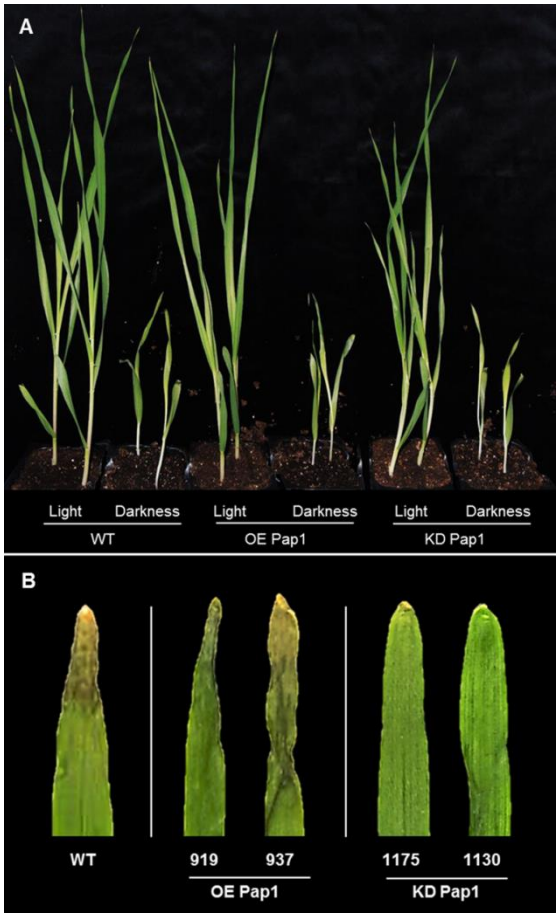


Figure 4.6. Phenotypes of barley plants grown in soil under darkness or light/dark photoperiod for 14 d. (A) Phenotype of whole plants grown under continuous darkness or 16 h/8 h photoperiod. (B) Detail of the oldest leaf apex grown under continuous darkness. Wild-type (WT), *HvPap-1* over-expressing (OE Pap1: 919 and 937 lines) and silencing (KD Pap1: 1130 and 1175 lines) barley plants.

HvPap-1, HvPap-19, HvPap-6 and HvPap-16 proteases were detected by immunoblot in protein extracts from control and dark-treated leaves of transformed and non-transformed plants, using specific antibodies. The HvPap-1 protein increased

not only in the over-expressing OE Pap1 lines in comparison with the WT, but also after the darkness treatment. In contrast, HvPap-1 diminished in all KD Pap1 lines (Fig. 4.7). A slight increase of HvPap-6 and HvPap-19 proteins was also observed in leaves grown under darkness. No alterations in the HvPap-16 protein levels were detected in OE Pap1 lines compared to WT plants. Nevertheless, a clear increase of the inactive form of the HvPap-16 protease (upper band) was detected in light-grown KD lines while this inactive form disappeared when plants were subjected to darkness. Additionally, Rubisco was slightly reduced in stressed leaves, being more pronounced in the over-expressing HvPap-1 lines (Fig. 4.7).

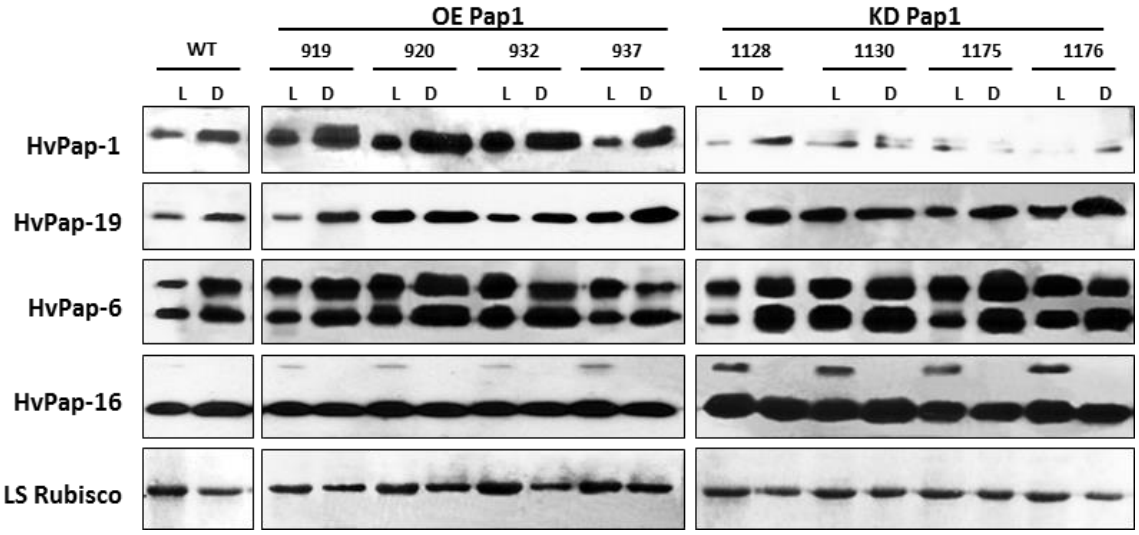


Figure 4.7. Protein patterns of C1A CysProt in transgenic and wild-type (WT) barley lines grown under darkness (D) or 16 h/8 h photoperiod (L) for 14 d and assayed by immunoblot. Proteins were extracted from leaves of WT, *HvPap-1* over-expressing (OE Pap1: 919, 920, 932, 937 lines) and silencing (KD Pap1: 1128, 1130, 1175, 1178 lines). Rubisco protein content was assayed using a specific antibody against its Large Subunit (LS Rubisco).

4.3.5. PHYSIOLOGICAL CHANGES ARE ASSOCIATED WITH STRESS MEDIATED BY DARKNESS IN HVPAP-1 TRANSGENIC BARLEY LINES

The total amount of soluble proteins was quantified in darkness-treated and non-treated transgenic lines as well as in non-transgenic controls. OE Pap1 lines did not show significant differences in protein content as compared to non-transgenic when grown under photoperiod but a slight protein reduction was appreciated in some

transgenic lines grown in darkness (Supplementary Fig. S 4.5A). By contrast, most KD Pap1 lines presented increased levels of protein in darkness-treated and non-treated leaves in comparison with their corresponding WT (Supplementary Fig. S 4.5B). Additionally, the proteolytic activity pattern (cathepsin L-/F- and B-like CysProt) of OE, KD and WT plants grown in the darkness or under control conditions was determined using specific substrates. No significant differences on the cathepsin L-/F-like activity were detected between transgenic and WT lines when plants were grown under light conditions. Under darkness, the cathepsin L-/F-like activity mostly decreased under darkness into KD lines (Supplementary Fig. S 4.6A). Similar data resulted from the measurements of cathepsin B-like activity (Supplementary Fig. S 4.6B).

Photosynthetic pigments were also determined in whole aerial biomass of the plants after 14 d of darkness *vs* control conditions. Differences in the chlorophyll *a* levels were nearly undetectable among OE, KD and WT when these plants were grown under photoperiod conditions. A decrease of chlorophyll *a* was observed in all darkness-treated leaves compared to the non-treated ones, particularly in the WT (Supplementary Fig. S 4.7A). Similar results were found for the quantification of carotenoids in most lines (Supplementary Fig. S 4.7C). In contrast, the amount of chlorophyll *b* was drastically reduced in treated and non-treated *HvPap1* amiRNA leaves in comparison with the WT and presented an undefined pattern in OE Pap1 lines (Supplementary Fig. S 4.7B).

Additionally, the total chlorophyll content of the oldest leaf was observed after 14 d of stress treatment, by detecting its auto-fluorescence under the confocal microscope. Since the apex of the leaf is older than the medium/basal part (segments 1 and 2, respectively, in Fig. 4.8), the highest chlorophyll fluorescence was generally found in the non-stressed segment 2 (Fig. 4.8). A lower fluorescence emission was detected in OE Pap1 lines than in WT. In contrast, auto-fluorescence levels were increased in the KD Pap1 lines. Similar patterns, although less remarkable, were found in leaf tissues grown under darkness which correlated with alterations in the tissue structures observed under bright field (Fig. 4.8). Besides, Table 4.1 shows that the total amount of starch in OE Pap1 and KD Pap1 transgenic leaves grown under photoperiod

was approximately half of the starch amount in WT leaves. As expected, the starch accumulation was strongly reduced in 14 d dark-treated leaves but no remarkable differences were detected between transgenic and WT samples.

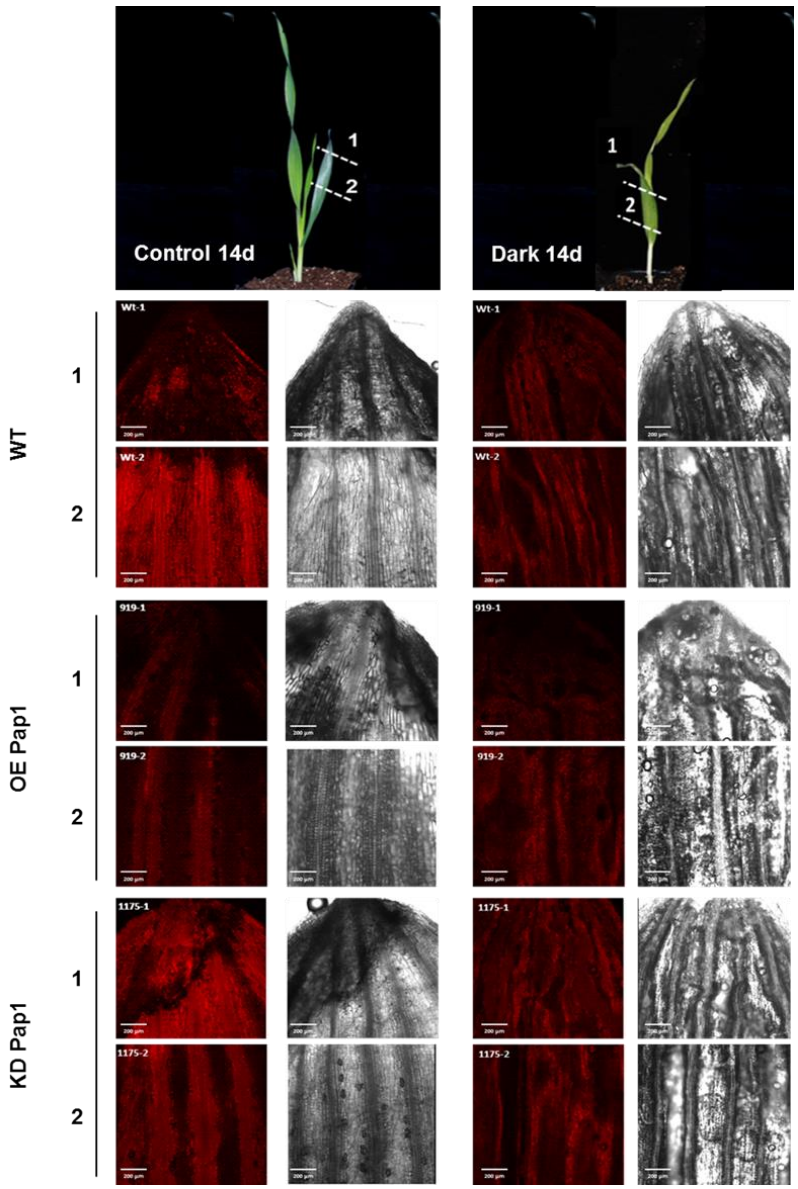


Figure 4.8. Chlorophyll detection in the oldest leaf of transgenic and wild-type barley lines grown under darkness or 16 h/8 h photoperiod for 14 d. Leaf fragments from *HvPap-1* over-expressing (OE Pap1: 919 line), silencing (KD Pap1: 1175 line) and wild-type (WT) plants were collected and observed under a Leica SP8 confocal microscope using the laser excitation lines 633 nm to detect the red auto-fluorescence from the chlorophyll. Same images were taken under light field conditions. Leaves were cut in two fragments (segments 1 and 2, corresponding to apical and medium-basal section of the leaf, respectively) as is indicated in the upper part of the figure. Scale bars: 200 µm.

	CONSTRUCT	LINE	STARCH CONTENT* (g/100 gFW)
Control	**Wild-type**	WT	$8.38 \pm 0.30_{A}$
	OE Pap1	919	$3.23 \pm 0.15_{B}$
		937	$3.58 \pm 0.15_{B}$
	KD Pap1	1130	$4.30 \pm 0.10_{B}$
		1175	$4.20 \pm 0.46_{B}$
Darkness 14d	**Wild-type**	WT	$0.84 \pm 0.17_{a}$
	OE Pap1	919	$0.83 \pm 0.11_{a}$
		937	$0.79 \pm 0.07_{a}$
	KD Pap1	1130	$0.90 \pm 0.10_{a}$
		1175	$0.73 \pm 0.09_{a}$

*Data, referred as grams of transformed starch per 100 grams of fresh weight, are means ± standard error of duplicate measurements of six independent replicates for each sample. Different letters indicate significant differences between lines. ($P < 0.05$, one-way ANOVA followed by HSD test).

Table 4.1. Starch content of the oldest leaf in the *HvPap-1* over-expressing (OE Pap1: 919, 937 lines), silencing (KD Pap1: 1130 and 1175 lines) and wild-type (WT) plants after 14 d of darkness or 16 h/8 h photoperiod.

4.4. DISCUSSION

Protein breakdown and mobilization are some of the major metabolic features associated with abiotic stresses, essential for nutrient recycling. Rubisco, the most abundant protein in plants, is likely the major target for proteases when proteolytic processes are activated (van der Hoorn, 2008; Theonen *et al.,* 2007; Krupinska *et al.,* 2012; Martinez *et al.,* 2012). The identification of these proteases is crucial to understand the physiological mechanisms behind the process in order to bioengineer plants with altered timing of senescence, which is closely related to grain quality and yield. The barley-C1A CysProt system is a promising model to analyze the role of proteases in plants subjected to abiotic treatments. C1A CysProt are strongly up-regulated in response to multiple stresses, barley is a model crop whose genome has been sequenced and transgenic technology is well established. Besides, the whole family of C1A CysProt and their inhibitors (cystatins) has been identified in this species (Martinez *et al.,* 2009; Diaz-Mendoza *et al*., 2014).

Slow growth rates and yellowish leaf apex were the first phenotypes displayed by barley plants under severe darkness and N starvation treatments (Supplementary Fig. S 4.1). Reduction in chlorophyll and carotenoid content was observed parallel to the dismantling of the cell structure (Fig. 4.1), probably due to microtubule rearrangements (Keech *et al.,* 2010). These parameters are considered reliable indicators of stress and senescence. In particular, chlorophyll abundance is a useful indicator of the chloroplast status because it tends to remain constant in photosynthetically active leaves (Sorin *et al.,* 2015). Major subcellular rearrangements in mesophyll cells undergoing severe stresses involved changes in chloroplast size and distribution within the cell. Chloroplasts lost their typical lenticular size and became more spherical and smaller. Under N starvation they still occupied the cell periphery with spaces among them. After the darkness treatment they formed aggregates (Fig. 4.1C). These results are in accordance with previous publications, and demonstrate that chlorophyll degradation is a common early event in stressed leaves and leaf senescence, closely related to plastid disassembly (Krupinska *et al.,* 2012; Carrion *et al.,* 2014; Hollmann *et al.,* 2014). Keech *et al.* (2007) reported that metabolism in whole darkened Arabidopsis leaves entered in a "stand-by mode" with low mitochondria activity to preserve active photosynthetic machinery, while in individual darkened leaves the high mitochondrial activity provided energy and carbon skeletons for a rapid degradation of cellular components. In this study, stress parameters measured in whole darkened barley plants or in N-depletion treated plants indicate that senescence was actually induced, since chlorophyll was degraded and chloroplasts were altered.

Another important parameter associated with abiotic stresses is the reduction of total protein content. The significant decrease in protein levels in darkness- and N-starved leaves in comparison to the controls (Fig. 4.1B) suggested either the inhibition of protein synthesis or/and the activation of protease activities associated to nutrient recycling. In this way, darkness and N starvation clearly induced cathepsin L-/F- and B-like activities in leaves (Fig. 4.2). These data were supported by the up-regulation of genes encoding barley C1A CysProt, particularly *HvPap-1, HvPap-12* and *HvPap-19* and the detection of these proteases by immunoblot assays under the assayed

experimental conditions (Fig. 4.3). The implication of different classes of CysProt cathepsin F, B-, L- and H-like, suggested a functional redundancy of these proteases in protein turnover upon treatments. So far, transcriptomic and proteomic data from different authors have consistently assigned a major role to members of all C1A CysProt groups during abiotic stresses induced in several plants species (Gregersen *et al.,* 2008; Martinez *et al.,* 2012; Diaz-Mendoza *et al.,* 2014; Hollmann *et al.,* 2014). Additionally, the subcellular localization of C1A CysProt revealed a dynamic trafficking of proteins to be degraded from the chloroplasts to the central vacuole. Senescence-Associated Vacuoles (SAVs) and the central lytic vacuole, both containing chloroplastic proteins and peptides, are enriched in CysProt activities during leaf senescence (Otegui *et al.,* 2005; Ishida *et al.,* 2008; Carrion *et al.,* 2013; 2014). The immunofluorescence signal of HvPap-1 and HvPap-19 CysProt was detected in small vesicles, probably SAVs, within parenchyma cells of leaves undergoing senescence (Fig. 4.4), which highly supports their involvement in the degradation of chloroplastic proteins. In contrast, HvPap-1 and HvPap-19 were less abundant and mainly localized in epidermal cells of control leaves.

HvPap-1 is a cathepsin F-like CysProt previously characterized with an important role in grain filling and germination. It actively participates in the hydrolysis and mobilization of storage proteins, mainly hordeins, controlling the grain amino acid composition (Cambra *et al*., 2012; Diaz-Mendoza *et al.,* 2016). Besides, *HvPap-1* gene is up-regulated in response to severe abiotic treatments. The functional relevance of this CysProt in response to darkness can be inferred from both forward and reverse genetic approaches. The alteration of its expression in over-expressing or silencing barley plants could disturb stress progress, and thereby nutrient mobilization. Transgenic over-expressing *HvPap-1* barley lines did not only exhibit high levels of mRNA and protein in control leaves but also in response to darkness-induced treatment, while opposite effects were observed in knock-down lines (Supplementary Fig. S 4.6 and Fig. 4.7). The accumulation of the HvPap-1 protease did not result in an increase in the proteolytic activity. This might be due to the lack of a specific substrate to be exclusively degraded by cathepsin F-like enzymes, since the one used in these proteolytic assays simultaneously targeted cathepsin F- and L-like activities, and to

compensating effects among protease activities. In senescing leaves of knock-down lines a clear decrease on protease activities, both cathepsin L-/F- and B-like classes (Supplementary Fig. S 4.8) also suggests that accumulation of other proteases (Fig. 4.7) and/or alternatively protease inhibitors is altered.

From a physiological point of view, the reduction of total protein paralleled to that of Rubisco was observed in most of the darkness-treated OE Pap-1 lines *vs* control. These results together with the reduced autofluorescence emission from the chlorophyll (Fig. 4.8) and the small amount of starch detected in the over-expressing leaves (Table 4.1) indicate that leaf senescence was sped up. In contrast, the high protein content and retarded loss of chlorophyll in the amiRNA leaves, mostly detected in the apex of the oldest leaf, indicated a delay in the senescence process. The carbohydrate content of barley leaves, mainly represented by low concentrations of sucrose, starch, fructans and hexoses (Sicher *et al.,* 1984), is completely remobilized in response to darkness-induced treatment and indicates that the photosynthetic partitioning was similar in transgenic and WT leaves. As shown in Fig. 4.5 and Supplementary information Fig. S 4.4, a clear delayed-senescence phenotype of *HvPap-1* amiRNA lines was observed both in barley plants grown either under light/dark photoperiod, corresponding to the natural lifespan, or under continuous darkness for 2 wk (Fig. 4.6). These phenotypes are presumably due to chloroplasts protection from degradation. Based on these results, it can be concluded that *HvPap-1* is a functional stress-associated gene and alterations in its expression bring about changes in barley abiotic stress responses. Previous reports have shown similar effects in plant behaviour by down-regulating the expression of senescence-related CysProt, in particular *BoCP5* and *CaCP* genes from broccoli and pepper, respectively (Eason *et al.,* 2005; Xiao *et al.,* 2014). In addition, the over-expression of the broccoli cystatin *BoCPI-1* significantly contributed to a delay in the yellowing of broccoli florets than did the suppression of *BoCP5* (Eason *et al.,* 2014). Many cystatins are strongly expressed upon exposure to abiotic stresses and the implication of the C1A CysProt-cystatin system has been reviewed (Martinez and Diaz, 2008; Martinez *et al.,* 2012; Diaz-Mendoza *et al.,* 2014; Kidric *et al.,* 2014). Very recently, the potential use of

phytocystatins in crop improvement to mitigate the negative impacts of climate change has been considered (Kunert *et al.,* 2015).

A positive correlation between chlorophyll content, cereal grain yield and total nitrogen content of the grain has been reported (Distelfeld *et al.,* 2014). Delay in leaf senescence, so-called 'stay green' phenotypes have a longer period of active photosynthesis and in consequence potentially higher plant productivity (Hörtensteiner 2007; Gregersen *et al.,* 2013). It has also been described that delayed leaf senescence leads to a high grain yield, inefficient nitrogen remobilization and a lower harvest index, whereas acceleration of senescence confers efficient nitrogen remobilization and high protein content, but also a lower total grain yield (Gregersen *et al.,* 2008). In this context, protein accumulation in barley grains is important depending on the end-product use of the harvested crop. Breweries do not require barley grains enriched in proteins while high levels of proteins are a particularly valuable character for food and animal feed. Further research is aimed to determine HvPap-1 CysProt responses to abiotic/biotic stresses able to induce leaf senescence and to characterize the functional role for other barley CysProt and cystatins.

In conclusion, our results clearly support that CysProt are associated with tolerance or sensitivity to environmental cues and consequently linked to leaf senescence, providing important basis for the improvement of small grain cereals, among other crops.

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4.6. SUPPLEMENTAL DATA

Figure S 4.1. Phenotypes of wild-type (WT) barley plants grown in vermiculite under continuous darkness or 16 h/8 h photoperiod, or in pots filled with Hoagland nutrient solution with or without N source for 3 and 7 d.

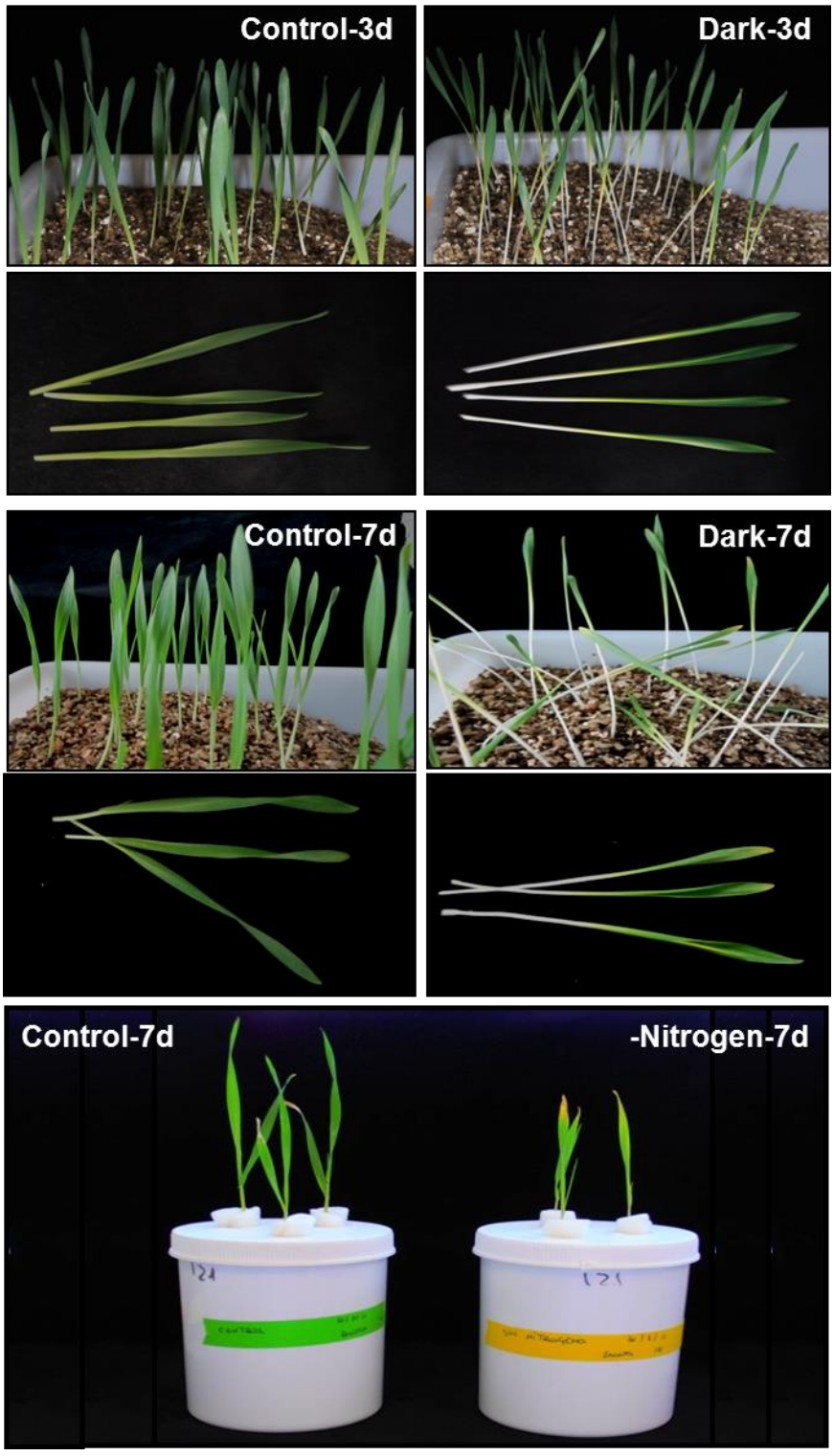


Figure S 4.2. Phenotype of barley plants at 3 (A), 5 (B), 7 (C) and 9 (D) wk of development. Wild-type (WT), *HvPap-1* over-expressing (OE Pap1: 919 and 937 lines) and silencing (KD Pap1: 1130 and 1175 lines) plants.

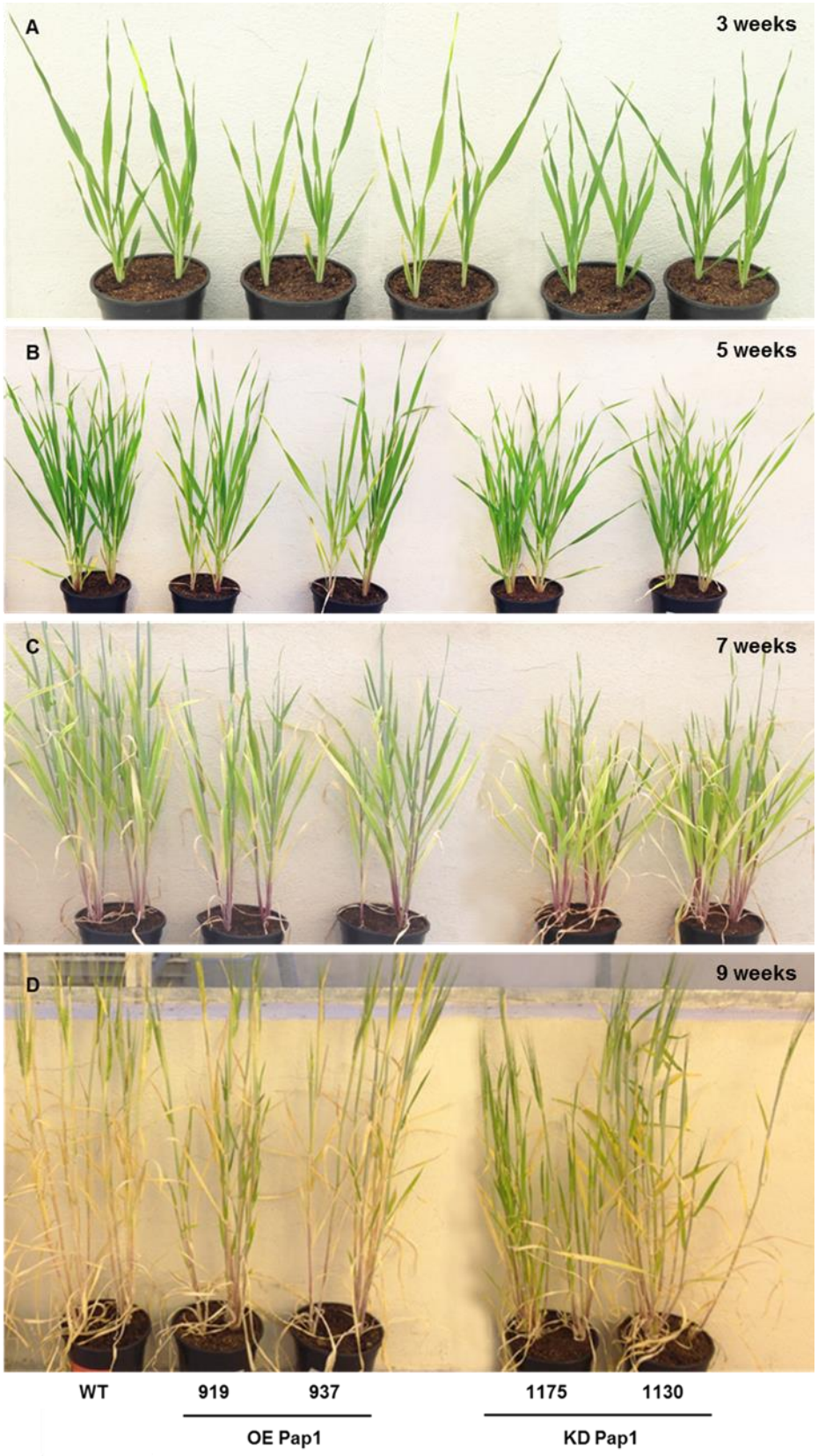


Figure S 4.3. Phenotype of wild-type (WT) barley plants grown in soil under continuous darkness or 16 h/8 h photoperiod for 3, 7, 14 and 21 d.

Figure S 4.4. Messenger expression levels of *C1A CysProt* genes in transgenic and wild-type (WT) barley lines grown under darkness or control treatment and assayed by RT-qPCR. Total RNA was extracted from leaves of WT, *HvPap-1* over-expressing (OE Pap1: 919, 920, 932 and 937 lines) and silencing (KD Pap1: 1175, 1176, 1128, 1130 lines) barley plants grown in soil under continuous darkness or with 16 h/8 h photoperiod for 14 d. Data were determined by RT-qPCR and expressed as relative mRNA levels of C1A CysProt genes normalized to barley *cyclophilin* mRNA content.

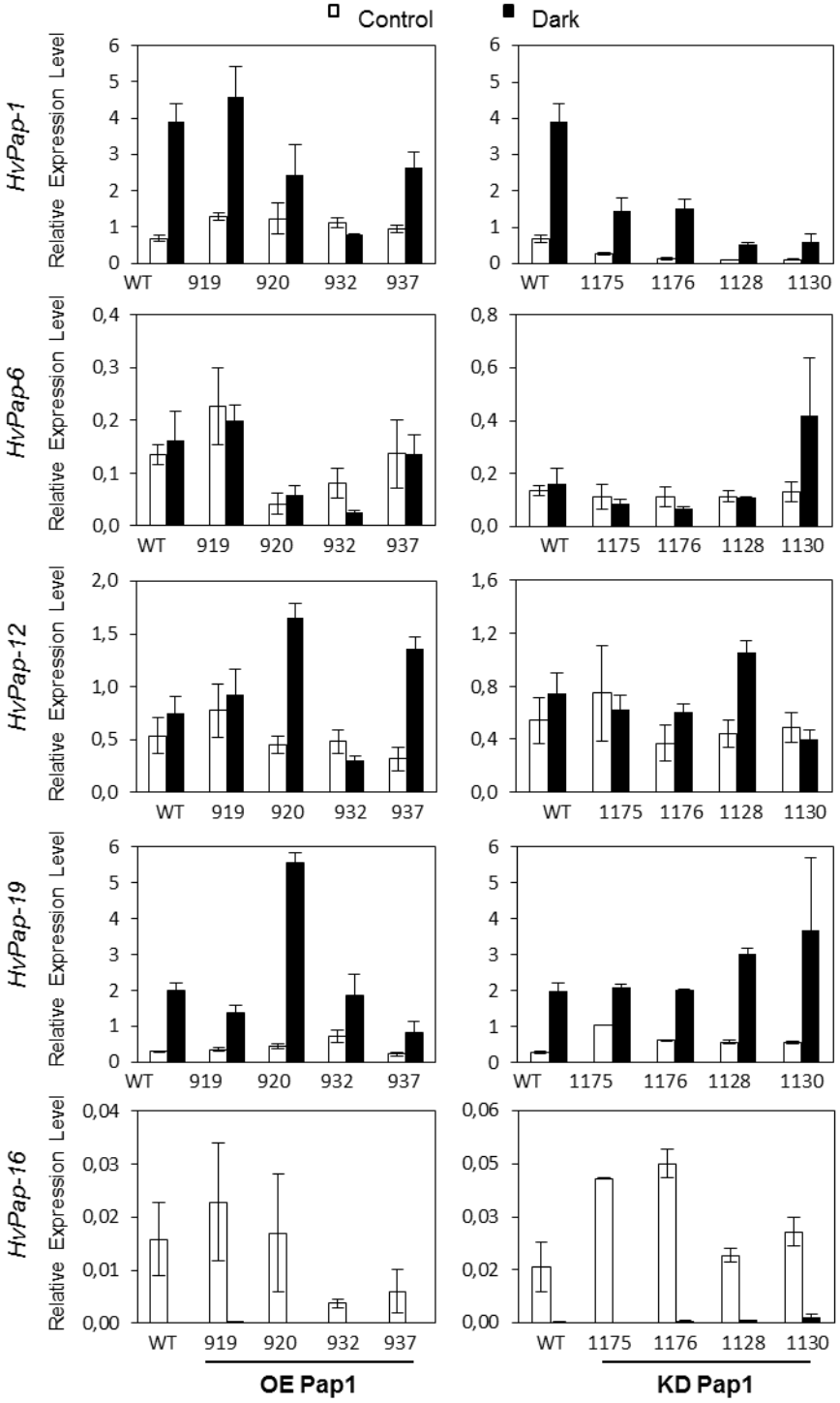


Figure S 4.5. Total protein content from HvPap-1 transgenic and wild-type lines. (A) *HvPap-1* over-expressing (OE Pap1: 919, 920, 932 and 937 lines) and wild-type (WT) barley plants. (B) *HvPap-1* silencing (KD Pap1: 1175, 1176, 1128, 1130 lines) and wild-type (WT) barley plants. Plants were grown in soil under continuous darkness or 16 h/8 h photoperiod for 14 d. All parameters are given in mg of total soluble protein per gram of initial fresh weight. Data are means ± standard error of at least three independent analyses. Different letters indicate statistical differences between lines ($P < 0.05$, HSD).

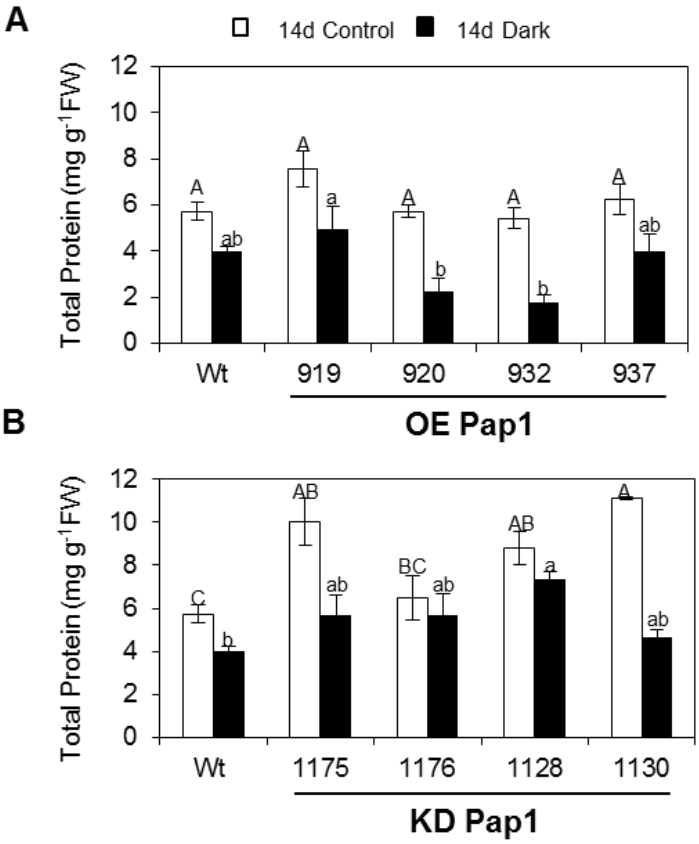


Figure S 4.6. Proteolytic activities of leaf extracts from *HvPap-1* transgenic and wild-type (WT) barley plants. (A) *HvPap-1* over-expressing (OE Pap1: 919, 920, 932 and 937 lines) and wild-type (WT) barley plants. (B) *HvPap-1* silencing (KD Pap1: 1175, 1176, 1128, 1130 lines) and wild-type (WT) barley plants. Plants were grown in soil without light or 16 h/8 h photoperiod for 14 d. Specific substrates to be degraded by cathepsin L-/F-like (a) and B-like (b) were used. Data are means ± standard error of at least three independent analyses. Different letters indicate statistical differences between lines ($P < 0.05$, HSD).

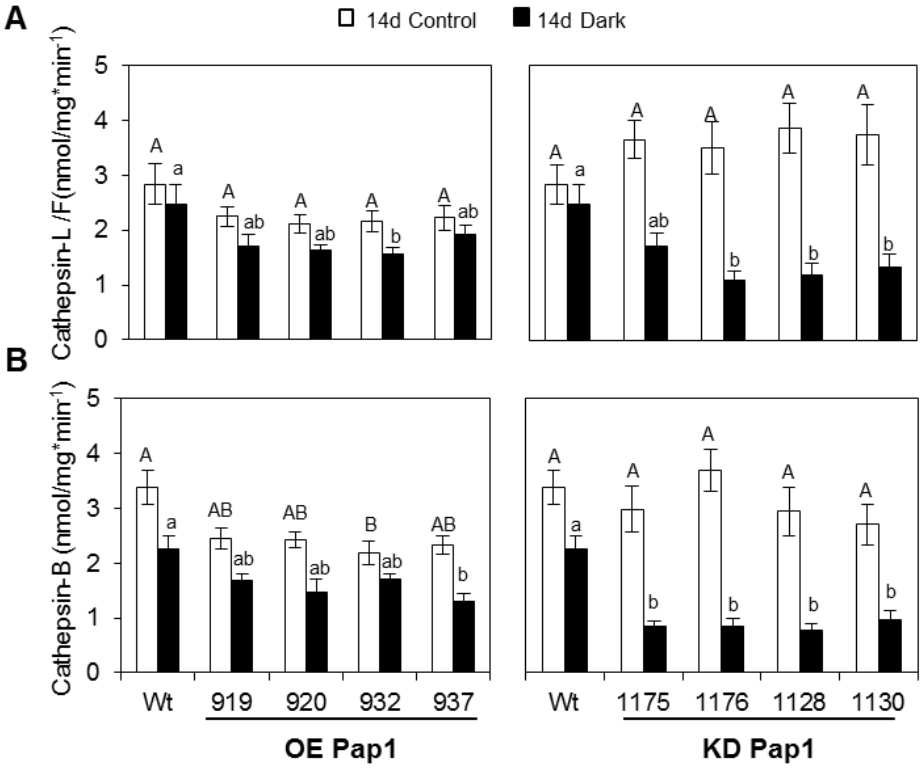


Figure S 4.7. Determination of photosynthetic pigments in whole plant leaves from *HvPap-1* over-expressing (OE Pap1: 919, 920, 932 and 937 lines), silencing (KD Pap1: 1175, 1176, 1128, 1130 lines) and wild-type (WT) barley plants grown in soil under darkness or 16 h/8 h photoperiod for 14 d. Chlorophyll *a* (A), chlorophyll *b* (B) and carotenoids (C) content. All parameters are referred in mg of pigment per gram of initial fresh weight. Data are means ± standard error of at least three independent analyses. Different letters indicate statistical differences between lines ($P < 0.05$, HSD).

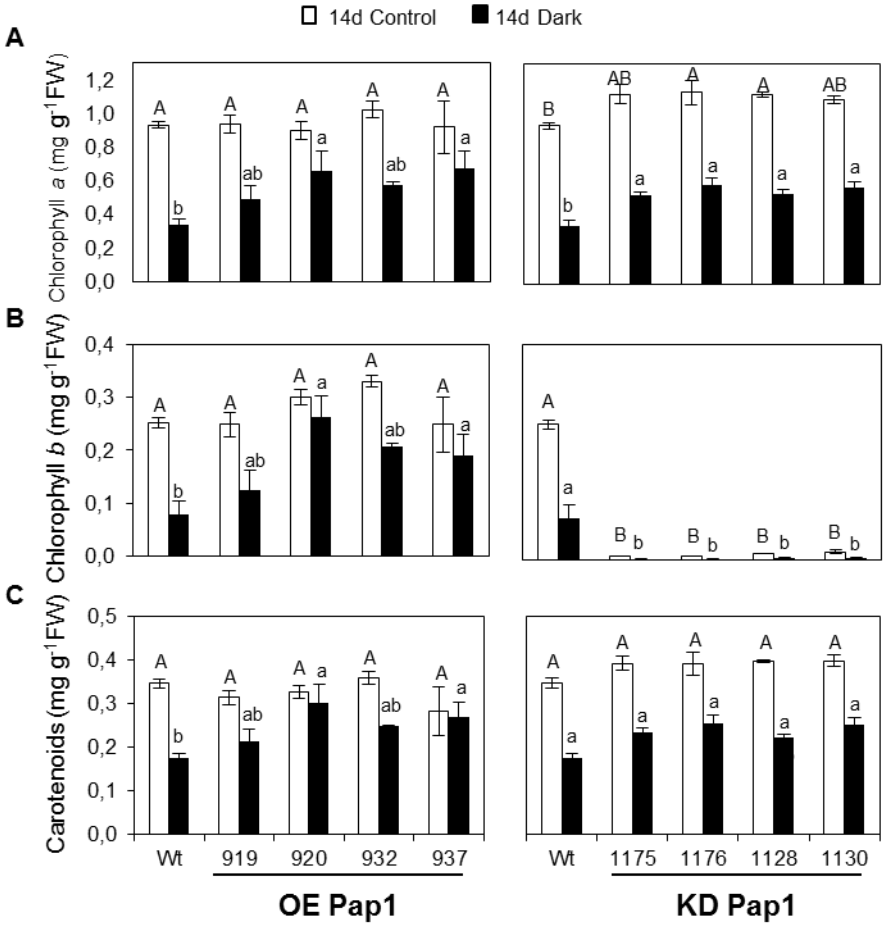
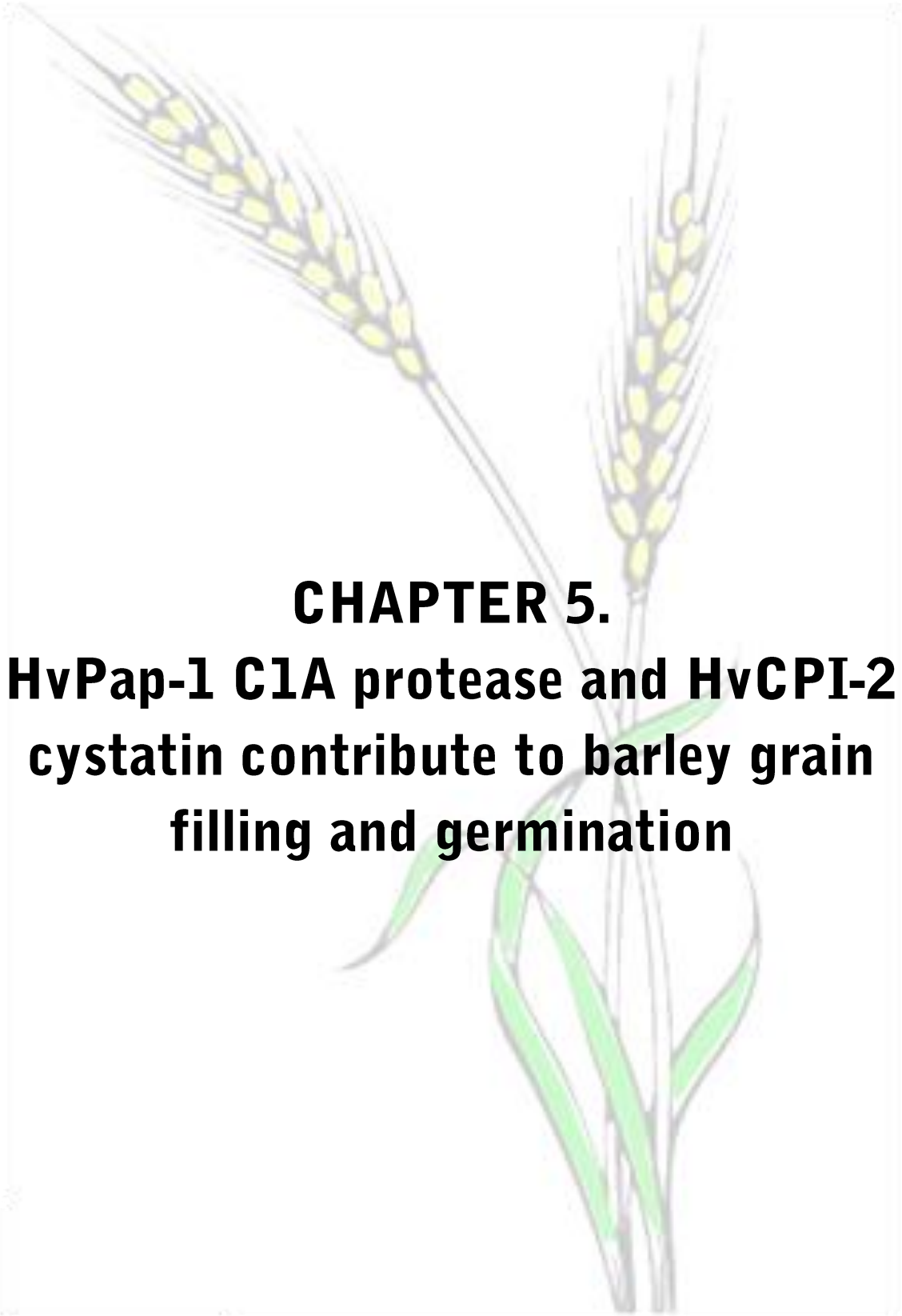


Table S 4.1 Primer sequences used for the amplification of barley genes by RT-qPCR assays. *HvPap-4, HvPap-6 and HvPap-16* genes (cathepsin L-like proteases), *HvPap-1* gene (cathepsin F-like protease), *HvPap-19* gene (cathepsin B-like protease) and *HvCycl* (cyclophilin).

Barley genes	Primers
HvCycl	forward: 5'-TCCACCGGAGAGGAAGTACAGT-3' reverse: 5'-AATGTGCTCAGAGATGCAAGGA-3'
HvPap-1	forward: 5´-TCCTGGAGTCGATCTTTGGTTTC-3' reverse: 5´-CAAGCATACTGTTGCGGCTTC-3'
HvPap-4	forward: 5´-CCTTGAGAGTCCTTGTTCCCGA-3´ reverse: 5´-CCATGTTCGTCGTTTTAACCGA-3´
HvPap-6	forward: 5´-TGCAATTGACGGCAAGAAGA-3´ reverse: 5´-TGGATCACCAGGTGATCATTTG-3´
HvPap-12	forward: 5´-ATGTGCGCTATTGCTACCTGC-3´ reverse: 5´-CACCTTATTCATGTCTGGCGAA-3´
HvPap-16	forward: 5´-CTGGATCGGTAAGAACTCGTGG-3´ reverse: 5´-TGATGGAGGTGCCATCATATGA-3´
HvPap-19	forward: 5´-CACCTTATTCATGTCTGGCGAA-3´ reverse: 5´-TGCCCGCTTAATTTGACAGG-3´

Table S 4.2. CysProt amino acid sequences used for specific antibody production.

Barley proteases	Peptides
HvPap-1	SGFAPSRFKEKPYWIIKN
HvPap-6	IDSEEDYPYKERDNRC
HvPap-16	RLRSYKKVTPPGNEAGLKE
HvPap-19	CQEKKHFSIDAYQVNSDPHD



CHAPTER 5.
HvPap-1 C1A protease and HvCPI-2 cystatin contribute to barley grain filling and germination

5.1. INTRODUCTION

Barley (*Hordeum vulgare* L.) is an annual, monocotyledonous plant of the Poaceae family, one of the world's earliest domesticated and most important crop plants. Nowadays it represents the fourth most abundant cereal in both surface and tonnage harvested. According to FAO the barley cultivated area in 2013 was 49,148,479 ha with a yield of more than 2,929 Kg/ha, meaning average world production of approximately 144 Mt (FAOSTAT, http://faostat3.fao.org/download/Q/QC/E). The high production of this crop is mainly due to its importance in the malting industry and breweries as well as to the nutritional intake for humans and livestock. Barley is considered as a model species for cereal research since its genome is fully sequenced (Mayer *et al*., 2012). Furthermore, it constitutes one of the best options to genetically improve cultivars to tackle climate change and guaranteeing cereal production (Nevo *et al*., 2012; Dawson *et al*., 2015).

During grain development and maturation proteins involved in germination are stored in the endosperm together with starch and lipids. The cereal grain protein concentration is about 10-12%, which is relatively low as compared to legume seeds. Nevertheless, cereals impact on humans and livestock nutrition is about three times higher than the effect of the most protein-rich legume seeds (Shewry and Halford, 2002). Storage, structural, metabolic and protective proteins are present in the grains. The major part corresponds to storage proteins, which represent near 80% of total proteins, falling into three different fractions based on the extraction method and their solubility: albumins (water soluble), globulins (soluble in salt solutions) and prolamins (soluble in alcohol/water mixtures) (Shewry *et al*., 1995). With the exceptions of oat and rice, the major endosperm storage proteins in all cereal grains are prolamins (Shewry and Halford, 2002), named hordeins in barley. Hordeins are highly hydrophobic molecules, rich in proline and glutamine and with a low content of charged amino acids, particularly the essential amino acid lysine. There are three broad groups of hordeins: B (sulphur-rich), C (sulphur-poor) and D (high molecular weight: HMW), with several subgroups within the B-group (Shewry *et al*., 1995). All of them are co-ordinately expressed during endosperm development (Sorensen *et al.*,

1989) and their expression is tightly regulated (Diaz *et al*., 2005).

Limited proteolysis mediated by peptidases is essential for the initiation of storage protein breakdown to nurture the developing embryo (Müntz, 1996). Peptidases were formerly classified as cysteine- (Cys), serine- (Ser), aspartic- (Asp) and metallo-proteases (metallic ion), according to the residue present in the active site of the enzyme. Papain-like cysteine proteases (CysProt), known as C1A (family C1, clan CA) according to the MEROPS database (Rawlings *et al*., 2014), constitute one of the most abundant groups of proteases responsible for the degradation and mobilization of storage proteins in seeds. Their role during germination has been reported in a wide range of both monocot and dicot plants (Grudkowska and Zagdańska, 2004; Tan-Wilson and Wilson, 2012). This group of proteases has a high number of members in angiosperms, ranking from 32 in Arabidopsis to 45 in rice, which are classified into cathepsins L-, B-, H- and F- like (Martinez and Diaz, 2008); in barley, 41 members have been identified (Diaz-Mendoza *et al*., 2014). A previous analysis reported that 27 C1A CysProt are among the 42 proteases involved in the germination of barley grain (Zhang and Jones, 1995).

A complete transcriptome analysis in two tissue fractions (starchy endosperm/ aleurone and embryo/scutellum) has shown the induction of a high number of CysProt genes during germination, most of them mediated by gibberellins (GA) (Sreenivasulu *et al.*, 2008). Some of the barley C1A proteases expressed in grain tissues have been characterized. Among them, several cathepsin L-like proteases of the scutellar epithelium and the aleurone layer were secreted to the endosperm upon germination in response to GA (Koehler and Ho, 1990; Mikkonen *et al*., 1996; Martinez *et al*., 2009). A cathepsin H-like protease isolated from GA-induced aleurone cells was targeted to vacuoles and a cathepsin B-like protein was expressed in the aleurone and induced by GA treatment (Holwerda and Rogers, 1992; Martinez *et al*., 2003). Recently, the HvPap-1, a cathepsin F-like peptidase, has been described to be involved in barley grain protein mobilization and to be modulated by its own propeptide and its inhibitors, the cystatins (Cambra *et al*., 2012). This protein is able to *in vitro* degrade different substrates, including barley endosperm proteins (hordeins, albumins and

globulins). It has been localized in protein bodies and vesicles of the embryo and it is induced by GA in aleurone cells (Cambra *et al*., 2012).

Cystatins are the endogenous proteinaceous inhibitors of C1A CysProt. They have several roles in plants and have been extensively related to the regulation of physiological processes in seeds (Benchabane *et al*., 2010). In barley, the whole cystatin family has been identified and characterized (Martinez *et al*., 2009). The functional relationship between barley cystatins and cathepsin L- and F-like proteases has been inferred from their common implication as counterparts during hordein storage protein mobilization upon barley grain germination (Martinez *et al*., 2009; Cambra *et al*., 2012). The barley cystatin HvCPI-2 encoded by the *Icy2* gene is a good inhibitor of different barley cathepsin L- and F-like CysProt, such as HvPap-1, -4, -6, -10, and -16. It is strongly expressed in the germinating embryo and repressed by gibberellic acid in aleurone layers. These results suggest a key role for HvCPI-2 in the regulation of the CysProt activity in barley grain (Martinez *et al*., 2009; Cambra *et al*., 2012).

Previous research indicates that a complex regulatory network including C1A CysProt and their inhibitors is involved in the regulation of the barley grain germination process. This work demonstrates how biotechnological modifications of the proteolytic machinery may affect grain composition and, consequently, germination in barley. For this purpose, *in planta* participation of the cathepsin F-like HvPap-1 and the cystatin HvCPI-2 proteins during grain filling and mobilization of stored proteins was analyzed in barley transgenic lines over-expressing the *HvPap-1* gene or knocking-down the expression of either the *HvPap-1* or *Icy-2* genes.

5.2. MATERIALS AND METHODS

5.2.1. PLANT MATERIAL

The samples used in this work were obtained from barley plants of *Hordeum vulgare* spring type cv. Golden Promise, grown at 22ºC under 16 h light/8 h dark photoperiod

in soil (Sanyo/Panasonic MLR-350-H). Barley transgenic lines over-expressing or silencing the barley *HvPap-1* gene (OE Pap1 and KD Pap1, respectively) and silencing the barley *Icy-2* gene (KD Icy2) were generated in collaboration with the IPK Gatersleben, Plant Reproductive Biology Group (Hensel *et al*., 2009). To generate over-expression lines, the *HvPap-1* gene was transferred into the intermediate vector pUbi-AB (DNA-Cloning-Service, Hamburg, Germany) and cloned using the *Sfi*I restriction sites into the p6U binary vector (DNA-Cloning-Service, Hamburg, Germany). This binary vector included the *HPT* (*HYGROMYCIN PHOSPHOTRANSFERASE*) selectable marker gene driven by the *Zea mays UBIQUITIN-1* promoter with first intron. The *HvPap-1* gene was driven by the same promoter. KD Pap1 and KD Icy2 knock-down lines were produced by artificial microRNA (amiRNA) technology developed in the MicroRNA Designer Web platform (WMD3, http://wmd3.weigelworld.org). The amiRNA constructs were engineered from pNW55 vector replacing the 21 bases of the natural Osa-MIR528 miRNA by 21 bases to silence specifically the *HvPap-1* or *Icy*-2 genes (5' TTATGCGGCATTGATACCGGT 3' and 5' TAAATTATTGTGTGGGGACTC 3', respectively). The final products of 554 bp were cloned into the p6d35S binary vector (DNA-Cloning-Service, Hamburg, Germany) using the pUbi-AB vector as intermediary. This binary vector included the *HPT* (*HYGROMYCIN PHOSPHOTRANSFERASE*) selectable marker gene driven by the doubled enhanced CaM35S-promoter. Immature embryos of barley cv "Golden Promise" were transformed with the binary vectors using the *Agrobacterium tumefaciens* strain AGL1 as described (Hensel et al., 2008). Homozygous transgenic plants were obtained by double haploid technology (Marthe *et al*., 2015). The presence of the antibiotic resistance marker was confirmed by PCR using specific primer pairs (Hensel *et al*., 2008). Homozygous transgenic lines for inserted gene constructions were characterized and used for grain analyses.

5.2.2. ANALYSIS OF THE COPY NUMBER IN TRANSGENIC BARLEY LINES

Total DNA was isolated from leaves of barley transgenic and non-transformed control lines. The RT-qPCR conditions were 40 cycles with 15 s at 95ºC, 1 min at 60ºC and 5 s at 65ºC. FastStart Universal SYBR Green Master (Rox, Roche) was used in a total volume of 20 µL. PCR amplification was performed in multiplates (Bio-Rad MLL9601)

using PCR sealers™ (Bio-Rad MSB1001). The reactions were carried out in a C1000™ thermal cycler with CFX96™ optical reaction module and results were analyzed with the CFX Manager Software 2.0 (Bio-Rad). Copy number was calculated by the $2^{-\Delta\Delta Ct}$ method to study gene expression (Livak and Schmittgen, 2001) adapted to estimate copy number (Ingham *et al*., 2001; Li *et al*., 2008). Cyclophilin and 4-hydroxyphenyl-pyruvate dioxygenase (*HvCycl* and *Hv4Hppd* genes) were used as endogenous and calibrator genes, respectively (Falk *et al*., 2002; Burton *et al*., 2004). The primers used are shown in Supplemental Table S 5.2. Analysis of dissociation curves was performed to check gene-specific amplification and reactions were performed in triplicate.

5.2.3. GRAIN PHENOTYPE AND STARCH ANALYSES

Grains from wild-type and transgenic barley plants were harvested and imbibed in double-distilled water. After 24 hours, grains were longitudinally dissected and starch was stained with Lugol's iodine staining reagent (Sigma). Stained and non-stained grains were visualized and photographed with a Leica MZ10 F stereo microscope and a Leica DFC420C CCD camera. For total starch quantification, six dry de-embryonated grains from transgenic and control lines were ground and 10 mg were assayed with STA20 Kit (Sigma) following the manufacturer recommendations. Wheat starch, included in the commercial kit, was used as control. Dilutions were carried out as necessary to fit into linearity of the glucose standard curve. Measurements were performed three times for each sample. After calculations, starch content was expressed as grams of transformed starch per 100 grams of initial kernel weight. Three independent biological replicates were used.

5.2.4. METABOLOMIC ANALYSES

Samples were obtained from dry de-embryonated grains. 50 mg of sample were re-suspended in 500 µL MeOH and disrupted by 3 cycles of frost/defrosting and processed with TissueLyser LT (Qiagen). After centrifugation at 19,300 x *g*, 20 min, the supernatant was transferred to a new tube and evaporated to dryness in a SpeedVac. The metabolite extracts were re-suspended in 0.1 mmol L^{-1} formic acid containing 0.2 mmol L^{-1} methionine sulfone (internal standard) by 1 min vortex mixing and then

centrifuged (19,300 x *g*, 15 min). Clear solution was analyzed by capillary electrophoresis coupled to a mass spectrometer detector (CE-ESI-MS-TOF). 19 nitrogen metabolites, mainly amino acids and other related compounds: Ala, sarcosine, Ser, Pro, Val, betaine, Ile, Leu, Asn, Asp, Gln, Lys, Glu, Met, His, Phe, Arg, Tyr and Trp, were analyzed as previously described (Moraes *et al*., 2011) with slight modifications. A CE System (7100 Agilent) coupled to a time-of-flight mass spectrometry system (Agilent 6224) was used. The separation occurred in a fused-silica capillary (Agilent) (total length, 100 cm; i.d., 50 µm). Separation was under normal polarity with a background electrolyte containing 1.0 mol L^{-1} formic acid in 10% (v/v) methanol at 20°C. Sheath liquid (6 µL min^{-1}) was methanol/water (1/1, v/v) containing 1.0 mmol L^{-1} formic acid with two reference masses to allow correction and higher accurate mass in the MS. Samples were hydrodynamically injected at 50 mbar for 35 s and stacked by injecting background electrolyte at 100 mbar for 10 s. The optimized MS parameters were: fragmentor 150 V, Skimmer 65 V, octopole 750 V, nebulizer pressure 10 psi, drying gas temperature at 200ºC and flow rate 10.0 L min^{-1}. The capillary voltage was 3,500 V. Data were acquired in positive ESI mode with a full scan from m/z 87 to 1,000 at a rate of 1.41 scan/s. The resulting CE-MS data files were cleaned of background noise and unrelated ions by the Targeted Feature Extraction tool with Profinder software (B.06.00, Agilent). Data were extracted using data mining algorithm based on the software. This software contains a list of standards used with their exact monoisotopic mass, migration time and molecular formula. Metabolites were previously identified in the wild-type barley samples by comparison of their migration time and spectra with pure standards. After their quantitation with standard solutions differences for individual metabolites were evaluated by a t-test comparison of every case group vs wild-type (where p values < 0.05 were considered significant). SIMCA-P+ 12.0.1 (Umetrics) and MetaboAnalyst v. 3.0 (Xia et al., 2012) were used for PCA plotting and Heatmap, respectively.

5.2.5. GRAIN PROTEIN ANALYSIS

Plant protein extracts were obtained from 20 dry de-embryonated grains and from 20 de-embryonated grains after 24 and 72 h of seed imbibition. Three independent

biological replicates were used. Samples were ground and around 100 mg were resuspended in 500 µl of protein extraction buffer (50 mM sodium phosphate pH 6.0, 15 mM NaCl and 2 mM EDTA pH 8.0). The suspension was centrifuged at 15,600 x *g* for 15 min at 4ºC. The protein content of the supernatants were quantified (Bradford, 1976) with bovine serum albumin (BSA) as a standard. Protein content was analyzed using SDS-PAGE technique. 20 µg of protein extracts were denatured using dissociation buffer (12.5 mM Tris-HCl pH 6.8, 0.25% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue and 3.75% (v/v) glycerol) for 5 min at 90ºC. Electrophoretic detection of proteins was performed using denaturing polyacrylamide gels at 15% (w/v). The electrophoresis was carried out using a BioRad Mini Protein Electrophoresis Cell system for 90 min at 130 V and prestained SDS-PAGE standard broad range (BioRad) was used as a weight molecular pattern. Gels were submerged into a staining solution 0.3% (w/v) Coomassie Blue G-250 in 40% (v/v) methanol and 10% (v/v) acetic acid to stain the proteins, and were distained in 25% (v/v) methanol and 10% (v/v) acetic acid.

5.2.6. FRACTIONATION AND ANALYSIS OF BARLEY GRAIN STORAGE PROTEINS

Albumins and globulins were sequentially extracted from 20 dry barley de-embryonated grains (Shi and Xu, 2009) and from 20 de-embryonated grains after 24 and 72 h of water imbibition. De-embryonated grains were completely crushed in a mortar, resuspended in distilled water, and continuously stirred for 12 h at 4ºC. Soluble and insoluble fractions were separated by centrifugation at 5,900 x *g* for 30 min at 4ºC. The supernatant, enriched in albumins, was isolated. Globulins were extracted from the pellet by adding 5% (w/v) NaCl in distilled water at 4ºC. After a second centrifugation step at 5,900 x *g* for 30 min, the recovered soluble fraction was enriched in globulins. Hordeins were extracted from a similar set of de-embryonated grains after incubation in a buffer containing 55% (v/v) 2-propanol and 1% (v/v) 2-mercaptoethanol, for 1 h at 60ºC and centrifugation for 10 min at 13,300 x *g* (Martinez *et al*., 2009). Protein concentrations and their electrophoretic bands patterns were analyzed as above described. Three independent biological replicates were used.

5.2.7. GERMINATION ASSAYS

Twenty barley grains from each transgenic line and from the wild-type cultivar were placed into a 150 mm petri dish with moistened, sterile filter paper. The germination process was followed by counting the number of germinated grains at 24 and 72 hours after imbibition (hai), considering as germinated grains those that had broken the grain coat or testa. Photographs were taken with an OLYMPUS VR-320 digital camera. The length of the emerging roots and coleoptiles was quantified using a measuring ruler. Three independent biological replicates were used.

5.2.8. ENZYMATIC ACTIVITY ASSAYS

The enzymatic activity of the same de-embryonated grain protein extracts used for grain protein quantification was determined using the fluorogenic substrates Z-FR-AMC (N-carbobenzoxy-Phe-Arg-AMC) and Z-RR-AMC (N-carbobenzoxy-Arg-Arg-AMC), susceptible to degradation by cathepsin L-/F- and B-like proteases, respectively. Mixtures of proteases and substrates were incubated in 100 mM sodium phosphate pH 6.0 buffer containing 10 mM cysteine, 1 mM EDTA, and 0.01% (v/v) Brij35 at 30ºC. Trypsin-like activity was also analyzed using the fluorogenic substrate Z-R-AMC (N-carbobenzoxy-Arg-AMC), assays were incubated in the buffer Tris-HCl 0.1 M, pH 7.5 at 30ºC. Emitted fluorescence was measured with a 365 nm excitation and a 465 nm emission wavelength filter. Triplicate assays were performed for determination of each value and the average was calculated. Blanks were used to account for spontaneous breakdown of substrates and results were expressed as nmol of hydrolyzed substrate by mg of protein by min (nmol/mg*min). The system was calibrated with known amounts of AMC (7-amido-4-methylcoumarin) hydrolysis product in a standard reaction mixture.

5.2.9. IMMUNOBLOT ANALYSES

The same de-embryonated grain protein extracts obtained for total protein quantification were used. After separation on an SDS–polyacrylamide gel, proteins were electrotransferred onto a nitrocellulose membrane (GE Healthcare).

Immunoblotting was performed with specific crude anti-HvPap-1, -6 and -19 peptide polyclonal antibodies produced in rabbits by Pineda Antibody Services. The sequences used to produce them were: HvPap-1-IgG, NH2-CSGFAPSRFKEKPYWIIKN-CONH2; HvPap-6-IgG, NH2-CGWSPVKDVNDPHVQEL-CONH2; and HvPap-19-IgG, NH2-CQEKKHFSIDAYQVNSDPHD-CONH2. Optimal dilution of the antibody was incubated in PBS (137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4) with 5% (w/v) milk. Peroxidase-conjugated anti-rabbit IgG (Sigma) diluted at 1:10,000 was used as secondary antibody for detection with ECL Plus (GE Healthcare). The specificity of these antibodies was confirmed by immunoblotting using the purified recombinant proteases HvPap-1, -6, and -19 (Supplemental Fig. S 5.7).

5.2.10. REAL-TIME QUANTITATIVE PCR ANALYSES

For real-time quantitative PCR (RT-qPCR) studies, isolated embryos from 24 h germinated grains were used. Twenty grains and three independent biological replicates per line were used. All samples were frozen in liquid nitrogen and stored at -80ºC until used for RNA extraction. Samples were ground using a mortar and a pestle. Total RNA was extracted by the phenol/chloroform method, followed by precipitation with 3 M LiCl (Oñate-Sánchez and Vicente-Carbajosa, 2008) and digestion with DNase. cDNAs were obtained from 2 µg of RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer´s instructions. RT-qPCR analyses were performed for duplicated samples by means of a CFX96 Real-time system (BioRad) using SYBR Green as a detection system. Relative expression values normalized to *Cyclophilin* gene (Burton *et al*., 2004) and expressed as $2^{-\Delta Ct}$. The primers used for PCR amplification are described in Supplemental Table S 5.3.

5.2.11. EMBRYO STRUCTURAL ANALYSIS AND IMMUNO-FLUORESCENCE DETECTION

Embryos from wild-type and transgenic plants were fixed in a freshly prepared solution of 4% (w/v) formaldehyde in PBS (phosphate buffered saline) first at room temperature under vacuum until the specimens sank, then overnight at 4ºC. Next day the samples were washed twice in PBS for 15 min. Dehydration in a series of increasing concentrations of methanol, resin infiltration, embedding and polymerization were

performed by Progressive Lowering of Temperature (PLT) in a Leica AFS. From methanol 30% to 70% (v/v) the temperature dropped from 0 to -20ºC at a speed of -70ºC/h, with changes every 30 min. Subsequent steps were performed at -20ºC. Further dehydration was achieved in 100% methanol for 90 min, with three changes. Resin infiltration was done in a series of mixtures methanol:LRwhite (Agar Scientific) with increasing concentrations of the resin (2:1; 1:1; 1:2, v:v), for 1 h each, and finally pure resin with 0.5% (w/v) bezoin-methyl-ether as a catalyst overnight. Polymerisation in plastic capsules was accomplished under UV light at -20ºC for 2 days and at 22ºC for 1 day. 1-2 µm thin sections were cut from the polymerized blocks in a Leica EM UC6 ultramicrotome, carefully collected on water drops on 10-well Teflon-printed slides (Fisher Scientific Inc.) and let dry down and stored at room temperature until further use.

To assess any possible structural rearrangements at the sub-cellular level in the transgenic samples *vs* the wild-type, the sections were stained with 0.3% (w/v) coomassie brilliant blue (Coomassie G-250, BioRad) or 20% (v/v) lugol (Lugol's iodine staining reagent, Sigma), rinsed in distilled water, mounted and observed on a Zeiss Axiophot microscope under bright field. Photographs were taken with a Leica DFC300 FX CCD camera using the Leica Application Suite 2.8.1 build 1554 acquisition software.

For immuno-fluorescence the sections on the 10-well slides were hydrated with PBS for 5 min and unspecific binding sites were blocked by 10 min incubation with 5% (w/v) BSA in PBS. Then, they were incubated with 20 µl drops/well of either a rabbit-raised antibody to the CysProt HvPap-1, HvPap-6 or HvPap-19, applied 1/50 in PBS, for 1 h at room temperature in a humid chamber. After two washes of 15 min in PBS, an Alexa Fluor 488 anti-rabbit antibody (Molecular Probes) was applied in a 1/25 solution in 2.5% (v/v) BSA in PBS, for 45 min at room temperature in a humid chamber and darkness. Subsequent to another two washes of PBS for 15 min each, the slides were mounted in a 50:50 solution of glycerol:PBS. Serial sections across the specimens were collected on a Leica SP8 confocal microscope using the laser excitation line of 488 nm to detect the proteases. All series were captured under the same conditions (pinhole size, gain, offset, magnification). The management of the series was performed with

either the LAS-AF-Lite 3.1.0_8587 or Fiji software. To composite the corresponding figure in Adobe Photoshop CS3, the maximum projections of the green channel were overlaid for each treatment shown. Only the automatic levels were adjusted.

5.2.12. DATA ANALYSIS

Statistical differences among treatments or lines were analyzed by one-way ANOVA followed by Tukey´s (HSD) multiple comparison test performed using the software R Project (v.3.1.2) package.

5.3. RESULTS

5.3.1. TRANSGENIC BARLEY LINES OVER-EXPRESSING OR SILENCING HVPAP-1 PROTEASE OR SILENCING ICY-2 CYSTATIN

Transgenic barley plants were obtained from immature embryos after *Agrobacterium* co-culture and selection on hygromycin-containing media. Transgenic barley plants ubiquitously over-expressing the *HvPap-1* gene were generated using p6U and p6d35s binary vectors. Silencing of the *HvPap-1* and *Icy-2* genes was generated using the artificial microRNA (amiRNA) technology. For each construct, 30 independent primary plants were generated. Afterwards, four to six T1 events were preliminary used for molecular characterization. Homozygous material was generated via embryogenic pollen cultures (Coronado *et al*., 2005) and obtained homozygous plants were analyzed in depth. Two *HvPap-1* over-expressing lines (OE Pap1: 919 and 937), two *HvPap-1* silencing lines (KD Pap1: 1130 and 1175) and two *Icy-2* silencing lines (KD Icy2: 1318 and 1399) were selected based on transgene copy number, transcript and protein content for further studies (Supplemental Fig. S 5.1A and Supplemental Fig. S 5.2A). Following these criteria, the over-expressing lines showed two copies of *HvPap-1* gene, the endogenous and the transgene, estimated by RT-qPCR assay and the $2^{-\Delta\Delta Ct}$ method (Supplemental Fig. S 5.1B) and presented higher accumulation of mRNAs and protein than the wild-type (Supplemental Fig. S 5.1C,D). The amiRNA lines contained a unique transgene insertion and the expression levels of their messengers and accumulation of

proteins were reduced in comparison with the wild-type (Supplemental Fig. S 5.1B-D and Supplemental Fig. S 5.2B-D). However, neither mRNA accumulation nor protein content was completely knocked out in the amiRNA plants.

5.3.2. GRAIN PHENOTYPE AND STARCH ACCUMULATION ARE ALTERED IN BARLEY TRANSGENIC LINES

Kernels from transgenic and control plants were obtained and their grains phenotypically compared 24 hours after imbibition (hai). The OE Pap1 and KD Pap1 grains were of similar size but were elongated and presented darker endosperms than the control grains. The KD Icy2 lines also showed grains with a slightly darker endosperm than wild-type ones (Fig. 5.1A). These phenotypic differences could be related to a different grain composition. The amount of starch can be inferred by the intensity of the dark-blue/black colour after Lugol staining. OE Pap1 and KD Icy2 grains presented a weaker colour than wild-type grains, indicating lower amount of starch. In contrast, the staining of KD Pap1 grains was stronger than the signal observed in wild-type grains (Fig. 5.1B). No differences in the balance of amylose/amylopectin could be detected since the typical amylopectin redish colouration was not observed (Fig. 5.1B). Quantification of the starch content in grains corroborated that the KD Pap1 silencing lines contained significantly higher quantities of starch than the control line (Supplemental Fig. S 5.3).

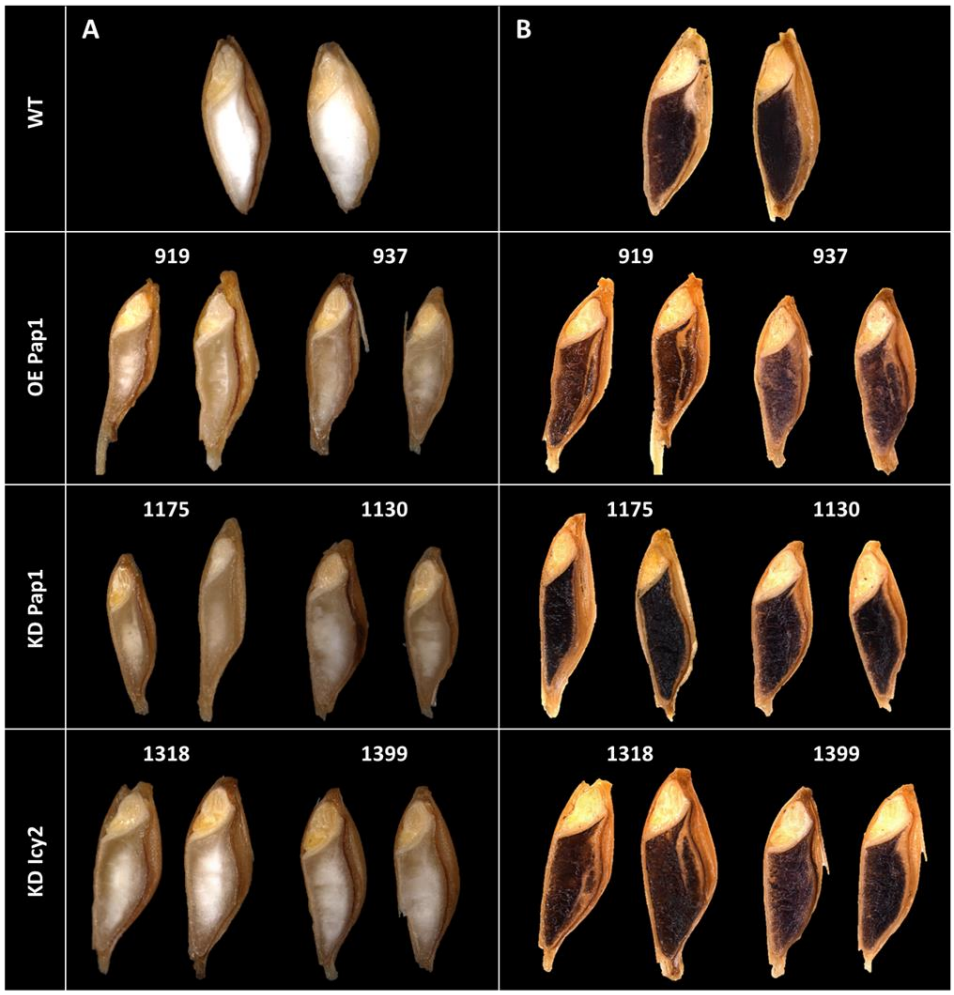


Figure 5.1. Phenotype and starch staining of barley grains. **A**, Structure of longitudinal dissected grains 24 hours after imbibition from wild-type and transgenic plants. **B**, Lugol's iodine staining of transgenic and wild-type barley grains.

5.3.3. GRAIN PROTEIN CONTENT IS MODIFIED IN BARLEY TRANSGENIC LINES

The protein quantity of de-embryonated grains was also quantified (Fig. 5.2A). Dry grains from all transgenic lines (OE Pap1, KD Pap1 and KD Icy2) presented similar quantities of protein between them, but significantly higher protein amounts per grain than the non-transformed line (Fig. 5.2A). These proteins are degraded as germination proceeds. At 24 and 72 hours after imbibition (hai), the amount of protein decreased in all transgenic and non-transgenic lines. However, the rate of remaining protein was different. About 40% of the grain protein was degraded at 72 hai for all lines with the exception of KD Pap1 lines, in which about 70% of the grain protein was broken down at 72 hai (Fig. 5.2A). These variations in the speed of protein degradation were

confirmed by electrophoretic band patterns, which were very similar for wild-type, OE Pap1 and KD Icy2 barley grains but different for KD Pap1 ones. KD Pap1 lines showed a fainter pattern for medium-high MW (molecular weight) protein bands (Supplemental Fig. S 5.4A).

Variations in the quantity of grain proteins could be related to alterations in the type of storage proteins in each transgenic line. Hordeins, albumins and globulins are the main storage proteins in barley and fractions enriched in each of these compounds were isolated. Quantification of these fractions revealed differences related to the storage and processing of these proteins (Fig. 5.2B-D). The total amount of hordeins was higher in OE Pap1 dry grains than in the rest of grains analyzed, but was more quickly degraded in these lines, reaching similar values at 72 hai than in the other transgenic and non-transgenic grains analyzed (Fig. 5.2B). These specific degradative patterns are shown by the appearance of intermediated MW bands in the electrophoretic gels for OE Pap1 (Supplemental Fig. S 5.4B). Globulins showed a striking pattern, with a significant higher accumulation in dry grain, in both OE Pap1 and KD Pap1 transgenic lines than in the wild-type or KD Icy2 lines (Fig. 5.2C). However, the rate of degradation was different. In the wild-type, OE Pap1 and KD Icy2 lines around 50% of stored globulins were broken down at 72 hai, whereas in the KD Pap1 lines approximately 75% of the stored albumins were degraded at the same time point. Electrophoretic gels showed this rapid degradation of most globulin bands in the KD Pap1 lines (Supplemental Fig. S 5.4C). Finally, albumins were also accumulated differentially in the dry grains from transgenic and non-transgenic lines, with a significantly higher quantity in the KD Icy2 lines and in the 919 OE Pap1 line (Fig. 5.2D). However, variations on protein degradation patterns among the different lines were not clearly detected (Fig. 5.2D and Supplemental Fig. S 5.4D).

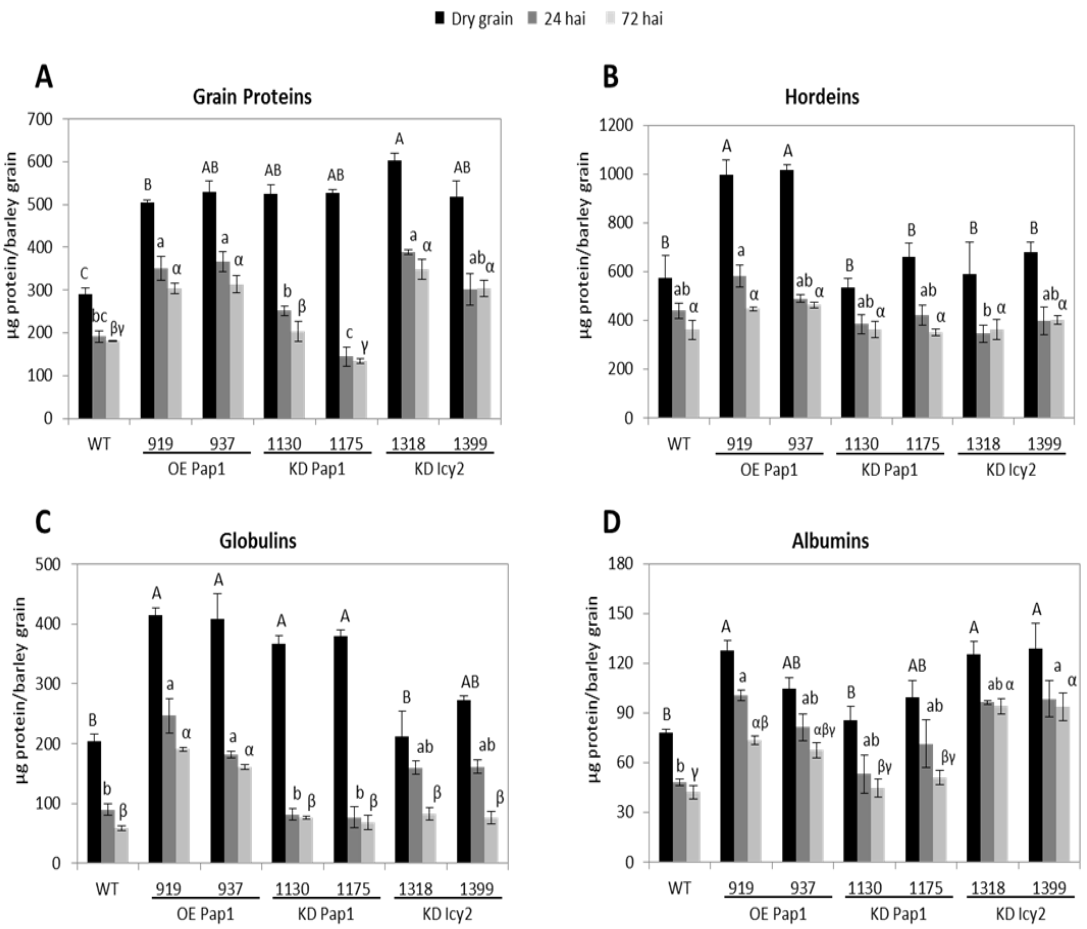


Figure 5.2. Quantification of the protein content per grain in wild-type and transgenic lines at different hours after imbibition (hai). **A**, Grain proteins. **B**, Hordeins. **C**, Globulins. **D**, Albumins. Different letters indicate significant differences between lines for each time point ($P < 0.05$, HSD). Capital, small and Greek letters are used for dry grains, 24 hai grains and 72 hai grains, respectively.

5.3.4. GRAIN METABOLOMIC ANALYSES SHOW CHANGES IN AMINO ACID ACCUMULATION IN BARLEY TRANSGENIC LINES

Differential protein accumulation in grains could be related to differences in the free amino acids composition in de-embryonated grains. To test that, a metabolomic analysis was performed comparing the free amino acids in transgenic and non-transgenic mature grains. PCA (principal component analysis) is an orthogonal linear transformation of possibly correlated variables into a smaller number of uncorrelated variables called principal components, where the greatest variance within the data is explained on the first coordinate. Samples that group together represent a specific

phenotype. According to the PCA scores plot, it can be observed that biotechnological proteolytic modifications induced specific amino acid perturbations. Samples originating from different lines clustered into different areas in the plots (Fig. 5.3A). Samples from KD Pap1 1130, and KD Icy2 1399 lines clustered closely and far from wild-type grains. OE Pap1 937 samples clustered together and separately from the rest of the lines. As expected, the plot showed that variation among the different groups was more pronounced on the component 1 which accounted for the highest variation in the models. Specific differences in the amino acid composition from grain extracts are illustrated in the heatmap (Fig. 5.3B). The heatmap readily shows changes in the concentrations of amino acids in the grains from the different transgenic and non-transgenic lines. The quantification and significance of these changes is reported in Supplemental Table S 5.1. In the samples coming from both KD lines, most amino acid molecules were significantly reduced, with the exception of proline in both lines and glutamine in the KD Icy2 line. On the contrary, in the OE Pap1 samples, the most remarkable difference in comparison to wild-type samples is a pronounced increase in the levels of two amino acids, proline and glutamine.

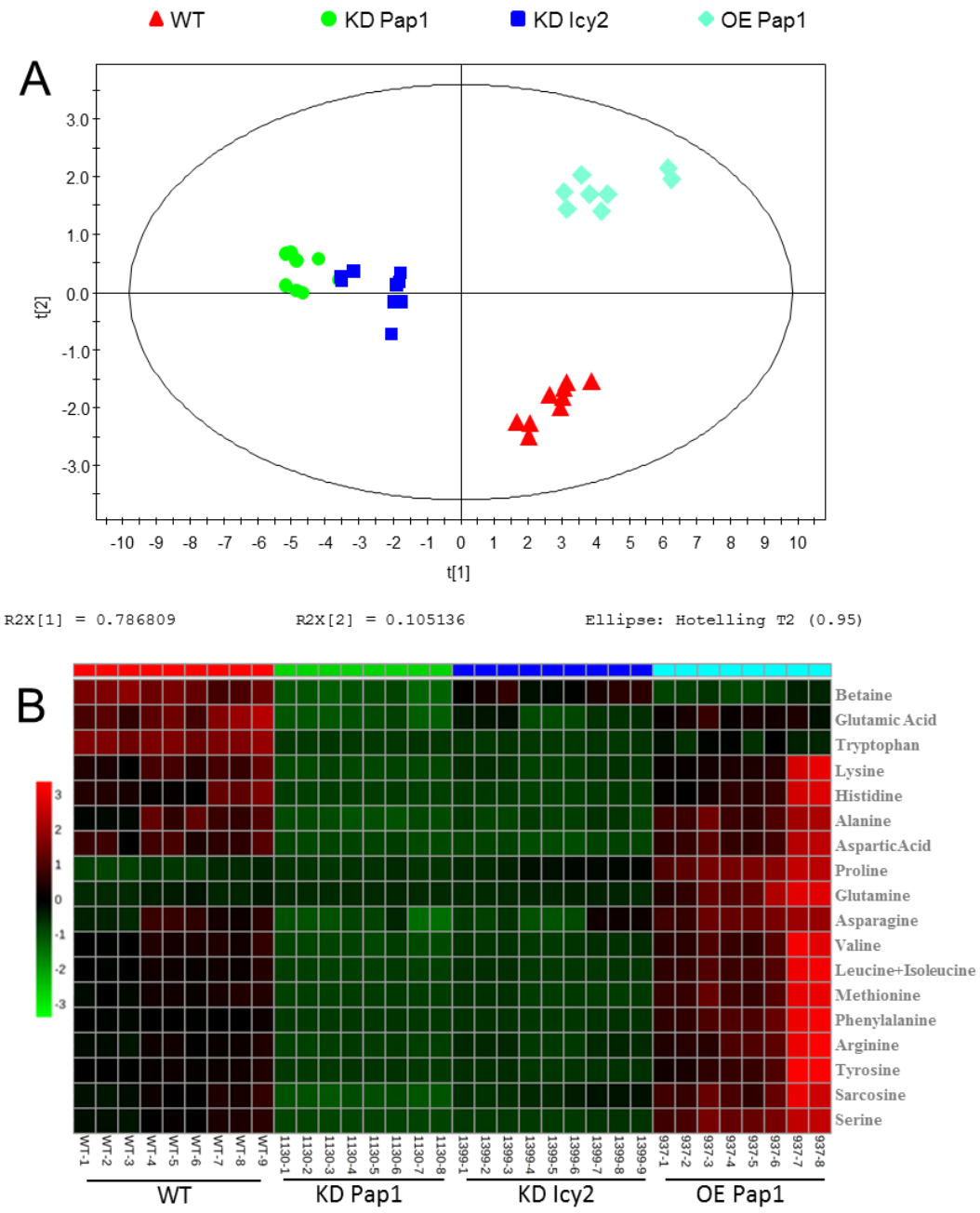


Figure 5.3. Principal component analysis (PCA) models and metabolite heatmap of amino acid composition of dry de-embryonated grains. **A**, PCA scores plot of all samples included in the study (red triangles, WT; green dots, KD Pap1 1130; blue squares, KD Icy2 1399; turquoise diamonds, OE Pap1 937). Cumulative R2 and Q2 were 0.892 and 0.803, respectively. **B**, Heatmap showing the relative contributions of each amino acid in the different type of samples. The colour code shows the abundance level.

5.3.5. GERMINATION IS DELAYED IN HVPAP-1 TRANSGENIC LINES

To analyze whether differences in grain composition affect germination ability, twenty barley grains per line were imbibed in watered paper on Petri dishes and germination was followed for 72 hours. A striking delay was observed for KD Pap1 lines (Fig. 5.4). The number of germinated grains was counted at 24 and 72 hai (Fig. 5.5). Whereas no differences were observed in the percentage of germinated grains between wild-type, OE Pap1 and KD Icy2 lines, a lower germination rate was detected in KD Pap1 lines, especially at 24 hai (Fig. 5.5A and B). Differences in development were also observed among germinated grains. All transgenic lines had shorter roots and coleoptiles than wild-type control line at 24 hai (Fig. 5.5C and E). However, a higher variability was observed for the transgenic lines at 72 hai (Fig. 5.5D and F). OE Pap1 grains had shorter coleoptiles but the length of their roots was similar to the wild-type. KD Pap1 roots and coleoptiles remained shorter than in the wild-type. Finally, KD Icy2 lines grew rapidly, with similar coleoptile lengths and longer roots than wild-type plants.

5.3.6. PROTEOLYTIC ACTIVITIES ARE AFFECTED IN DRY AND GERMINATING GRAINS

Differences in germination between wild-type and transgenic lines may be due to variations in grain composition but also to a distinct mobilization of the storage compounds. Thus, the main proteolytic activities in barley de-embryonated grains (cathepsin L-/F- and B-like CysProt and trypsin-like serine protease) were analyzed at different germination times. Cathepsin L-/F-like activity was detected in all transgenic and non-transgenic lines in the dry grain, being significantly higher in the lines over-expressing the *HvPap-1* gene (Fig. 5.6A). At 24 hai this activity did not increase and was significantly lower in all transgenic lines than in the wild-type. However, a sharp increase of cathepsin L-/F-like activity was shown in all lines at 72 hai, reaching similar levels of this peptidase activity. Dynamics of cathepsin B-like activities was similar to that of cathepsin L-/F-like (Fig. 5.6B), appearing in all dry grains at similar levels, without a significant increase in all transgenic lines at 24 hai, and showing a strong increase in all lines at 72 hai.

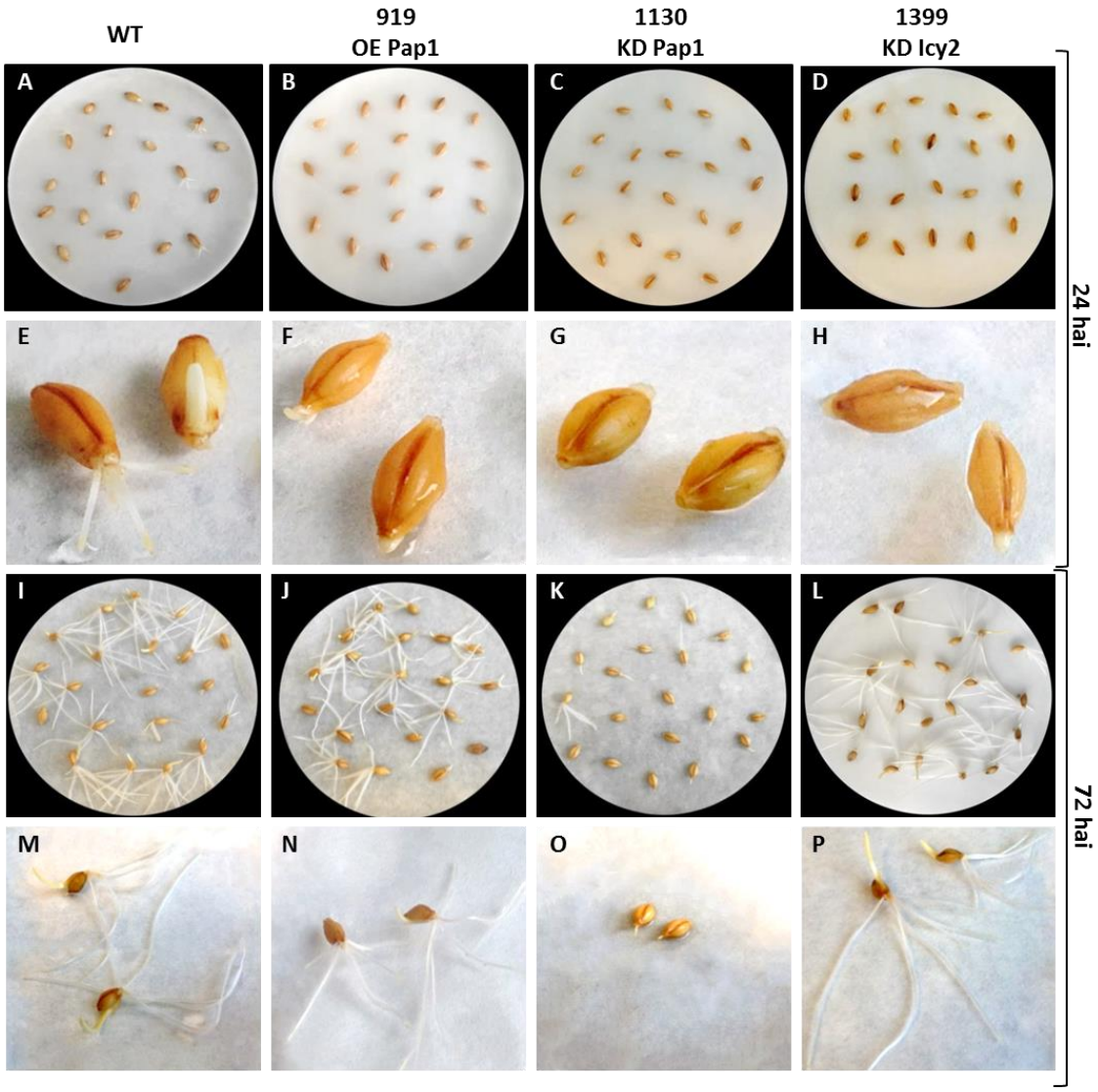


Figure 5.4. Photographs of the germination process in wild-type and transgenic lines. **A-D**, **I-L**, General appearance of the germination process of grains 24 and 72 hours after imbibition (hai), respectively. **E-H**, **M-P**, Detailed appearance of two grains per line at 24 and 72 hai, respectively.

However, the activity levels reached at this time point were distinct among different lines. All transgenic lines showed a significantly lower activity than the wild-type, especially KD Pap1 lines. Finally, trypsin-like activity behaved differentially (Fig. 5.6C). It increased progressively during the germination of wild-type grains, although maximum levels were lower than CysProt activities at 72 hai. This pattern was similar for OE Pap1 and KD Icy2 grains, but the activity in these lines did not increase at 24 hai and the activity at 72 hai was significantly lower than in the wild-type. In contrast, KD Pap1 lines reached more trypsin-like activity in the dry grain than wild-type; this activity

peaked up at 24 hai at levels similar to that observed in wild-type grains at 72 hai, and then the activity decreased.

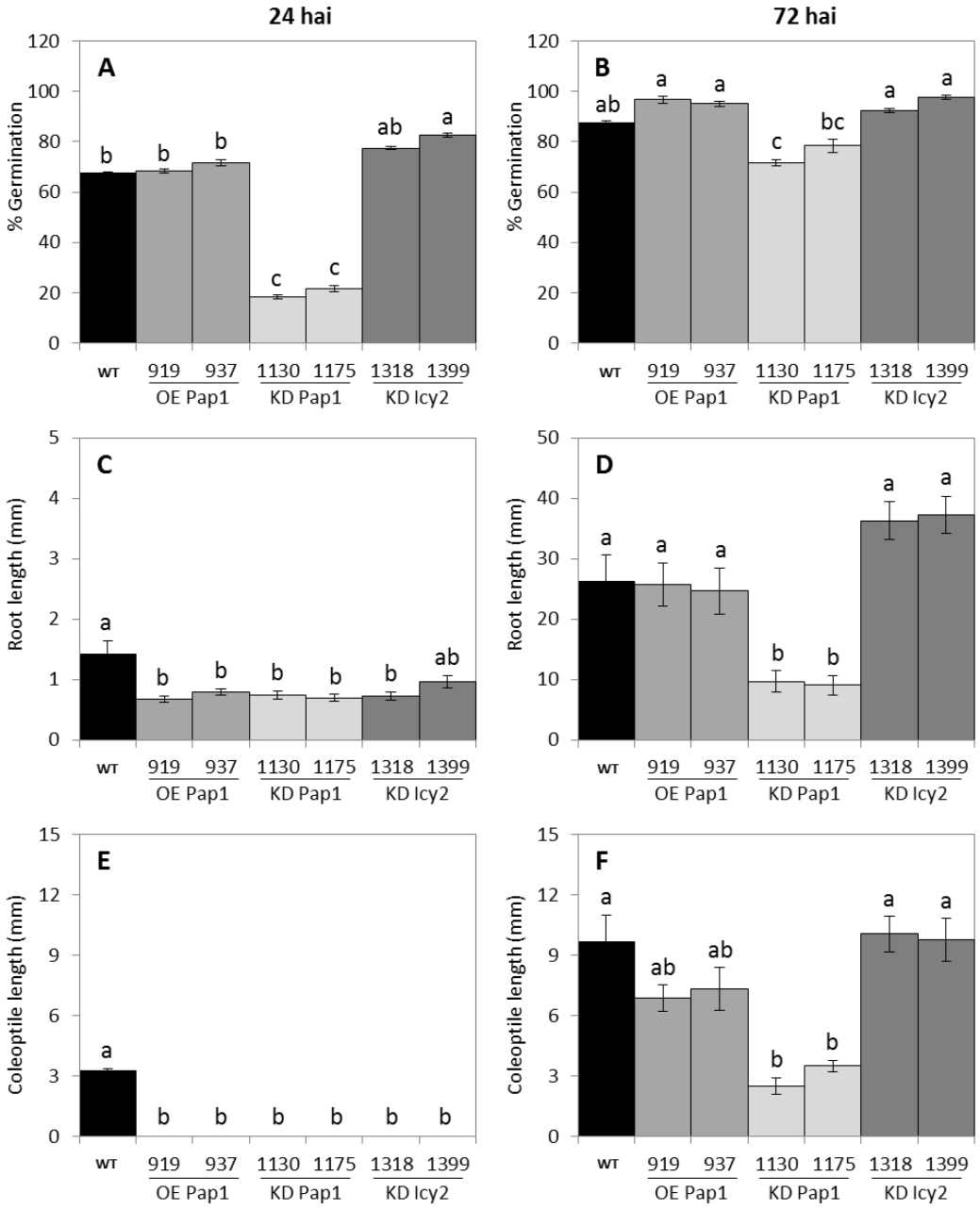


Figure 5.5. Quantification of the germination process for wild-type and transgenic grains at 24 and 72 hours after imbibition (hai). **A-B**, Percentage of germinated grains. **C-D**, Root length. **E-F**, Coleoptile length. Different letters indicate significant differences between lines ($P < 0.05$, HSD).

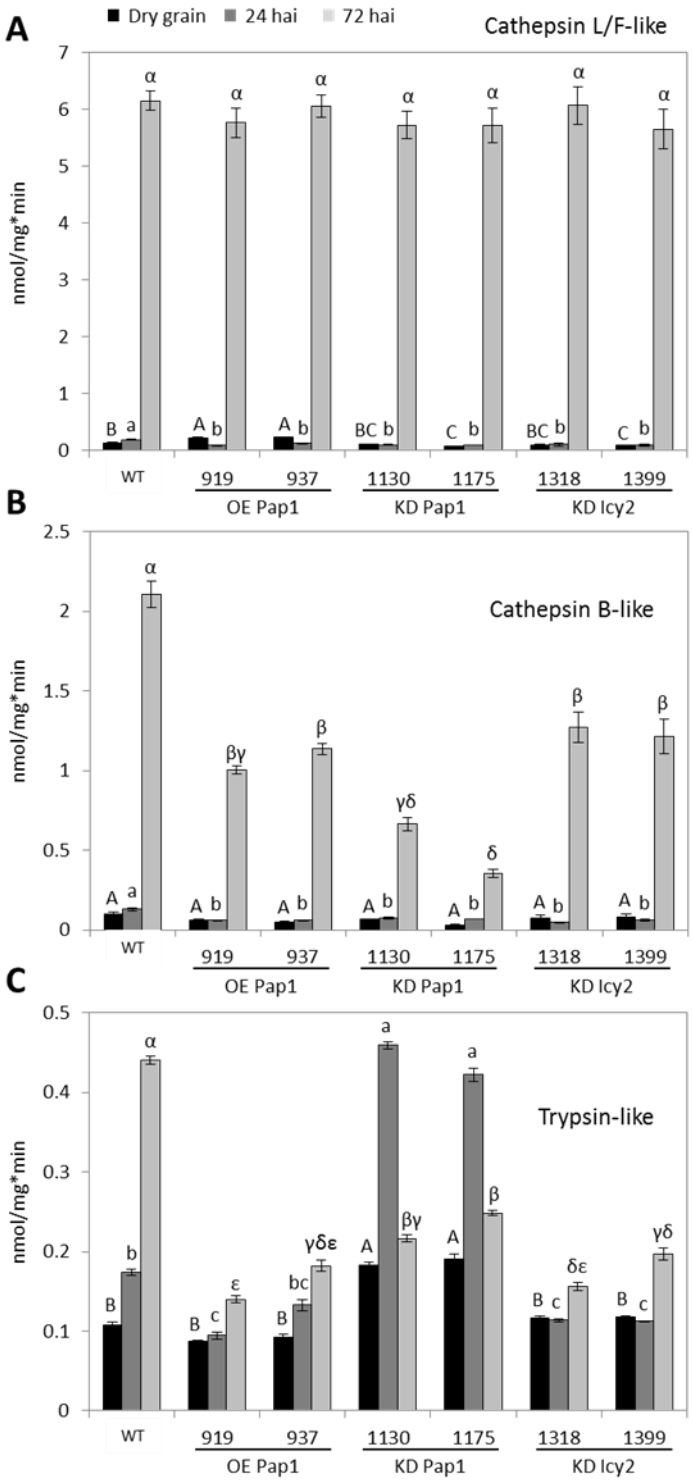


Figure 5.6. Proteolytic activities in de-embryonated grains of wild-type and transgenic lines. **A**, Cathepsin L-/F-like CysProt activity. **B**, Cathepsin B-like CysProt activity. **C**, Trypsin-like serine protease activity. Different letters indicate significant differences between lines for each time point ($P < 0.05$, HSD). Capital, small and Greek letters are used for dry grains, 24 hours after imbibition (hai) grains and 72 hai grains, respectively.

5.3.7. C1A CYSPROT PATTERNS ARE ALTERED IN TRANSGENIC BARLEY GRAINS

The protein profiles of CysProt HvPap-1, HvPap-6 and HvPap-19 were analyzed by immunoblot assays using total protein purified from de-embryonated grains (Fig. 5.7). The active (mature protein) and inactive (including the inhibitory propeptide) forms of these CysProt were observed. HvPap-1 active and inactive forms increased progressively during germination in all analyzed lines, although different levels for this protein were observed. As expected, OE Pap1 plants showed a higher quantity of this protein than wild-type whereas this protein was clearly reduced in KD Pap1 lines. The HvPap-19 active form was mainly present in wild-type dry grains. Its protein quantity increased during germination, reaching elevated levels at 72 hai in all lines. In KD Pap1 samples, the accumulation of HvPap-19 CysProt was lower. The active form of the HvPap-6 CysProt increased in all transgenic lines at 72 hai but in wild-type grains the inactive form was predominant at the same time point.

In addition, grain embryos at 24 hai were used to analyze the expression of several proteases of the C1A family by RT-qPCR. De-embryonated grains were not used since the quantity and quality of the RNA obtained from this tissue was insufficient for an accurate analysis. Ten proteases were analyzed: two cathepsin F-like (*HvPap-1* and -*2*), five cathepsin L-like (*HvPap-4*, *-6*, *-9*, *-10* and -*17*), one cathepsin H-like (*HvPap-12*) and two cathepsin B-like (*HvPap-19* and -*20*). The results indicated a great variability in the expression levels for these genes in embryos from wild-type and transformed plants (Supplemental Fig. S 5.5). Whereas several genes, such as *HvPap-1*, *-4*, *-10* and -*19,* were highly expressed, others, such as *HvPap-2*, *-9,* were poorly expressed. Comparing transgenic with wild-type embryos, several genes were repressed in all transgenic lines (*HvPap-4*, *-9* and -*10*); *HvPap-6* and -*12* were induced in KD Icy2 lines; *HvPap-17* was repressed in OE Pap1 and KD Icy2 lines; and the two cathepsin B-like genes *HvPap-19* and -*20* were repressed in KD Pap1 lines. As expected, *HvPap-1* was induced in OE Pap1 lines and repressed in KD Pap1 lines.

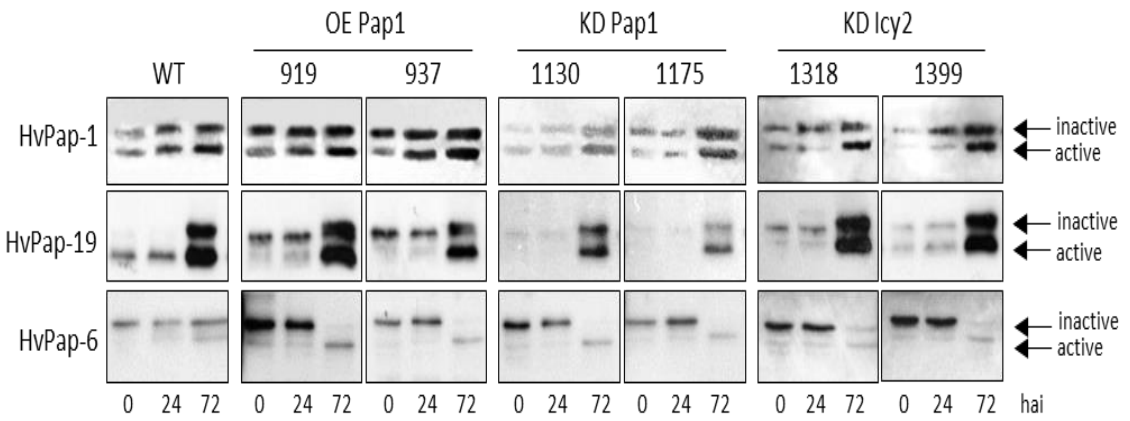


Figure 5.7. Immunoblot analyses in de-embryonated grains of wild-type and transgenic lines. The accumulation of three different C1A CysProt, HvPap-1, -6, and -19 was determined at different times during grain germination (0, 24 and 72 hours after imbibition).

5.3.8. C1A CYSPROT ARE DIFFERENTIALLY LOCATED IN EMBRYOS OF TRANSGENIC AND WILD-TYPE BARLEY LINES

Embryos were histochemically characterized at 24 hai (Supplemental Fig. S 5.6). De-embryonated grains were not used since the features of this tissue prevent the obtaining of thin sections for an accurate analysis. Higher protein content was revealed by Coomassie blue staining in OE Pap1 and KD Icy2 embryos (Supplemental Fig. S 5.6A-D). Starch was almost absent in OE Pap1 embryos after Lugol staining (Supplemental Fig. S 5.6E-H).

C1A CysProt HvPap-1, HvPap-19 and HvPap-6 were localized by immunofluorescence in wild-type and transgenic embryos at 24 hai (Fig. 5.8). A punctate or ring-shaped pattern of labelling was observed, the latter probably corresponding to the location of the proteases at the periphery of the protein bodies identified by the histochemical study. HvPap-1 was strongly detected in OE Pap1 and KD Icy2 lines. HvPap-19 was localized to all four specimens analyzed but it was particularly strong in KD Icy2 embryos. HvPap-6 showed a weak punctate labelling in amiRNA events, KD Pap1 and KD Icy2 lines.

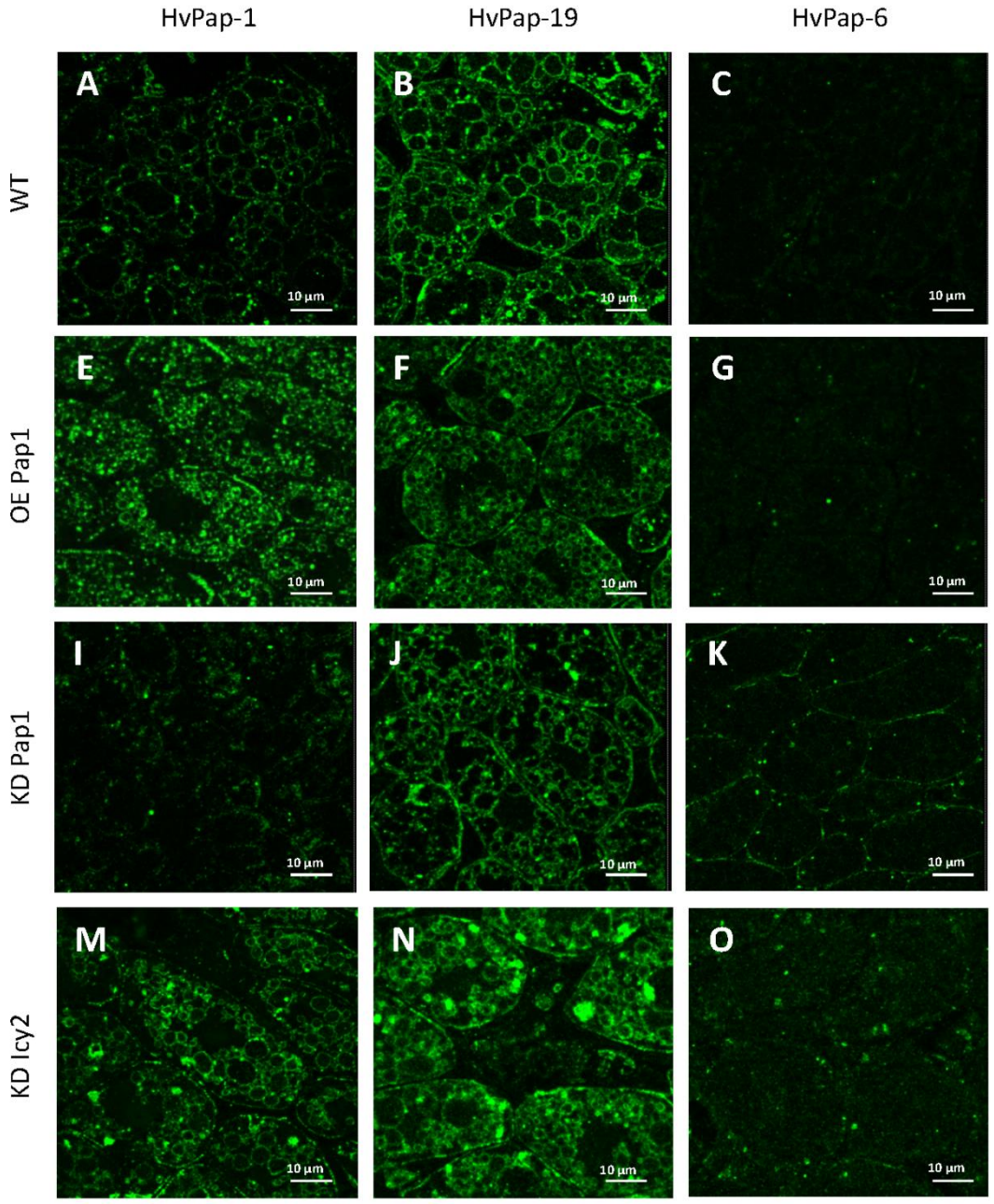


Figure 5.8. Maximum projections of confocal series of the immuno-fluorescence localization of barley CysProt HvPap-1 (**A**, **E**, **I**, **M**), HvPap-19 (**B**, **F**, **J**, **N**) and HvPap-6 (**C**, **G**, **K**, **O**) in embryos from transgenic and non-transgenic grains at 24 hours after imbibition.

5.4. DISCUSSION

Barley germination involves the activity of several proteases and amylases that hydrolyze and mobilize storage compounds. To date, the mechanisms regulating the

action of these hydrolases are poorly understood. Various studies have been focused at the transcriptional level, comprising a complex network of regulatory pathways (An and Lin, 2011). The participation of several barley C1A CysProt during germination has been predicted since members of the C1A subgroups L-, B-, H, and F-cathepsins, such as HvPap-6, HvPap-19, HvPap-12 and particularly HvPap-1 are induced by GA treatment in barley grain (Holwerda and Rogers, 1992; Martinez *et al*., 2003; Martinez *et al*., 2009; Cambra *et al*., 2012). The cathepsin F-like protease HvPap-1 was firstly identified in barley grains during germination by a transcriptomic analysis (Sreenivasulu et al., 2008). *HvPap-1* was expressed in grain tissues during germination and it was able to efficiently degrade stored hordeins *in vitro* (Cambra *et al*., 2012).

To demonstrate the *in vivo* involvement of this peptidase during germination we have generated over-expression and knock-down barley transgenic plants for the *HvPap-1* gene. If HvPap-1 is one of the responsible enzymes to *in vivo* degrade stored proteins, a delay in the germination process should be expected for the silencing lines as well as an acceleration of the event should occur in the over-expressing ones. Several experiments were carried out to identify alterations during this process (Fig. 5.4 and Fig. 5.5). A decrease in the number of germinated grains over time was found in HvPap-1 knock-down plants, especially at 24 hai. The germination percentage was similar at 72 hai compared to the wild-type, although the knock-down grains were notably in an earlier stage of development. Although we could expect an increase in the germination rate in over-expressing plants, the number of germinated grains was similar to the wild-type and, in terms of developmental stage; they even had a slight delay as compared to wild-type. There are two possible explanations for these observations: i) over-expression or silencing of the *HvPap-1* gene leads to significant modifications in the grain composition that subsequently affect the germination process; ii) over-expression or silencing of the *HvPap-1* gene alter the expression of some other hydrolytic activities crucial in the mobilization of stored compounds.

The capacity to store different molecules in the grain is related to these two hypotheses. An increased capacity to uptake sucrose was previously related to a higher content of storage proteins in wheat (Weichert *et al*., 2010), and inactivation of

cytosolic ADP-Glc pyrophosphorylase resulted in decreased starch and storage proteins in barley endosperms (Faix *et al*., 2012). Modifications of grain composition have been found for both plants with altered *HvPap-1* expression, OE Pap1 and KD Pap1, in terms of a dissimilar accumulation of starch and free amino acids and a higher quantity of protein in the dry grains (Fig. 5.1, Fig. 5.2 and Fig. 5.3). These differences and additional variations in some other stored molecules (Bowerman *et al*., 2015), not tested in this work, imply a differential specificity in the source of nutrients that the embryo can use to develop in a new plant.

Likewise, alteration in the genetic content of *HvPap-1* implies changes in the expression of some other genes. Transcriptomic analyses of several C1A CysProt in embryos at 24 hai revealed that alterations in the expression of the *HvPap-1* gene were associated with variations in the expression of some other C1A CysProt (Supplemental Fig. S 5.5). This result is corroborated by immunolocation analyses performed in OE Pap1 and KD Pap1 embryos, in which differences on protein accumulation for HvPap-1, -6 and -19 have been found (Fig. 5.8). Besides, in de-embryonated grains, immunoblot analyses show that the accumulation of the same HvPap-1, -6 and -19 C1A CysProt varies between the different lines (Fig. 5.7).

Alterations in the expression for these proteolytic enzymes should be correlated to variations in the enzymatic activity showed by the de-embryonated grain during germination. Enzymatic activities measurements confirmed this circumstance (Fig. 5.6) and highlight the fact that enzymatic compensations would be involved in the response to alterations in the proteolytic mechanisms of the plant. However, an increase in the activity of the proteolytic machinery was not translated into a higher germination rate. KD Pap1 lines had a lower cathepsin L-/F-like and B-like enzymatic activity at 24 hai than wild-type plants but, in contrast, they had a higher trypsin activity. Although C1A CysProt is the main activity related to degradation of stored proteins during late germination, trypsin activities are important during early germination events (Wrobel and Jones, 1992). Thus, a quick and effective degradation of stored proteins could be correlated to a rapid germination in the KD Pap1 lines, which has not been shown. In fact, several other hydrolytic enzymes are necessary for

a proper degradation of the stored compounds, and genes encoding enzymes involved in the degradation of cell wall, lipids, starch and nucleic acids are also transcribed during this process (Sreenivasulu *et al*., 2008). Modifications in the levels of these enzymes will also affect the onset and speed of barley grain germination.

Furthermore, the proteolytic activity in the grain should be correlated to the degradation of different fractions of stored proteins. Several peptidases with cathepsin L-/F-like proteolytic activity had the capacity to degrade stored hordeins *in vitro* (Martinez *et al*., 2009; Cambra *et al*., 2012), and they are probably involved in the mobilization of the amino acid content of albumins and globulins. Different C1A CysProt could have specific capacities to degrade storage proteins. HvPap-1 has the ability to mainly degrade hordeins (Cambra *et al*., 2012), and a higher rate of degradation for this protein fraction was observed in OE Pap1 lines (Fig. 5.2). However, as mentioned above, a direct relationship between gene over-expression and physiological activity cannot be concluded since perturbations in the expression of other proteolytic enzymes may change the expected effect on protein degradation.

Thus, the changes observed in the speed of the germination process in both OE Pap1 and KD Pap1 lines should be globally considered as a consequence of both grain composition and the machinery necessary to mobilize the stored compounds.

Cystatins are key members in the regulation of C1A CysProt during barley grain germination (Martinez *et al*., 2009; Cambra *et al*., 2012). According to this, silencing of a cystatin in the grain could lead to an acceleration of the germination process, since inhibition of the C1A CysProt would be minor. The results obtained in KD Icy2 lines reinforce the importance of the complex network modulating mobilization of stored proteins. Compensating effects implying proteases such as HvPap-6 and HvPap-19 and probably some other enzymes (Fig. 5.7 and Fig. 5.8, Supplementary Fig. S 5.5) led to altered proteolytic activities (Fig. 5.6) and modified grain composition in this line (Fig. 5.1, Fig. 5.2 and Fig. 5.3). These perturbations would explain the distinct germination process observed in KD Icy2 grains (Fig. 5.4 and Fig. 5.5).

Free amino acids are major determinants related to grain processing, quality and food safety (Halford *et al*., 2015). Although most of the nitrogen in the grain is incorporated into proteins, free amino acids are crucial during germination. Genetic modifications leading to variations in the accumulation of storage proteins may alter the amino acid composition in the grain. For example, antisense C-hordein barley grains had metabolic changes leading to alterations in amino acid biosynthesis (Schmidt *et al*., 2015). Extensive changes have been detected in the free amino acid composition in all the different transgenic barley grains (Fig. 5.3). The lowest accumulation of most amino acids in both the KD Pap1 and KD Icy2 lines could be also related with the slow start of germination showed in both lines, which was most remarkable in KD Pap1 line. Besides, OE Pap1 line strongly accumulates the amino acids proline and glutamine. Hordeins are enriched in these two amino acids. Their abundance in the dry grain could be used as a source to get a higher accumulation of hordeins in OE Pap1 lines. However, a higher concentration of this storage protein does not correlate to an accelerated germination process.

In conclusion, the importance of HvPap-1 and HvCPI-2 proteins during grain germination has been demonstrated. Delayed germination phenotype observed in silencing *HvPap-1* plants agrees with a role for this protease in degrading grain stored proteins. However, caution should be taken when plants are modified over-expressing or silencing a peptidase or an inhibitor, since the plant tries to compensate the modified proteolytic effect by modulating the expression of some other peptidases or their inhibitors. The non-expected phenotypes during grain germination for overexpressing *HvPap-1* and silencing *HvCPI-2* plants are examples of this proteolytic reprogramming. Future work will aim to carry out similar analysis on other C1A proteases and their inhibitors in order to demonstrate the existence of a regulatory network during the germination process.

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5.6. SUPPLEMENTAL DATA

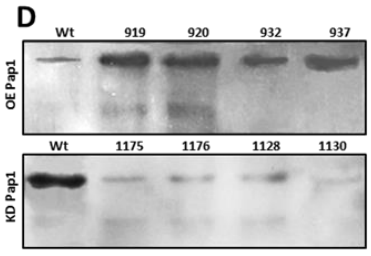
Figure S 5.1. Selection of *HvPap-1* transgenic homozygous lines of barley generated by double haploid technology. Over-expressing (OE Pap1) and knock-down (KD Pap1) plants were selected following a double criteria, single transgene integration and high mRNA and protein content. A, Number of independent homozygous lines per transformation event, number of events per construct and final selected lines used for molecular characterization. B, Estimation of transgene copy number by RT-qPCR assays coupled to the $2^{-\Delta\Delta Ct}$ method. Values are expressed as the average ± standard error of triplicate measurements. *Hv4hppd* and *HvCycl* genes were used as references for single copy and endogenous calibrators, respectively. CN: copy number for each group. C, Expression levels of the *HvPap-1* gene in transgenic barley lines by RT-qPCR technology, referred as relative mRNA levels of C1A CysProt genes normalized to barley *cyclophilin* mRNA content. D, Expression of HvPap-1 proteins in transgenic barley lines by western-blot assays.

A

	Construct	Event	Number of lines	Selected lines
Over-Expressing lines (OE)	OE Pap1	E04	12	-
		E06	15	-
		E07	13	-
		E13	6	**919, 920**
		E15	20	**932, 937**
		E27	20	-
amiRNA silencing lines (KD)	KD Pap1	E22	14	-
		E23	4	**1175, 1176**
		E24	4	-
		E25	14	**1128, 1130**

B

Lines	$2^{-\Delta\Delta Ct}$ ± SE	CN
Wt	0.93 ± 0.09	**1**
919	2.45 ± 0.25	**2**
920	1.81 ± 0.38	**2**
932	1.56 ± 0.18	**2**
937	2.06 ± 0.23	**2**
Wt	0.00 ± 0.00	**0**
1128	0.81 ± 0.15	**1**
1130	1.07 ± 0.06	**1**
1175	1.00 ± 0.08	**1**
1176	0.86 ± 0.16	**1**



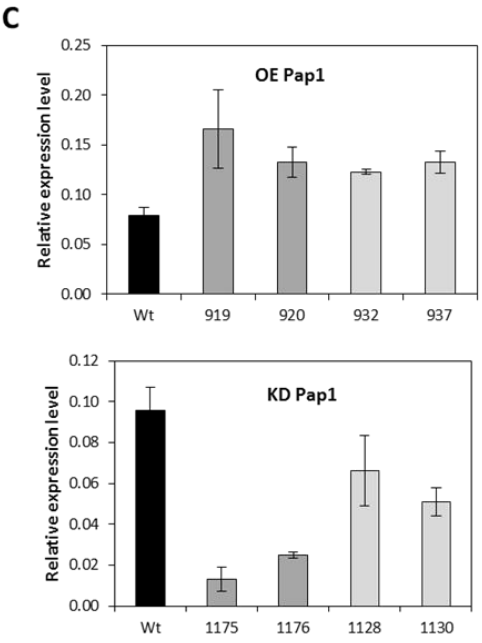


Figure S 5.2. Selection of *Icy-2* transgenic homozygous lines of barley generated by double haploid technology. Knock-down (KD Icy2) plants were selected following a double criteria, single transgene integration and low mRNA and protein content. **A**, Number of independent homozygous lines per transformation event, number of events per construct and final selected lines used for molecular characterization. **B**, Estimation of transgene copy number by RT-qPCR assays coupled to the $2^{-\Delta\Delta}$Ct method. Values are expressed as the average ± standard error of triplicate measurements. *Hv4hppd* and *HvCycl* genes were used as references for single copy and endogenous calibrators, respectively. CN: copy number for each group. **C**, Expression levels of the *HvIcy-2* gene in transgenic barley lines by RT-qPCR technology, referred as relative mRNA levels normalized to barley *cyclophilin* mRNA content. **D**, Expression of HvCPI-2 protein in transgenic barley lines by immunoblot assays.

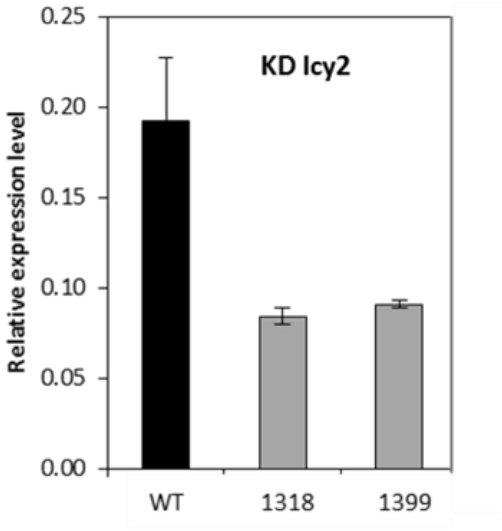
A

	Construct	Event	Number of lines	Selected lines
amiRNA silencing lines (KD)	KD Icy2	E02	11	**1318**
		E03	13	-
		E04	17	**1399**
		E05	21	-

B

Lines	$2^{-\Delta\Delta Ct}$ ± SE	CN
WT	0.00 ± 0.00	**0**
1318	0.74 ± 0.08	**1**
1399	1.03 ± 0.08	**1**

C



D

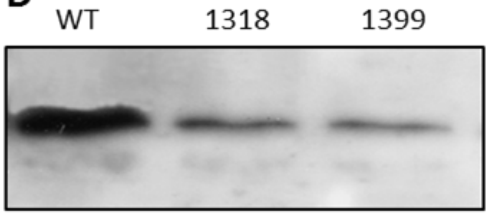


Figure S 5.3. Starch content of de-embryonated dry grain of OE Pap1: 919, 937 lines, KD Pap1: 1130 and 1175 lines, KD Icy2: 1318 and 1399 lines, and wild-type (WT) plants. Data, referred as grams of transformed starch per 100 grams of fresh weight, are means ± standard error of three independent replicates. Significant differences between wild-type and transgenic lines are indicated with capital letters ($P < 0.05$, HSD).

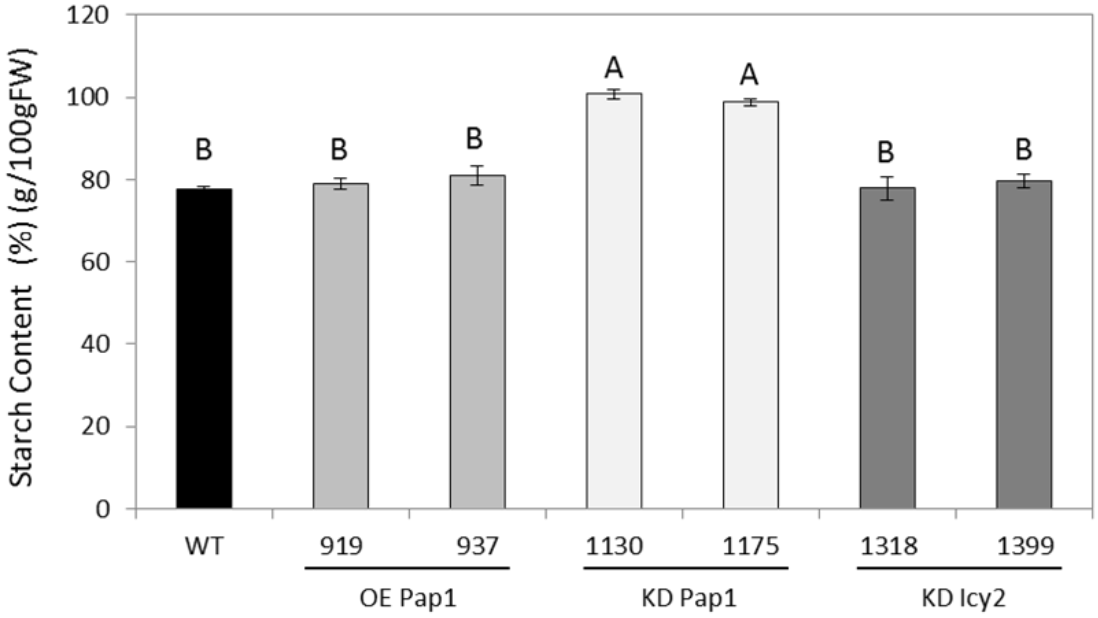


Figure S 5.4. Protein patterns of de-embryonated grains at different germination times (0, 24 and 72 hours after imbibition) of wild-type and transgenic OE Pap1, KD Pap1 and KD Icy2 lines carried out by SDS-PAGE and stained with Coomassie Brilliant Blue G-250. Twenty µg of each grain protein extract and of each hordein, globulin and albumin enriched fraction were used. **A**, Grain protein. **B**, Hordein fraction. **C**, Globulin fraction. **D**, Albumin fraction.

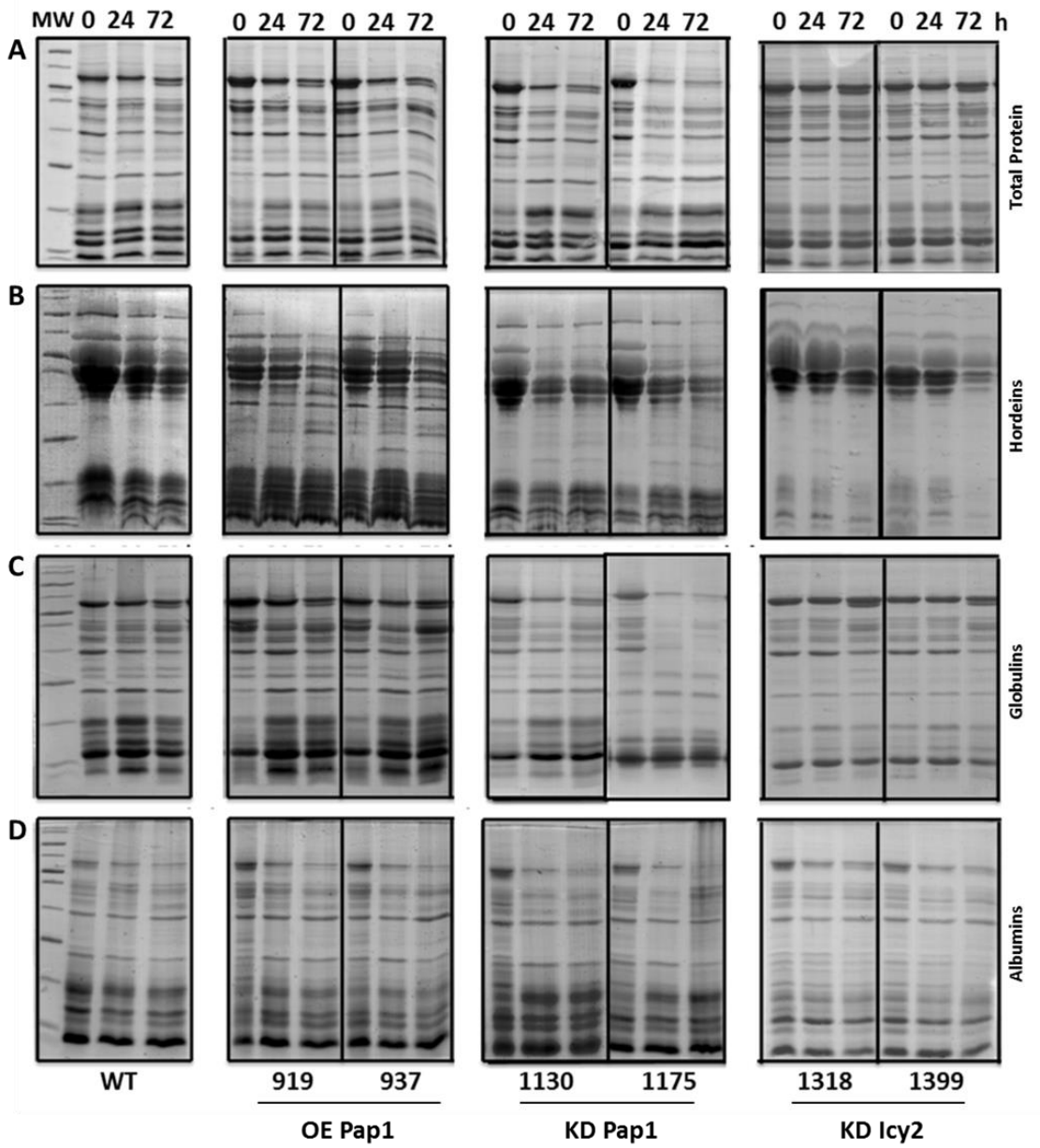


Figure S 5.5. RT-qPCR analyses of the mRNA accumulation in embryos of wild-type and transgenic lines at 24 hours after imbibition. Ten different C1A CysProt were analyzed (HvPap-1, -2, -4, -6, -9, -10, -12, -17, -19, -20). Relative expression was normalized to barley *cyclophilin* mRNA content.

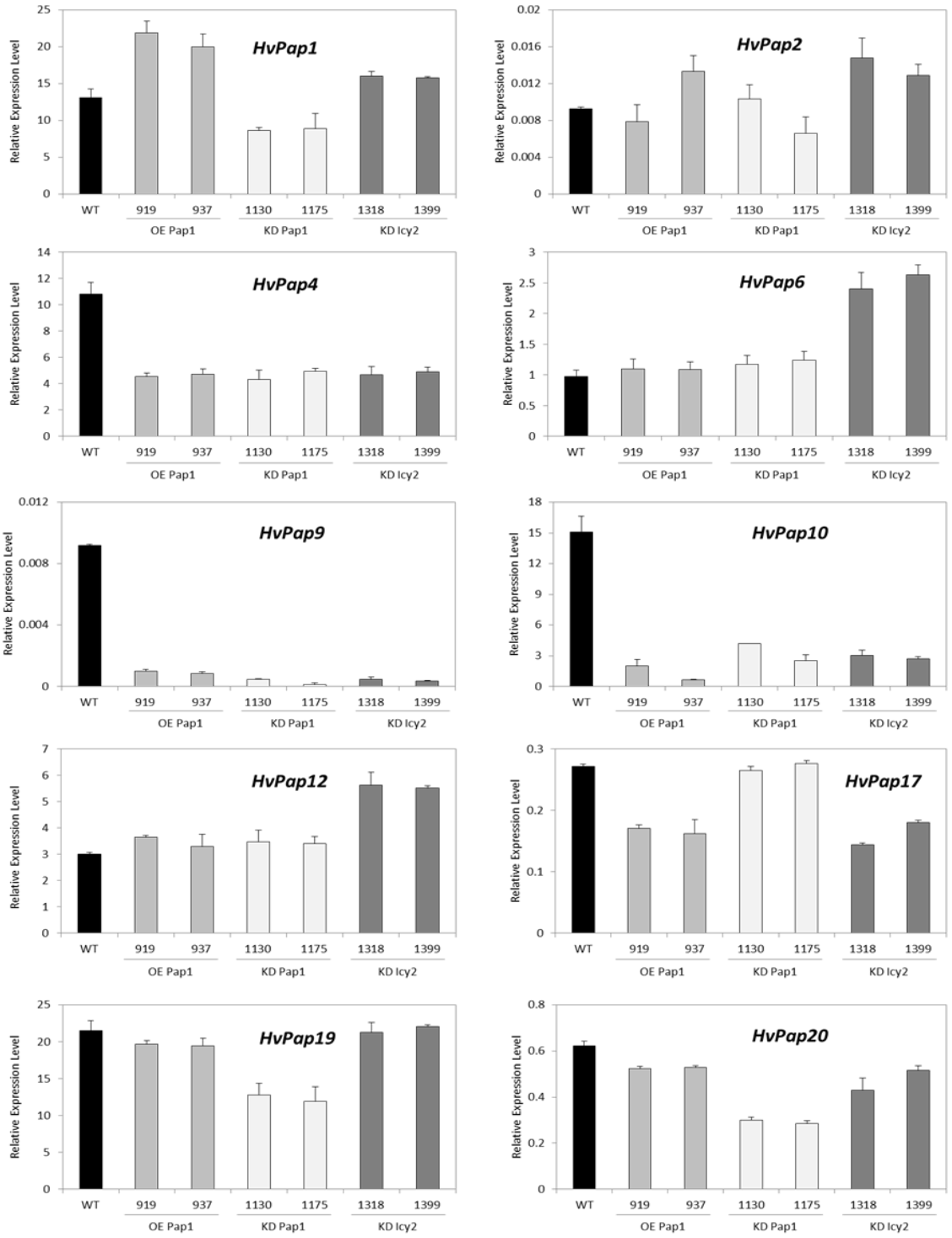


Figure S 5.6. Structural characterization of wild-type, OE Pap1, KD Pap1 and KD Icy2 embryos at 24 hours after imbibition. **A-D**, Protein structures stained with Coomassie Brilliant Blue G-250. **E-H**, Starch accumulation stained with Lugol. Images were observed on a Zeiss Axiophot microscope under bright field.

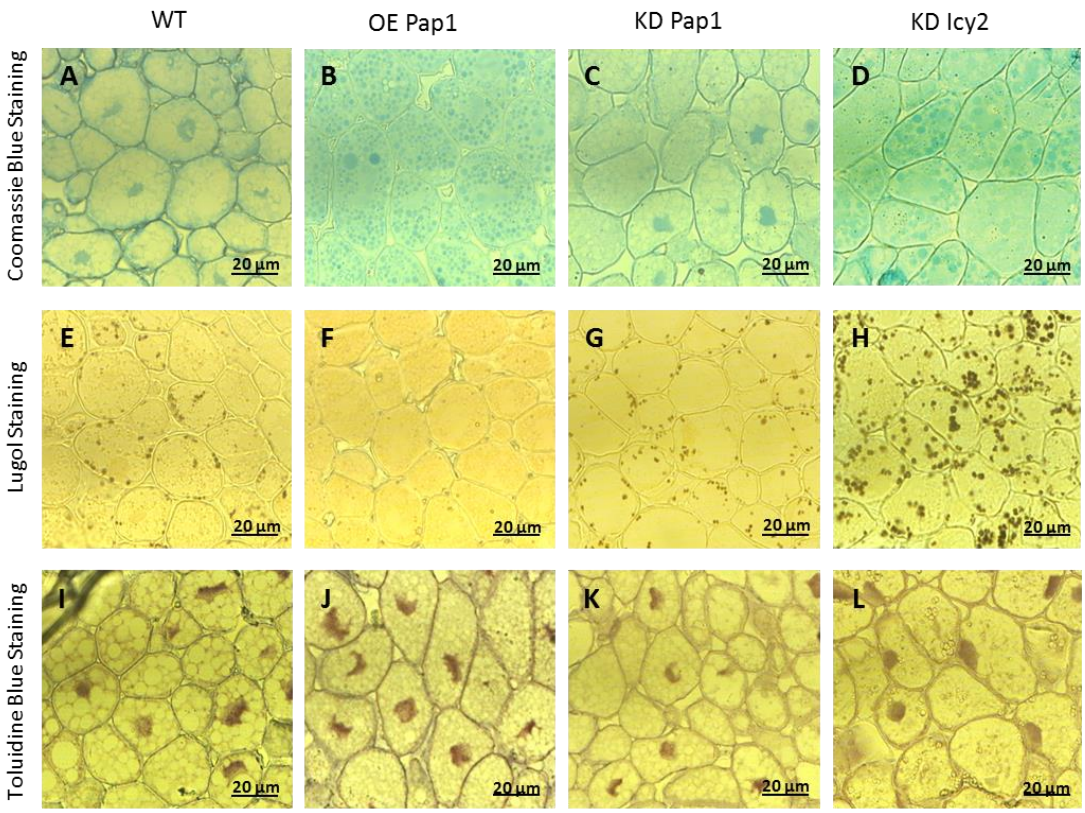


Figure S 5.7. Immuno-blot of recombinant barley CysProt (HvPap-19, 1, and 6) purified from *E. coli* cultures used to check peptide specificity. Only protein bands corresponding to the inactive form of each CysProt were observed. Protein detection was analyzed by using specific antibodies.

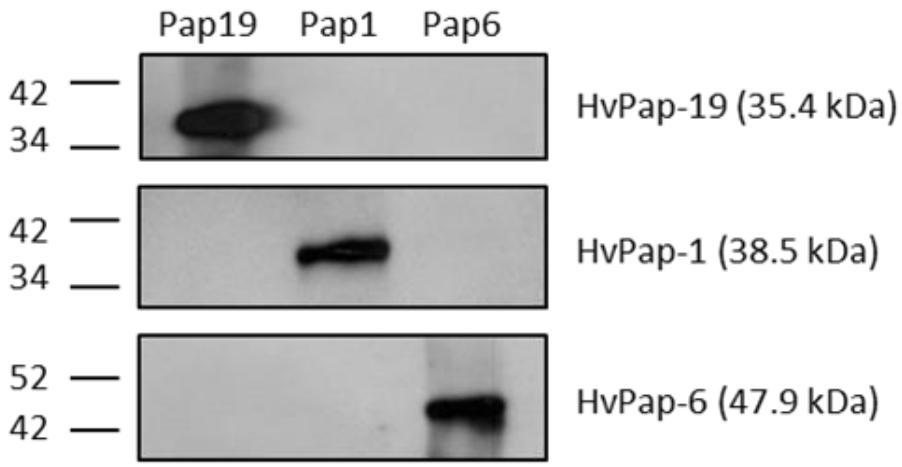


Table S 5.1. Identified compounds with statistical significance and their variation tendency for each of the comparisons. RMT (relative migration time *versus* IS). % change ([average concentration in the case group-average concentration in the WT group] *100/ average concentration in the WT group).

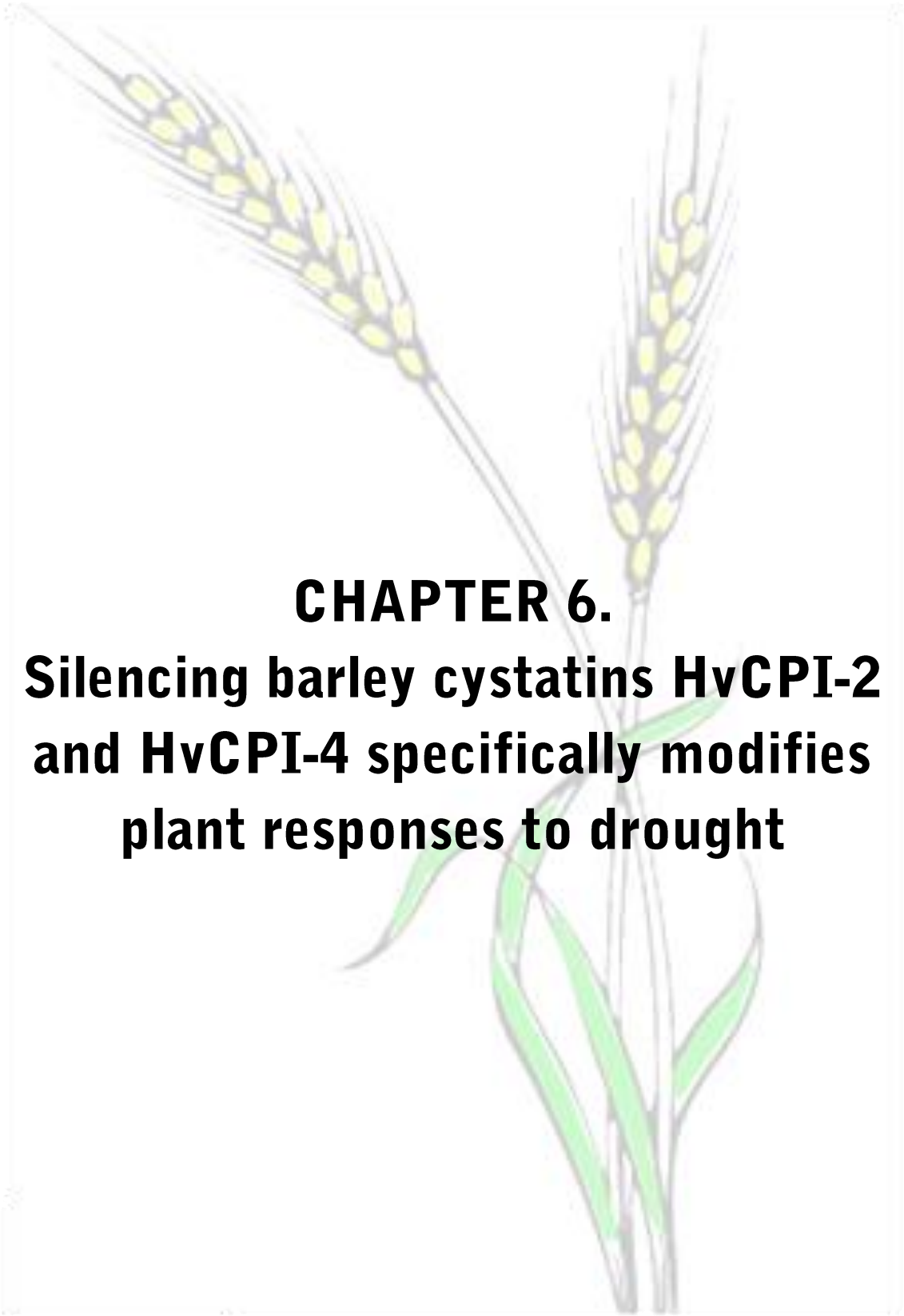
Compound	Formula	Mass	RMT	KD Pap1 1130 vs WT		KD Icy2 1399 vs WT		OE Pap1 937 vs WT	
				% Change	p-Value	% Change	p-Value	% Change	p-Value
Alanine	C3 H7 N O2	89.0477	0.67	-84.4	5.42E-04	-73.0	1.05E-03		
Sarcosine	C3 H7 N O2	89.0477	0.79	-77.0	1.39E-03	-36.4	1.64E-02		
Serine	C3 H7 N O3	105.0426	0.86	-86.4	1.55E-03	-73.2	3.03E-03	134.5	9.22E-03
Proline	C5 H9 N O2	115.0633	0.92			144.3	4.31E-02	810.2	3.21E-07
Valine	C5 H11 N O2	117.0790	0.86	-80.8	4.67E-04	-65.2	1.22E-03		
Betaine	C5 H11 N O2	117.0790	0.96	-77.7	6.65E-06	-36.7	2.36E-04	-66.2	1.47E-05
Leucine + Isoleucine	C6 H13 N O2	131.0946	0.88	-84.5	3.30E-04	-72.0	6.82E-04		
Asparagine	C4 H8 N2 O3	132.0535	0.90	-59.0	1.20E-03	-34.7	9.44E-03		
Aspartic acid	C4 H7 N O4	133.0375	0.97	-83.3	1.85E-04	-84.1	1.74E-04		
Glutamine	C5 H10 N2 O3	146.0692	0.92	-89.6	1.94E-02			1758.9	6.43E-04
Lysine	C6 H14 N2 O2	146.1055	0.63	-88.7	5.09E-04	-76.5	9.88E-04		
Glutamic acid	C5 H9 N O4	147.0532	0.93	-82.2	8.85E-04	-66.5	2.10E-03	-37.3	1.39E-02
Methionine	C5 H11 N O2 S	149.0514	0.91	-91.0	5.25E-04	-83.8	7.44E-04		
Histidine	C6 H9 N3 O2	155.0695	0.67	-92.6	3.87E-03	-91.4	4.07E-03	23.2	6.84E-01
Phenylalanine	C9 H11 N O2	165.0795	0.94	-86.1	1.30E-04	-78.4	2.06E-04	175.2	1.29E-02
Arginine	C6 H14 N4 O2	174.1117	0.65	-80.4	6.49E-04	-61.8	1.91E-03		
Tyrosine	C9 H11 N O3	181.0739	0.96	-84.9	3.80E-04	-77.6	5.79E-04		
Tryptophan	C11 H12 N2 O2	204.0899	0.94	-96.3	4.35E-05	-95.6	4.54E-05	-79.5	9.16E-05

Table S 5.2. Primer sequences used for RT-qPCR amplification to analyze the copy number in transgenic barley plants. *HvCycl* gene (cyclophilin), *Hv4Hppd* gene (4-hydroxyphenyl-pyruvate dioxygenase), *HvPap-1* gene (cathepsin F-like protease), *Icy-2* gene (cystatin) and *miR* (Osa-MIR528 miRNA gene).

Barley genes	**Primers**
HvCycl	forward: 5´-CCTGTCGTGTCGTCGGTCTAAA-3´ reverse: 5´-ACGCAGATCCAGCAGCCTAAAG-3´
Hv4Hppd	forward: 5´-GCTCCAAATCTTCACCAAGC-3´ reverse: 5´-CTCTTCCCCTCTCTCGTCCT-3´
HvPap-1	forward: 5´-TCCTGGAGTCGATCTTGGTTTC-3´ reverse: 5´- CAAGCATACTGTTGCGGCTTC-3´
Icy-2	forward: 5´-TCCTGGAGTCGATCTTGGTTTC-3´ reverse: 5´-CAAGCATACTGTTGCGGCTTC-3´
miR	forward: 5´-AGTTATGCGGCATTGATACCGGTCAGGAGATTCAGTTTGA-3´ reverse: 5´-AATTATGCGGCATAGATTCCGGTAGAGAGGCAAAAGTGAA-3´

Table S 5.3. Primer sequences used for the amplification of barley genes in RT-qPCR assays. *HvPap-4*, *HvPap-6*, *HvPap-9*, *HvPap-10* and *HvPap-17* genes (*c*athepsin L-like protease), *HvPap-1* and *HvPap-2* genes (cathepsin F-like protease), *HvPap-12* gene (cathepsin H-like protease) *HvPap-19* and *HvPap-20* genes (cathepsin B-like protease) and *HvCycl* (cyclophilin).

Barley genes	Primers
HvCycl	forward: 5'-TCCACCGGAGAGGAAGTACAGT-3' reverse: 5'-AATGTGCTCAGAGATGCAAGGA-3'
HvPap-1	forward: 5´-TCCTGGAGTCGATCTTTGGTTTC-3' reverse: 5´-CAAGCATACTGTTGCGGCTTC-3'
HvPap-2	forward: 5'-ATGGCTCGCCTCCGCCTCCGC-3' reverse: 5'-CTATTCCTTCTTAGAGGTATG-3'
HvPap-4	forward: 5´-CCTTGAGAGTCCTTGTTCCCGA-3´ reverse: 5´-CCATGTTCGTCGTTTTAACCGA-3´
HvPap-6	forward: 5´-TGCAATTGACGGCAAGAAGA-3´ reverse: 5´-TGGATCACCAGGTGATCATTTG-3´
HvPap-9	forward: 5'-ACTGCGACAACGTCAACAAC-3' reverse: 5'-TCTTCTGGATGAACTGGAAGGC-3'
HvPap-10	forward: 5'-TCGATCCATGTGCTTATCCGA-3' reverse: 5'-AACACACGCCTAATCCTTGGC-3'
HvPap-12	forward: 5´-ATGTGCGCTATTGCTACCTGC-3´ reverse: 5´-CACCTTATTCATGTCTGGCGAA-3´
HvPap-16	forward: 5´-CTGGATCGGTAAGAACTCGTGG-3´ reverse: 5´-TGATGGAGGTGCCATCATATGA-3´
HvPap-17	forward: 5'-AGCTGCGTGTGCATTTATCATG-3' reverse: 5'-GCGGTGAAATATGCAACCCA-3'
HvPap-19	forward: 5´-CACCTTATTCATGTCTGGCGAA-3´ reverse: 5´-TGCCCGCTTAATTTGACAGG-3´
HvPap-20	forward: 5'-GGAGGTCACGCTGTCAAGTT-3' reverse: 5'-GTATCCGTCATCACCCCATC-3'



CHAPTER 6.
Silencing barley cystatins HvCPI-2 and HvCPI-4 specifically modifies plant responses to drought

6.1. INTRODUCTION

Drought is expected to represent the leading environmental stress in agriculture threatening world´s food security by directly reducing yields of major crops in the current climate change framework. Detrimental effects may be even amplified due to synergistic events during the prevalence of biotic factors (Gupta *et al*., 2016; Valim *et al.,* 2016). Plants respond to water deprivation by activating specific molecular and physiological changes to minimize damage. Generally, stomata are closed to prevent transpiration, photosynthesis and respiration are inhibited, osmotically active compounds and protective proteins are accumulated, sink/source allocation is adjusted, senescence is accelerated and changes in the expression of phytohormones, mainly abscisic acid as regulator of the global process, are synthesized (Golldack *et al*., 2011; Thomas, 2013). A significant relationship within genes involved in leaf senescence and those participating in drought response has been described (Wehner *et al.*, 2016). Accelerated leaf senescence when a stress is perceived usually leads to a decreased in the canopy as an initial strategy, but normally this determines a reduced yield in annual crops. In most drought-conducted research works, authors hypothesize that it is possible to enhance drought tolerance without reducing harvest indexes by delaying leaf senescence during drought episodes (Rivero *et al.*, 2007). In several crops, drought stress resistance coincides with a stay-green trait (Gregersen *et al.*, 2013). Among the transgenic approaches to achieve stay-green phenotypes, expression of the isopentenyltransferase (IPT), an enzyme that catalyses the rate-limiting step in cytokinin synthesis under the control of senescence-associated promoters, has been the most successful (Gan and Amasino, 1995). This approach resulted in enhanced drought tolerance through a delay in leaf senescence, presumably through enhanced expression of dehydrins and heat shock proteins (Rivero *et al*., 2007; Zhang *et al.*, 2010; Qin *et al*., 2011). In most crops transformed with such constructs, the stay-green character has led to increased biomass, but this is not always translated into improved yields (Borrell *et al.*, 2014).

Enhanced expression of genes coding for proteases and their inhibitors is a common related event necessary for the reorganization of plant metabolism and

nutrient recycling (D.E. Martinez *et al*., 2007; Diaz-Mendoza *et al.*, 2016a,b; Velasco-Arroyo *et al.*, 2016). Although proteases involved in drought may differ from those specifically expressed in senescence (Khanna-Chopra *et al.*, 1999; Beyene *et al.*, 2006), experimental evidences suggest that drought-sensitive plants have higher proteolytic activity compared to resistant ones (Simova-Stoilova *et al.*, 2010). In this scenario, it is essential a fine-tuning regulation of protease activities mediated by specific inhibitors, cofactors and the activation of zymogens in response to specific environmental conditions (Cambra *et al.,* 2012a; Martinez *et al*., 2012). Protease inhibitory proteins are the main regulators of protein degradation caused by proteases. Among them, phytocystatins (PhyCys) are members of the plant subgroup to the ubiquitous family of cystatins (MEROPS identifier I25). PhyCys are proteins that tightly and reversible bond to the cysteine proteases (CysProt) from the C1A papain-like family. Some members contain a carboxy-terminal extension involved in the inhibition of a second family, the C13 legumain-like CysProt (M. Martinez *et al*., 2007; Martinez and Diaz, 2008). The structural complexity of cystatin complements in plant genomes typically encoding multiple inhibitory variants with distinct expression patterns and complementary affinity spectra for CysProt, suggests a diversity of functions (Benchabane *et al.*, 2010; Martinez *et al.*, 2016). Several findings support that PhyCys are involved in plant response to abiotic stresses. A multicystatin, VuC1, was induced by drought-stress in cowpea (*Vigna unguiculata*) leaves (Diop *et al*., 2004). Christova *et al*. (2006) reported the induction of a cystatin in winter wheat (*Triticum aestivum* L.). Drought repressed the expression of protease inhibitors (cystatins and serpins) in roots, while variable levels were detected in leaves when comparing two winter wheat varieties with differing drought sensitivity (Vaseva *et al.*, 2014). Ectopic cystatin expression suggested that this response implies a protective function for cystatins against abiotic stresses. Transformed tobacco plants expressing the rice cystatin oryzacystatin-I (OCI) were more resistant to the negative impacts of chilling stress on photosynthesis (Van der Vyver *et al*., 2003), showing delayed senescence and higher protein contents than WT at late stages. The OCI-dependent inhibition of CysProt also enhanced drought tolerance in soybean and Arabidopsis through strigolactones-mediated signaling (Quain *et al.*, 2014). Moreover, over-expression of two cystatins in Arabidopsis, AtCYSa and AtCYSb, increased resistance to drought, salt, cold and oxidative stress (Zhang *et*

al., 2008). Also, cystatin AtCYS4 seemed to be involved in thermotolerance under the control of the DREB2C cascade, leading to a decline of the endogenous CysProt activity (Je *et al.*, 2014). All described effects are a consequence of the direct inhibition of protease targets. Thus, manipulation of CysProt activities by altered PhyCys expression might improve resilience and quality towards the obtaining of crops with the capacity to adapt and/or tolerate restricted water regimes while rendering appropriate yields under these stressful conditions (Kunert *et al.*, 2015). However, pleiotropic effects of recombinant protease inhibitors *in planta* demonstrate that the current knowledge of plant proteolytic processes is still incomplete.

Barley represents a perfect model to study leaf stress, senescence and cereal improvement in the climate change scenario, specifically during drought events. It is paramount to generate stress-resistant varieties without neglecting quality (Perez-Lopez *et al.*, 2010; Dawson *et al.*, 2015). In barley, 41 C1A and 8 legumain CysProt have been identified, as well as 13 cystatins (Martinez *et al.,* 2009; Julian *et al.,* 2013; Diaz-Mendoza *et al*., 2014). The relationship of barley CysProt with senescence associated to abiotic stimuli has been reported. Several C1A and C13 CysProt differentially respond to abiotic stresses such as drought, low nitrogen, darkness or abscisic acid treatments (Parrot *et al.,* 2007; Julian *et al.,* 2013; Diaz-Mendoza *et al*., 2014; Velasco-Arroyo *et al.,* 2016). Among PhyCys, HvCPI-2 and HvCPI-4 present the highest expression in leaves. While the expression of HvCPI-4 and HvCPI-3 was induced by darkness treatment, the expression of HvCPI-2 was not altered (Diaz-Mendoza *et al.,* 2014). All these cystatins are able to inhibit C1A CysProt, but their efficiency depends on the specific CysProt involved (Martinez *et al*., 2009). HvCPI-4 is the only barley PhyCys that presents a long C-terminal extension which is also able to inhibit legumain-like proteases (M. Martinez *et al.,* 2007). The functional relationship between barley cystatins and C1A CysProt is reinforced from their common implication as counterparts during hordein storage protein mobilization upon barley grain germination (Cambra *et al.,* 2012b; Diaz-Mendoza *et al.,* 2016a).

The current work analyzes the implications of PhyCys under drought conditions, assessing the impact of silencing the barley´s *Icy-2* and *Icy-4* genes encoding cystatins

HvCPI-2 and HvCPI-4, respectively. The ultimate aim of this study is to decipher their putative *in vivo* roles and to deepen on the existing knowledge about molecular events underlying proteolysis-induced senescence.

6.2. MATERIALS AND METHODS

6.2.1. PLANT MATERIAL AND GROWTH CONDITIONS

Transgenic barley lines (*Hordeum vulgare* L. cv. Golden Promise) silencing *Icy-2* and *Icy-4* genes (KD Icy2 and KD Icy4 hereafter) were generated by amiRNA technology in collaboration with the IPK Gatersleben, Plant Reproductive Biology Group, according to Hensel *et al*. (2009). The partial molecular characterization for the homozygous KD Icy2 lines was recently reported in Diaz-Mendoza *et al*. (2016a) and it has been completed in the current research. Genetically fixed KD Icy4 transgenic plants were obtained and evaluated (gene copy number, mRNA expression levels), as described in Diaz-Mendoza *et al*. (2016a). The amiRNA construct for KD Icy4 lines was likewise engineered from pNW55 vector replacing the 21 bases of the natural Osa-MIR528 miRNA to specifically silence the *Icy-4* gene (5'-TCAAACTACATTACTGTGGGC-3'). Grains of transgenic and non-transformed (WT) barley lines were germinated in pots of 8x8x8cm size with a mixture of soil and vermiculite (3:1), at 22°C under a 16 h light/8 h darkness photoperiod, for 7 days in Sanyo MLR-350-H chambers. At this point, they were subjected to drought stress imposed by continuous water deprivation for 7, 10, 14 or 21 days, watering in alternate days in the case of controls. Pots were placed over plastic plates to individualize watering. For characterization and selection experiments, 7 day-old plants were used. Whole plant leaves were harvested, frozen into liquid nitrogen and stored at −80 ºC until further analysis. Each experiment was set up three times (independent biological replicates).

6.2.2. PHENOTYPICAL ASSESSMENT

A monitoring of senescence symptoms was performed with drought experiments, comparing control *vs* treated WT and transgenic lines. In addition, the natural

senescence progression for all lines growing under controlled greenhouse conditions in individual pots (15cm Ø) was weekly documented. Likewise, the oldest leaf of each sample was examined under a Leica MZ10 F stereomicroscope (Leica, Wetzlar, Germany) and serial sections were collected on a Leica SP8 confocal microscope (Leica, Wetzlar, Germany) to detect the red auto-fluorescence from the chlorophyll (laser excitation line 633 nm).

6.2.3. PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS

Drought-induced senescence progression was assessed by determining several physiological parameters. Aerial plant biomass was measured by weighting (Precisa XB 2200 C) at the end of each treatment (fresh weight), and after drying in a stove at 70ºC (dry weight). Aerial plant water content (PWC) was obtained from these measurements (fresh - dry weight). Soil water content (SWC) was analyzed with a soil moisture sensor (SM150 Delta-T-Devices, Cambridge, UK). Stomatal conductance (gs) was determined with a leaf porometer (SC-1 Decagon-T, Pullman, USA).

Photosynthetic pigments, total chlorophyll and carotenoids contents were quantified in treated and non-treated WT and transgenic lines following Velasco-Arroyo *et al*. (2016). Results were expressed as milligrams per gram of dry weight.

6.2.4. REAL-TIME QUANTITATIVE PCR ANALYSES

Leaves from WT and transgenic plants were used for RT-qPCR analyses. RNA extraction, cDNA synthesis and RT-qPCR conditions were performed as described in Velasco-Arroyo *et al*. (2016). Primers are specified in Supplementary Tables S 6.1 and S 6.2.

6.2.5. ELECTROLYTE LEAKAGE

Electrolyte leakage (EL) was determined in WT and transgenic barley lines under 14 days of drought *vs* control conditions following Rolny *et al*. (2011). Four freshly cut

apical old leaves (3 cm length) were floated on 15 mL of deionized water with continuous shaking. EL content in the solution was measured immediately (C0) and after 3 h (C3) of incubation at room temperature using a conductimeter (EC-Metro BASIC 30, CRISON). Total electrolyte content was determined in the same way after boiling for 10 min (TC). Results were expressed as percentage of EL: % EL= 100 * (C3-C0)/TC.

6.2.6. PROTEIN QUANTIFICATION AND PROTEASE ACTIVITIES

Total soluble protein was extracted from barley WT and transgenic lines at different time points, following the procedures detailed in Velasco-Arroyo *et al*. (2016). Protein quantification was carried out on a microplate reader (Varioskan Flash, Thermo Scientific) according to Bradford (1976), with bovine serum albumin as standard. Results were expressed as milligrams of protein per gram of dry weight.

In vitro enzymatic assays with the obtained crude protein extracts were performed to test cathepsin L-/F-, B- and legumain-like activities. Fluorogenic substrates, buffers and assays were as in Velasco-Arroyo *et al*. (2016). Results were expressed as nanomoles of substrate per milligram of protein hydrolysed in a minute. Blanks were used to account for spontaneous breakdown of the commercial substrates.

6.2.7. METABOLOMICS ANALYSES

For metabolomic assays, all samples were obtained from dried grinded barley leaves after 14 days of drought or watering treatments. Sugars were analyzed at the Gas Chromatography Service at CIB (CSIC, Madrid, Spain), as previously described by Bernabe *et al.* (2011) with slight modifications. After extraction in methanol, polysaccharides were hydrolysed with 3 M trifluoroacetic acid (TFA). The resulting metabolites were identified and quantified by gas-liquid chromatography (GLC), after derivatization of the monosaccharides released to their corresponding TMS-oxymes. The absolute configuration of the sugars was determined by gas-liquid

chromatography-mass spectrometry (GC-MS). A list of standards with their exact monoisotopic mass, migration time, and molecular formula was used.

Amino acids were analyzed at the Protein Chemistry Service at CIB (CSIC, Madrid, Spain), following the procedure of Spackman *et al.* (1958), with slight modifications. After MeOH resuspension and disruption, samples were centrifuged and the supernatant was evaporated to dryness in a SpeedVac. The metabolite extracts were resuspended in 6 N hydrochloric acid containing a known concentration of norleucina (used as internal standard), vortexed and then centrifuged. Finally, samples were injected on a Biochrom 30 Amino Acid Analyser (Biochrom, USA) and amino acids were post-column derivatised with ninhydrin reagent and detected by absorbance at 440 (proline) or 570 (rest of amino acids) nm. A mixture of amino acids at known concentrations (Sigma) was used as standard. After quantitation, differences for individual metabolites were evaluated, values were transformed to a $\log_2$ scale and used for Heat map representation using the software R Project (v.3.1.2) package.

6.2.8. STATISTICAL ANALYSES

A t-Student test was used to assess differences between control and drought for each tested barley *Icy*-gene in the correspondent RT-qPCR experiments. Data related to characterization experiments were analyzed by One-Way ANOVA followed by a Student-Newman-Keuls (SNK) test. Two-Way ANOVA analysis was used to evaluate the effects of drought during timing-based experiments with WT, and among treatment, lines and their interaction on all dependent variables when using transgenic material. Means were compared using Tukey's (HSD) test. P-values less or equal to 0.05 were considered statistically significant.

A linear trend line was drawn through EL (%) and total protein content in the different plant lines. The R2 value indicates how well data fits the line. To test the statistical significance of the correlation between EL (%) and total protein in each line, a Pearson Product Moment Correlation test was performed. A negative correlation

coefficient (r) and a p value lower than 0.05 described a negative correlation. The software GraphPad Prism 6 was used for all statistical analyses.

6.3. RESULTS

6.3.1. SOIL WATER DEFICIT ALTERS PLANT PHYSIOLOGICAL STATUS AND DROUGHT-ASSOCIATED BIOMARKERS

To establish a time point to further analyze the effects of drought, seven day-old barley plants were subjected to water deprivation and results at 7, 10, 14 and 21 days of treatment were analyzed. Phenotypic observations showed a progressive decrease on aerial biomass, leaf number and turgor leading to the appearance of senescence symptoms at 10 days of treatment (Suppl. Fig. S 6.1a). After 14 days, an incipient senescence-like status was displayed as indicated by the yellowish of the oldest leaf. Besides, 21 days of drought induced an acute phenotype indicating cell death. As expected, plant and soil water contents (PWC and SWC, respectively) decreased over time in stressed samples and limited moisture was detected in soil after 14 days of water deprivation (Suppl. Fig. S 6.1b,c). A significant reduction in other drought markers, such as stomatal conductance, chlorophyll and carotenoids content, and total soluble protein, were also detected when drought-stressed and watered plants were compared after 7 days of treatment. These parameters progressively decreased over time and were hardly measurable in 21 days-treated plants (Suppl. Fig. S 6.1d-g). Finally, control and stressed plants showed similar levels of cathepsin L-/F- and B-like enzymatic activities after 7 days of treatment but a continuous reduction of these activities was observed in treated plants (Suppl. Fig. S 6.1h,i). Altogether, these results suggest that 14 days of water deprivation was the time point to perform drought experiments and analyze its effects. At this time of water deprivation, the stress was evident and there was limited water in the soil, while severe symptoms, as those observed after 21 days of treatment, were not detected yet.

6.3.2. BARLEY CYSTATINS HVCPI-2 AND HVCPI-4 ARE INDUCED BY DROUGHT

Since proteolysis is a key process associated to senescence induced by abiotic stresses, the expression of protease inhibitors should be regulated to tightly control protease activity. A RT-qPCR analysis of the whole barley cystatin family was conducted at 14 days of drought. Only two (*Icy-2* and *Icy-4*) out of the 13 members were significantly induced in stressed *vs* control plants (Fig. 6.1). To obtain further insights on the role of these two PhyCys, we decided to use silencing transgenic plants for the mentioned cystatins, using soil water deprivation as the stressor factor.

Transgenic *Icy-2* knock-down barley plants (KD Icy2) were previously obtained by artificial microRNA (amiRNA) technology (Diaz-Mendoza *et al*., 2016a). Homozygous transgenic barley plants for *Icy-4* gene (KD Icy4) were generated using the same amiRNA approach via *Agrobacterium*-mediated transformation. Two additional KD Icy2 silencing lines and four KD Icy4 silencing lines belonging to separated transformation events were selected for further characterization (Suppl. Fig. S 6.2a). The selection was based on transgene copy number, transcript abundance and CysProt activities. All transgenic lines showed a unique transgene insertion, estimated by RT-qPCR assays and the $2^{-\Delta\Delta Ct}$ method (Suppl. Fig. S 6.2b). Their transcript levels were reduced in comparison to WT, although mRNA accumulation was not completely knocked out (Suppl. Fig. S 6.2c). The proteolytic activity pattern assessed in KD Icy4 lines revealed variations between transgenic and WT lines, as display the results on cathepsin L-/F-, B- and legumain-like proteolytic activities (Suppl. Fig. S 6.3).

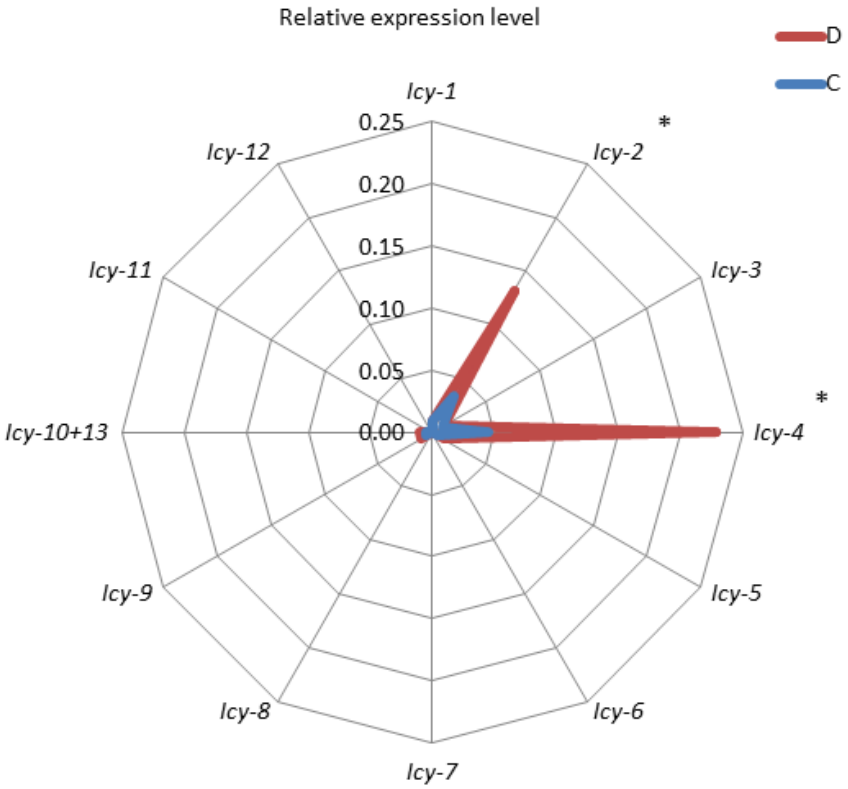


Fig. 6.1. Expression of barley cystatin genes in leaves after 14 days of water-deprivation (drought) and under optimal watering regime (control) treatments. Data were determined by quantitative real-time reverse-transcription PCR (RT-qPCR) and are expressed as relative mRNA levels of cystatin genes (*Icy*), normalized to barley *cyclophilin* mRNA content. Data represent the mean ± SE of at least 6 technical replicates from three independent experiments. Asterisks (*) indicate significant differences between control and drought (t-student, $p<0.05$).

6.3.3. KD ICY2 AND KD ICY4 LINES DISPLAY OPPOSITE PHENOTYPES DURING NATURAL AND DROUGHT-INDUCED SENESCENCE

The *in vivo* behaviour of cystatin knock-down plants was firstly analyzed by controlling their phenotypes during the whole plant life cycle (natural senescence) and at the initial growing stages under controlled water deprivation. Natural growth revealed a greener phenotype and higher biomass for KD Icy2 lines in comparison to WT during all monitored stages (Suppl. Fig. S 6.4a-d). In contrast, KD Icy4 lines showed an earlier lighter green coloration, and a general smaller size and thinner stems (Suppl. Fig. S 6.4e-h). In addition, a striking phenotype was observed in the last natural senescence

stage (12 weeks). KD Icy2 lines presented a stay-green-like phenotype in which senescence was initiated on schedule but the photosynthetically active phase was expanded (Fig. 6.2). KD Icy4 plants showed a quite similar senescence related phenotype at the plant life end compared to WT, aside from differences in size and tiller abundance (Fig. 6.2).

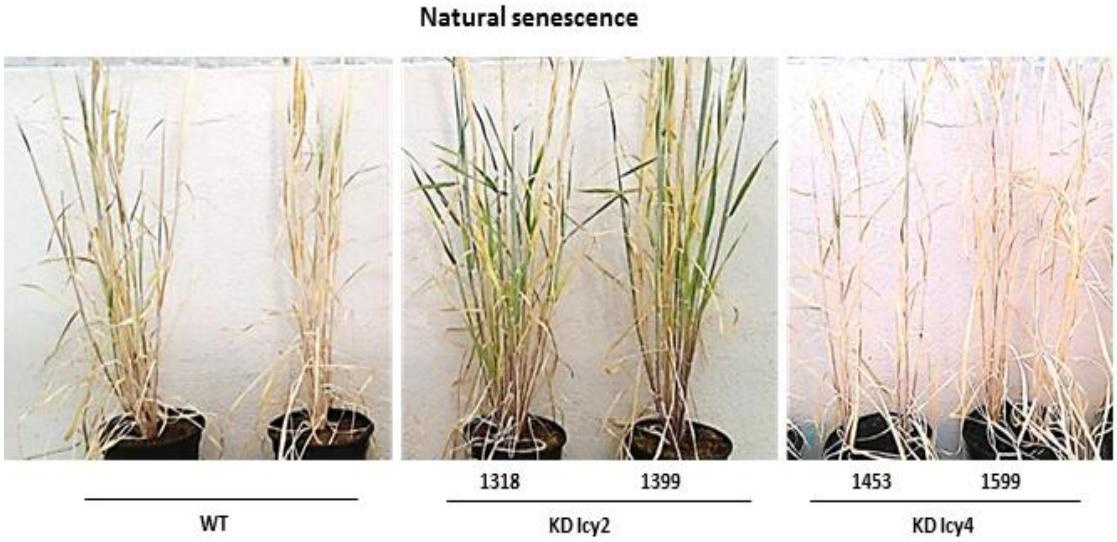


Fig. 6.2. Natural senescence phenotypes of 12 week-old wild-type (WT), KD Icy2 (1318 and 1399) and KD Icy4 (1453 and 1599) silencing barley plants.

Phenotypes were likewise recorded after drought experiments. KD Icy2 lines presented a similar size than WT plants but exhibited a healthier aspect concerning leaf turgor than WT and KD Icy4 plants during drought treatments. Conversely, KD Icy4 plants always displayed shorter leaves after drought than WT (Fig. 6.3a). Apical senescence in the oldest leaf was clearly drawn in drought-stressed WT while no apparent symptoms were detected over KD Icy2 samples. In contrast, an advanced stage of brownish and yellowish symptoms was detected in the oldest leaf apex of the KD Icy4 stressed plants, even greater than in WT (Fig. 6.3b). Chlorophyll auto-fluorescence in drought-treated samples was assessed in the medium part of the oldest leaf, and observed under the confocal microscope (Fig. 6.3c). Whereas the lowest fluorescence signal was recorded in KD Icy4 lines, KD Icy2 ones emitted a strikingly higher signal than WT. Concomitantly, observed chlorophyll alterations were

coincident with the monitored status in the tissue structures under bright field (Fig. 6.3c).

6.3.4. TRANSGENIC BARLEY LINES SHOW SLIGHT VARIATIONS RELATED TO PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS AFTER DROUGHT TREATMENT

To test how genetically modified plants responded to soil water deficit from a physiological aspect, various parameters were measured. Regarding stomatal conductance and fresh weight of the aerial part, small differences were observed among lines. However, when comparing treated *vs* control conditions, all plants evinced a significant decrease in both parameters (Suppl. Fig. S 6.5a,b). In contrast, KD Icy2 plants retained significantly more water in the plant than WT or KD Icy4 plants after drought (Suppl. Fig. S 6.5c). Likewise, whereas limited moisture was recorded in the soil of WT and KD Icy4 plants after the stress, some water was retained in the substrate of KD Icy2 plants (Suppl. Fig. S 6.5d).

The chlorophyll content of KD Icy2 and KD Icy4 plants was significantly higher or lower, respectively, in well-watered plants in comparison to WT (Suppl. Fig. S 6.5e). A significant reduction in the total amount of chlorophyll was observed in treated plants. Interestingly, KD Icy2 lines maintained significantly higher chlorophyll levels than KD Icy4 ones (Suppl. Fig. S 6.5e), which corroborates chlorophyll auto-fluorescence observations (Fig. 6.3c). Carotenoids levels showed a great variability independently of the transgene insertion in both treated and non-treated lines, and they were only significantly reduced in WT plants after drought treatment (Suppl. Fig. S 6.5f).

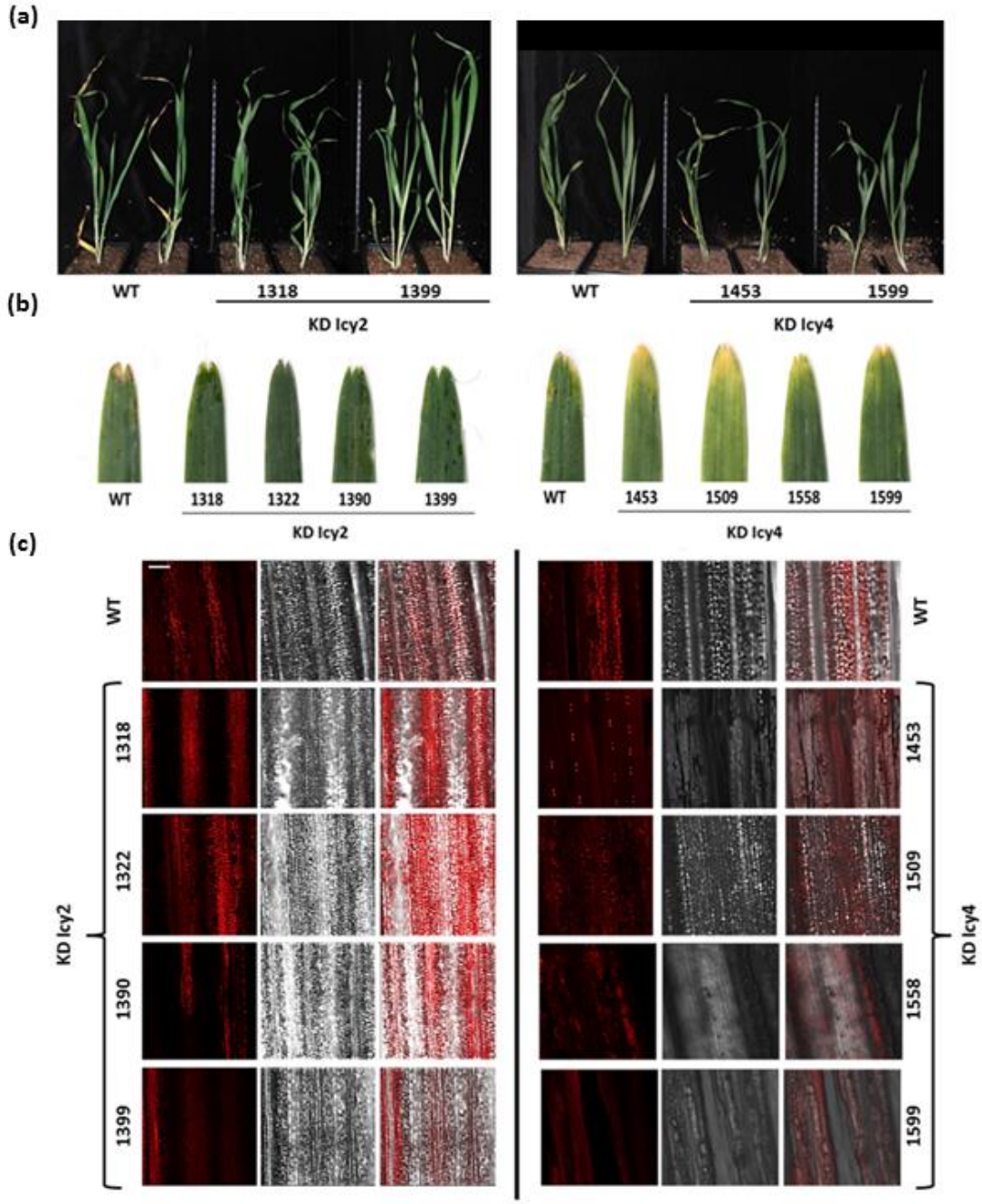


Fig. 6.3. Phenotypes of wild-type (WT), KD Icy2 (1318, 1322, 1390 and 1399) and KD Icy4 (1453, 1509, 1558 and 1599) silencing barley plants after 14 days of water deprivation. (a) Whole plant phenotypes; (b) detail of the oldest leaf apex; (c) chlorophyll detection in the oldest leaf of transgenic and WT barley lines. Plants were collected and observed under a Leica SP8 confocal microscope using the laser excitation lines 633 nm to detect the red autofluorescence from the chlorophyll. The same images were taken under light field conditions, and the fluorescence signal overlap is likewise documented. Scale bar, 200 µm.

6.3.5. PROTEIN CONTENT AFTER DROUGHT TREATMENT IS RELATED TO PROTEASE ACTIVITY AND MEMBRANE STABILITY

To test how PhyCys-silenced barley plants modify their protein content status, the total amount of soluble protein was quantified in drought-treated and non-treated transgenic and WT lines. Small differences were found in the protein content of non-treated plants (Fig. 6.4a). After drought, all plants, except KD Icy2 1399 line, showed a significant reduction in the protein content (Fig. 6.4a). However, soluble protein concentration was significantly higher in KD Icy2 plants than in WT or KD Icy4 plants. In order to assess the concomitant protein degradation associated to leaf senescence, CysProt activities were measured using specific substrates for cathepsin L-/F-, B- and legumain-like proteases (Fig. 6.4b-d). Some differences on these activities were detected between transgenic and WT lines when plants were grown under optimal conditions. After applying a drought treatment, protease activities were significantly lower in all lines with the exception of the L-/F- and B-like activities in KD Icy4 plants. These plants rendered a similar proteolytic activity to that observed in well-watered plants, whose proteolytic activity was significantly higher than that observed in drought-stressed WT and KD Icy2 plants.

Protein content may be related to membrane stability. Thus, leaf electrolyte leakage and its correlation with protein content were analyzed in control and drought-stressed WT and transgenic lines (Fig. 6.5). The electrolyte leakage inversely correlated with the total protein content in the WT and KD Icy4 plants. Samples from control and drought treatments grouped in two quite separated clusters, with control plants showing lower values of electrical conductivity and higher values of total protein content whereas drought samples presented the opposite pattern. Conversely, regardless of the treatment and the protein content, KD Icy2 lines showed low electrolyte leakage which ended with a loss of the correlation between both parameters in treated/non treated samples.

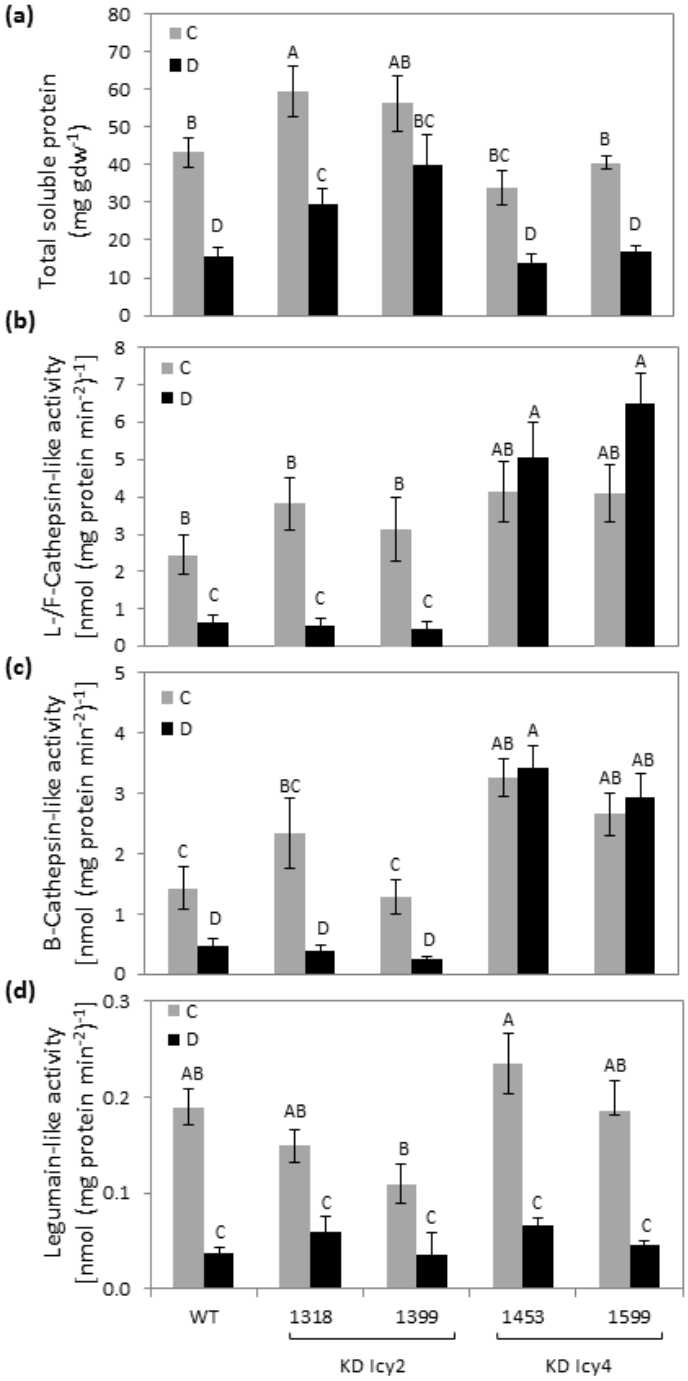


Fig. 6.4. Total soluble protein content and proteolytic activities of leaf extracts from wild-type (WT), KD Icy2 (1318 and 1399) and KD Icy4 (1453 and 1599) silencing barley plants at 14 days of water deprivation (black bars) or under optimal watering regime (gray bars). (a) Total protein content in milligrams per gram of dry weight (gdw). Proteolytic patterns using specific substrates to be degraded by (b) L-/F- cathepsin-like, (c) B- cathepsin-like and (d) legumain-like, expressed as nanomoles per milligram of protein and minute. Data represent the mean ± SE of at least 6 technical replicates from three independent experiments. Different letters indicate significant differences between plant lines and treatment, as determined by a Two-way ANOVA test (Tukey´s, HSD, at $p<0.05$).

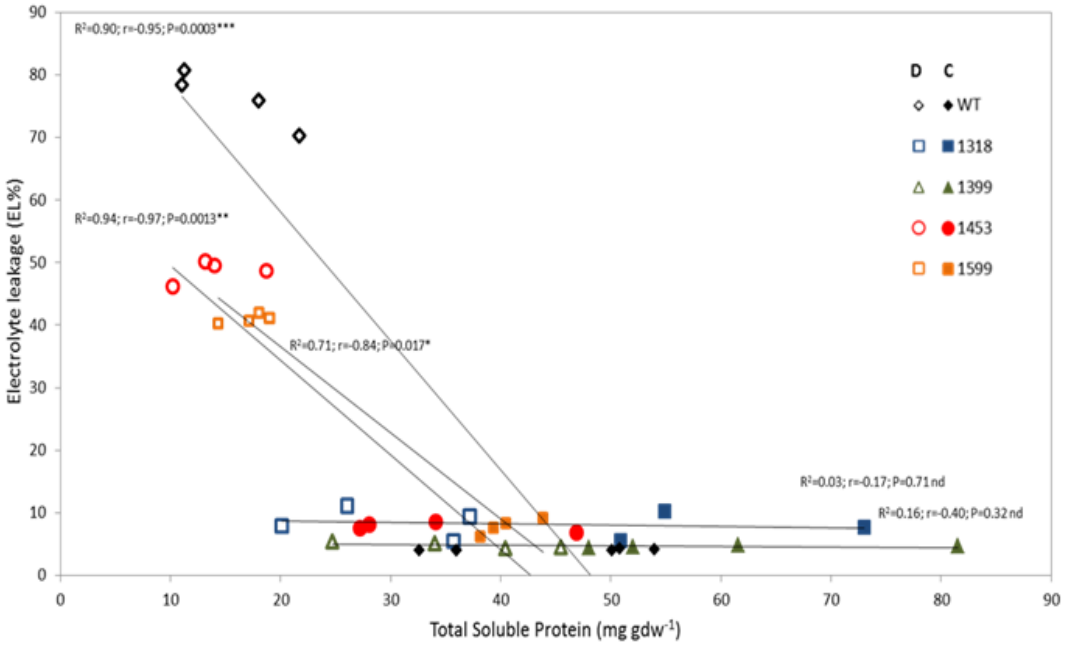


Fig. 6.5. Correlations between electrolyte leakage (EL) and total protein. Dispersion graphs showing the linear trend between the two parameters represented per line, the correspondent correlation coefficient (R^2) and the statistical result (r; P). Data for different lines are indicated in different colours and shapes. Wild-type (WT; black rhombus), 1318 (blue square), 1399 (green triangle), 1453 (red circle) and 1599 (orange square). Drought and control conditions are indicated by empty/filled shapes respectively.

6.3.6. MOLECULAR ALTERATIONS WITHIN TRANSGENIC LINES SHOW STRIKING COMPENSATION EFFECTS AT THE TRANSCRIPTIONAL LEVEL

Since *Icy-2* and *Icy-4* transcripts were upregulated after drought stress in non-transformed plants, their expression patterns were likewise studied by RT-qPCR under the tested conditions within all transgenic lines. A significant increase in *Icy-2* transcripts was observed in KD Icy4 lines in comparison to WT and KD Icy2 drought-treated samples (Fig. 6.6a). Interestingly, a mutual pattern was repeated in KD Icy2 background, in which *Icy-4* transcripts significantly increased during drought in comparison to WT and KD Icy4 lines (Fig. 6.6b). Although both genes were induced after drought-stress in their correspondent knock-down backgrounds, as expected, their expression levels were always below those observed in the WT plants.

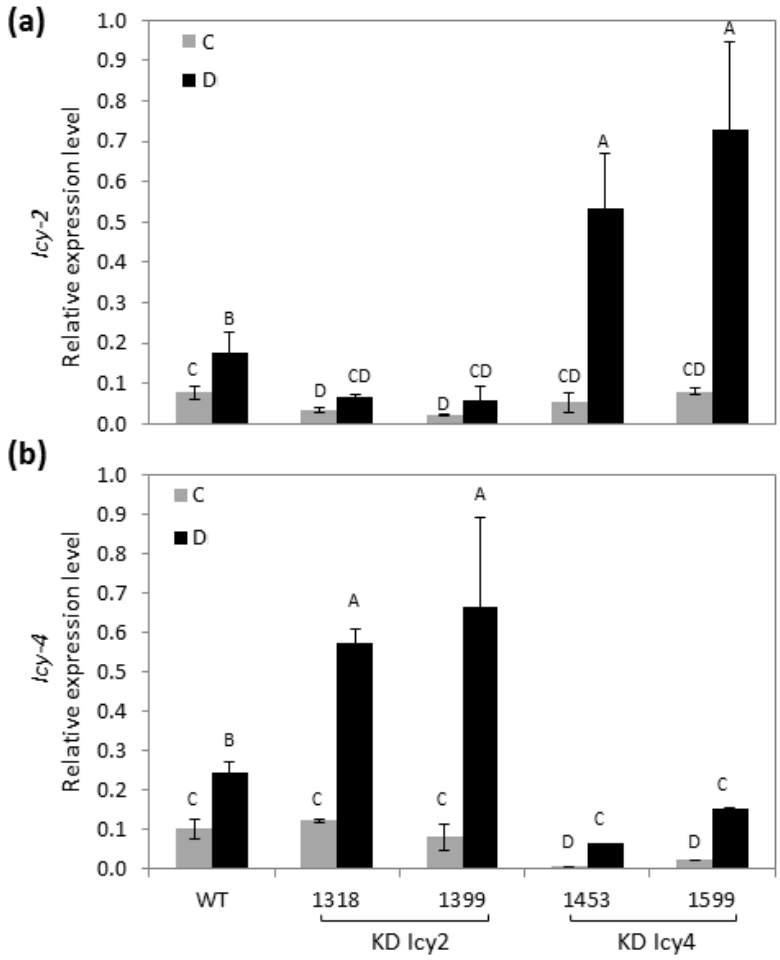


Fig. 6.6. Expression of barley cystatin genes *Icy-2* and *Icy-4* in wild-type (WT), KD Icy2 (1318 and 1399) and KD Icy4 (1453 and 1599) silencing barley plant leaves after 14 days of water deprivation (drought, black bars) or under optimal watering regime (control, grey bars) treatments. Data were determined by RT-qPCR and are expressed as relative mRNA levels of cystatin genes (*Icy-2* and *Icy-4*), normalized to barley *cyclophilin* mRNA content. Data represent the mean ± SE of at least 6 technical replicates from three independent experiments. Different letters indicate significant differences between plant lines and treatment, as determined by a Two-way ANOVA test (Tukey´s, HSD, at $p<0.05$).

Variations in the transcript content of PhyCys may have an effect on the expression of their targets. Thus, the expression patterns for several CysProt belonging to different C1A subgroups (F-, L-, H-, and B-like cathepsins) were analyzed by RT-qPCR under the same conditions (Suppl. Fig. S 6.6). Transcripts of *HvPap-1*, *HvPap-12* and *HvPap-19* significantly increased in stressed plants independently of the transgene insertion. To highlight, *HvPap-1* levels were significantly higher in WT and KD Icy4 than in KD Icy2 plants after drought. On the othe hand, *HvPap-4* and *HvPap-16* showed a

general repression pattern after drought. Based on these results, the observed great variability among and within genotypes and treatments may underlie compensation effects at the transcriptional level.

6.3.7. SLIGHT CHANGES IN METABOLITES CONTENTS ARE DETECTED IN BARLEY TRANSGENIC LINES SUBJECTED TO DROUGHT

Differences on protein content in the leaves for the examined lines may be reflecting changes in specific amino acids' metabolism. To test this hypothesis, metabolomics analyses were performed comparing transgenic and control treated and untreated leaf samples (Fig. 6.7a, Suppl. Table S 6.3). The most remarkable result is that the drastic reduction in total amino acids observed in drought-stressed WT in comparison to non-treated WT plants was not reproducible in any of the transgenic lines (Fig. 6.7a). However, the results revealed a differential global pattern among lines with minor variations when comparing the up/down individual trends of all the amino acids for a specific line. KD Icy4 lines (mainly KD Icy4 1599) had higher amino acid content than WT and KD Icy2 lines in control conditions. However, the response of each genotype to drought largely varied. All amino acids, except the sulfur-containing residues cysteine and methionine, decreased their content in WT plants. KD Icy2 plants (mainly KD Icy2 1318) increased their amino acid content, which was significantly higher than in WT drought-stressed plants for all amino acids except cysteine and arginine. On the contrary, the amino acids content of KD Icy4 plants was not altered after drought treatment and the quantity of every amino acid, except cysteine, was higher than in WT stressed plants.

Recycling along leaf senescence may alter reserve molecules other than proteins. Accordingly, several monosaccharides (glucose, galactose, arabinose and xylose) were quantified in a separated metabolomics assay (Fig. 6.7b, Suppl. Table S 6.3). Small differences were found in their content among all tested lines in control or drought conditions and no significant differences for both lines of a specific genotype could be detected within control and/or drought conditions when comparing all tested samples.

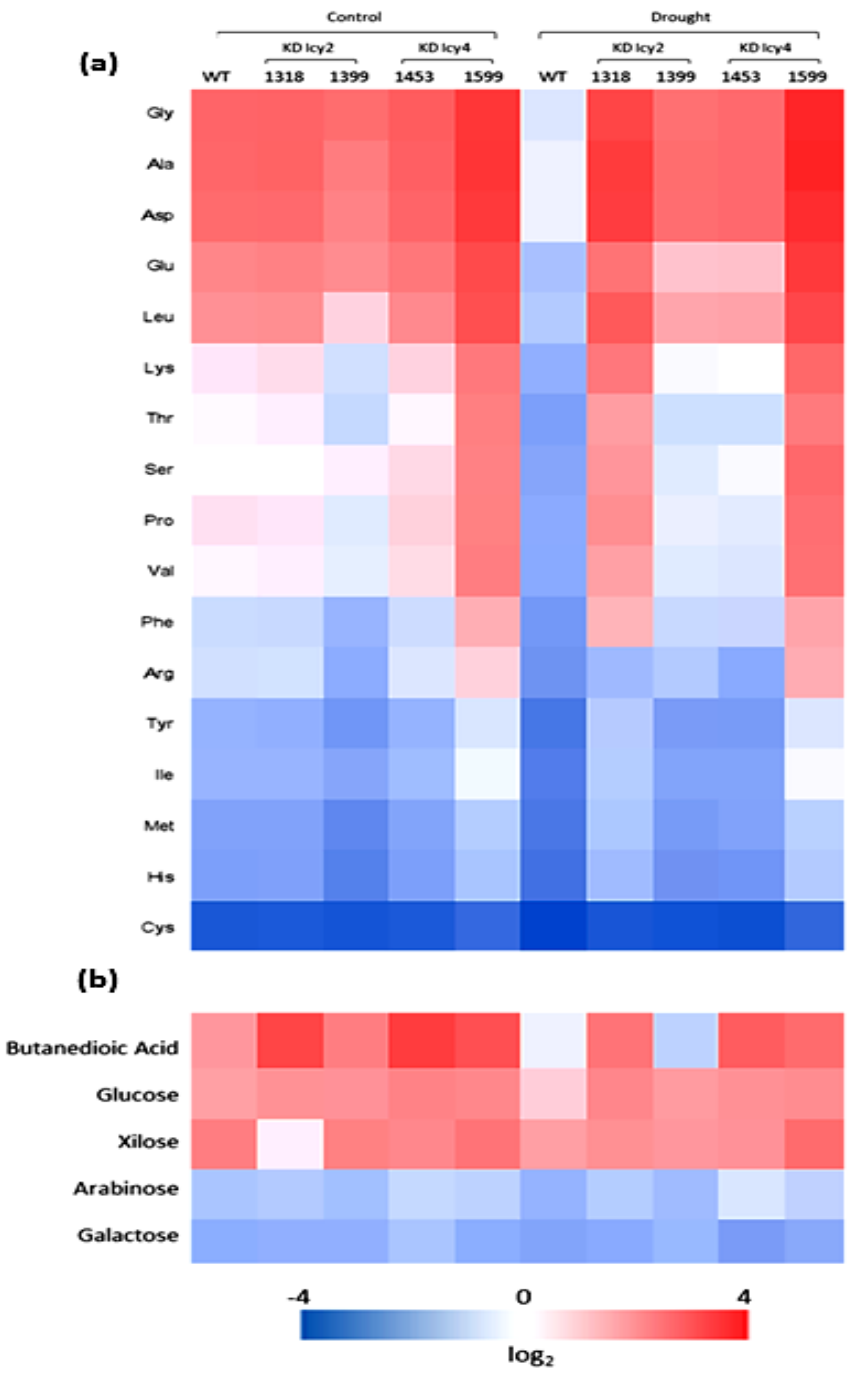


Fig. 6.7. Heat maps showing drought-induced changes in the accumulation levels of metabolites identified in wild-type (WT), KD Icy2 (1318 and 1399) and KD Icy4 (1453 and 1599) silencing barley leaves after 14 days of water deprivation (drought) or under optimal watering regime (control) treatments. (a) Amino acids, (b) carbohydrates and others components. Results of quantitative analyses for identified barley metabolites are normalized to an internal standard, averaged over three technical replicates from two independent experiments and transformed to a $\log_2$ scale. Data were presented in red (higher abundance) and blue (lower abundance), with the scale below the heat map. Quantitative results and statistical significance are shown in supplementary Table S 6.1.

Interestingly, the last metabolomics released data revealed the possible implication of one compound that participates in the Kelvin cycle, the butanedioic or succinic acid. This compound largely accumulates in both KD transgenic genotypes in comparison to WT, either under watering or drought conditions, but with a clear decrease during dehydration in all treated samples.

6.4. DISCUSSION

Altered phytocystatin expression has been postulated as a way to improve crops in the face of climate change (Kunert *et al.,* 2015). Since proteolysis is a crucial event involved in the response to abiotic stresses, such as drought, the modification of PhyCys expression patterns should be correlated to a modulation of CysProt activity.

Genetically modified plants have been used to understand how the overexpression of a PhyCys affects the response of the plant to a specific abiotic stress. For example, enhanced tolerance to drought has been achieved by overexpressing PhyCys in soybean and Arabidopsis (Prins *et al.,* 2008; Zhang *et al.,* 2008; Quain *et al.,* 2014). However, there is only one report that analyzes the response to an abiotic stress in a PhyCys silenced transgenic plant. Knock-down rice plants for the bifunctional OcXII inhibitor exhibited slower growth under alkali stress (Christoff *et al.,* 2016).

The phenotype of the genetically modified plant depends on the strength and the specificity of the inhibition of the endogenous proteases. For example, most of the 13 barley PhyCys are able to inhibit the same cathepsin L-like proteases, such as papain and the endogenous HvPap-4, -6, -10 and -16 C1A CysProt, but with different K_i values (Martinez *et al.,* 2009). This inhibitory redundancy could be translated into a stronger phenotype in overexpressing transgenic plants, since most PhyCys are able to inhibit several endogenous CysProt. Conversely, in knock-down transgenic plants, other PhyCys could overcome the loss of the inhibitory capacity of a single PhyCys. The exception would be the specific inhibition of a CysProt by a PhyCys. In this case, a strong phenotype would be found. Then, whereas the overexpression of a PhyCys is

the way to obtain broader responses against stresses, the silencing of a PhyCys is the way to evaluate the actual role of a PhyCys against a specific stress.

The final response to an abiotic stress is a consequence of the length and severity of the treatment. To detect a strong response, we selected 14 days of continuous water deprivation as the time point, since plants had an obvious deleterious phenotype but they were healthy enough to allow the extraction of proteins and nucleic acids. The importance of a gene in the plant´s reaction to a stress or stimulus usually relies on variations in its expression. The increase in mRNA levels of a gene is often related to an acquisition of the correct cellular state to ensure the best physiological condition. When we tested the changes in the expression levels of the thirteen barley PhyCys after the drought treatment, a strong induction for two of them, *Icy-2* and *Icy-4*, was detected, which implies a putative role of these PhyCys in the control of the specific proteolytic mechanisms triggered by drought stress.

Interestingly, the phenotypes of barley knock-down plants for *Icy-2* and *Icy-4* were different. The natural phenotype of KD Icy2 plants was characterized by a higher biomass than WT plants and a delayed senescence, leading to a stay-green stage. Conversely, KD Icy4 plants presented lesser biomass than WT ones and exhibited an earlier appearance of a pale green coloration. In agreement with these features, whereas KD Icy2 kept more chlorophyll than WT plants, KD Icy4 lines lost chlorophyll quickly along drought. Since C1A CysProt have been largely associated to the cellular dismantling occurring during natural or stress- induced senescence processes (Diaz-Mendoza *et al.,* 2014, 2016b), the phenotypes observed for KD Icy2 and KD Icy4 plants should be related to the differential inhibition of drought-associated C1A CysProt. Three out of the five C1A proteases analyzed, a cathepsin F-like, HvPap-1, a cathepsin H-like, HvPap-12, and a cathepsin B-like, HvPap-19, were up-regulated after drought treatment. Interestingly, whereas these three proteases and the cathepsin L-like protease HvPap-4 were induced after darkness and low nitrogen treatments (Velasco-Arroyo *et al.,* 2016), HvPap-4 was repressed after drought. Differential induction/repression of C1A CysProt and their inhibitors may be the responsible for the global cathepsin activities, which were increased after girdling, darkness and low

nitrogen treatments (Parrott *et al.,* 2010; Velasco-Arroyo *et al.,* 2016), but decreased after drought. Therefore, the distinguishing element between KD Icy2 and KD Icy4 could rely on the inhibitory properties of the silenced inhibitors. HvCPI-2 is able to efficiently inhibit HvPap-1, as well as several cathepsin L-like barley proteases (Martinez *et al.,* 2009; Cambra *et al.,* 2012b). HvCPI-4 is a worse inhibitor of these proteases, but it is the only barley PhyCys able to inhibit C13 legumain proteases because of its C-terminal extra domain (Martinez *et al.,* 2007; Julian *et al.,* 2013). In the physiological context, HvCPI-4 showed stronger inhibitory features than HvCPI-2 against the proteolytic activity of barley leaf extracts. HvCPI-4 is a better inhibitor of cathepsin L-/F- like activity and, in contrast to HvCPI-2, it is also able to efficiently inhibit cathepsin H-like activity (Martinez *et al.,* 2009).

At this point it is important to address the consequences of the induction of PhyCys in the context of a process whose success relies on the accuracy of specific sequential protein degradation. PhyCys induction by water-deficit has been extensively reported suggesting a key role for these inhibitors in the regulation of the plant response (Diop *et al.,* 2004; Christova *et al.,* 2006; Valdes-Rodriguez *et al.,* 2007; Zhang *et al.,* 2008; Megdiche *et al.,* 2009; Tan *et al.,* 2014; Chojnacka *et al.,* 2015; Wang *et al.,* 2015). Therefore, the induction of *Icy-2* and *Icy-4* genes should be linked to the control of the CysProt activities during the recycling process, avoiding sudden protein degradation by inhibiting some/all CysProt expressed along the drought treatment.

When *Icy-2* gene is silenced, the barley plant responds by reducing the expression of *HvPap-1* CysProt and by increasing the expression of the broad range inhibitor *Icy-4* gene after drought treatment. These alterations, probably together with additional modifications in the expression of other proteases and/or inhibitors lead to a decrease in the protease activity, a lower protein degradation, a conservation of amino acid levels and a lower alteration of membrane permeability as measured by electrolytic leakage. As a consequence, the senescence process is delayed, KD Icy2 plants remain greener after the treatment, and the soil is able to retain some water as the plants reduce water losses. On the contrary, when *Icy-4* gene is silenced, a higher induction of *Icy-2* and *HvPap-12* than in WT plants is triggered by drought. The

combination of these alterations with presumable modifications in the expression of other proteases and/or inhibitors differentially affects the response of KD Icy4 plants. The cathepsin activities do not decrease and proteins are degraded reaching levels as in WT plants. This steady proteolytic activity does not lead to an even stronger senescence phenotype in KD Icy4 plants, probably because the high basal accumulation of amino acids in these plants allows a quick replacement of degraded proteins. Metabolite accumulation could also be related to the known role of some amino acids (such as proline) or sugars (such as glucose) as osmolytes to maintain cell turgor (Jorge *et al.,* 2016). Thus, the differential levels of metabolites, mainly amino acids, showed by WT and transgenic plants could be linked to their own capacity to maintain cell turgor and viability.

Altogether, these observations lead to hypothesize a cooperative role behind the induction of *Icy2* and *Icy4* genes. The broad range of targets for HvCPI-4, that includes cathepsins F-, L-, and H- like and legumains, along with the high efficiency of HvCPI-2 in the inhibition of some drought-induced proteases, would permit a tight modulation of the protein degradation in response to water deprivation. Changes in the expression of other proteases/inhibitors and pleiotropic effects associated to the silencing of any of these cystatins leads to a quicker/slower response to the abiotic treatment. In many cases, drought tolerance, stay-green phenotype and yield are closely related (Gregersen *et al.,* 2013). Whether differential responses to silencing PhyCys plants have consequences in grain yield and composition is a question that remains to be elucidated and that would shed light on the suitability of these plants to be used as biotechnological tools to face stressful environmental conditions.

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6.6. SUPPLEMENTAL DATA

Figure S 6.1. Phenotypical, physiological and biochemical parameters of wild-type barley plants at 7, 10, 14 and 21 days (D) of water-deprivation (drought, black line) or under optimal watering regime (control, grey line). (a) Phenotype, (b) plant water content (PWC, %), (c) soil water content (SWC, %) and (d) stomatal conductance (*gs*). (e) Total soluble protein content, (f) chlorophyll and (g) carotenoid content in milligrams per gram of dry weight (gdw). (h) L-/F-cathepsin-like and (i) B-cathepsin-like activities. Data represent the mean ± SE of at least 6 technical replicates from three independent experiments. An asterisk (*) indicates significant differences between control and drought within each time (Two-way ANOVA test, Tukey´s, HSD, at $p<0.05$).

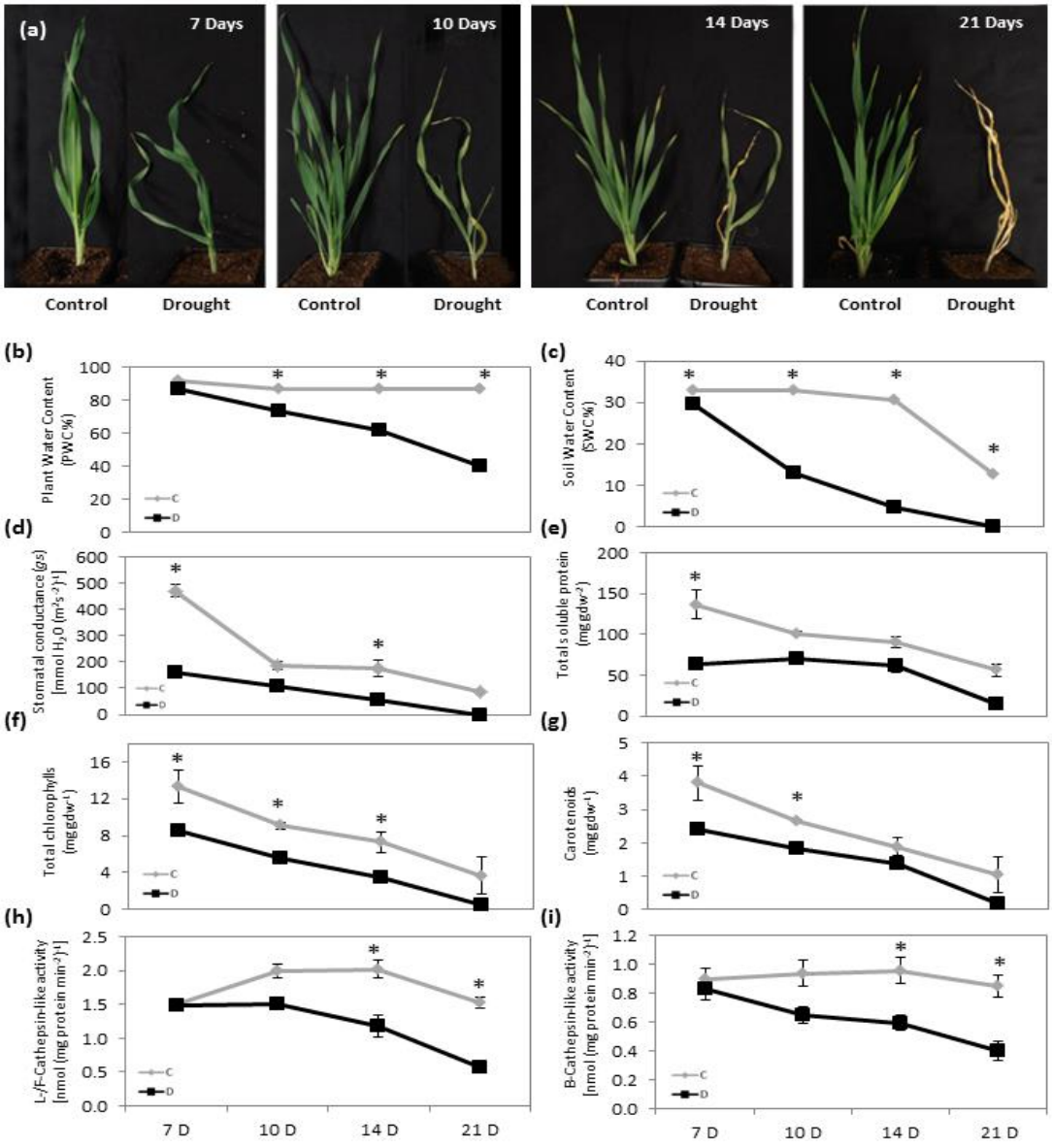


Figure S 6.2. Selection of Knock-down *Icy-2* (KD Icy2) and *Icy-4* (KD Icy4) transgenic homozygous barley lines generated by artificial microRNA and double haploid technology. Knock-down plants were selected following a double criteria, single transgene integration and low mRNA content. (a) Construct, number of events per construct, number of independent homozygous lines analyzed per transformation event and final selected lines used for molecular characterization. (b) Estimation of transgene copy number by RT-qPCR assays coupled to the $2^{-\Delta\Delta Ct}$ method. CN: copy number for each group. (c) Expression levels for *Icy-2* or *Icy-4* genes in wild-type (WT), KD Icy2 and KD Icy4 transgenic barley lines by RT-qPCR technology, referred as relative mRNA levels normalized to barley *cyclophilin* mRNA content. Data represent the mean ± SE of triplicate measurements from three independent experiments. Different letters indicate significant differences (One-way ANOVA, Newman-Keuls test, $p<0.05$).

(a)

	Construct	Event	Number of lines analysed	Selected lines
amiRNA silencing lines (KD)	KD Icy2	E02	11	1322
		E04	17	1390
	KD Icy4	E01	14	1453
		E02	15	1509
		E03	9	1558
		E04	11	1599

(b)

KD Icy2	$2^{-\Delta\Delta Ct}$ ± SE	CN
WT	0.00 ± 0.00	**0**
1322	0.77 ± 0.28	**1**
1390	0.82 ± 0.09	**1**
KD Icy4	**$2^{-\Delta\Delta Ct}$ ± SE**	**CN**
WT	0.00 ± 0.00	**0**
1453	1.45 ± 0.63	**1**
1509	0.67 ± 0.19	**1**
1558	1.52 ± 0.08	**1**
1599	1.37 ± 0.11	**1**

(c)

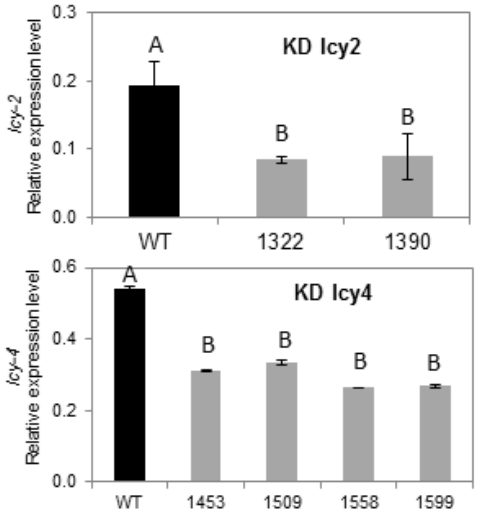


Figure S 6.3. Proteolytic patterns of barley transgenic lines (KD lcy4) and non-transformed wild-type (WT) from 7 day-old barley plants expressed as nanomoles per milligram of protein and minute, using specific substrates to be degraded by (a) L-/F-cathepsin-like, (b) B-cathepsin-like and (c) legumain-like. Data are means ± SM of triplicate measurements from three independent experiments. Different letters indicate significant differences (One-way ANOVA, Newman-Keuls test, $p<0.05$).

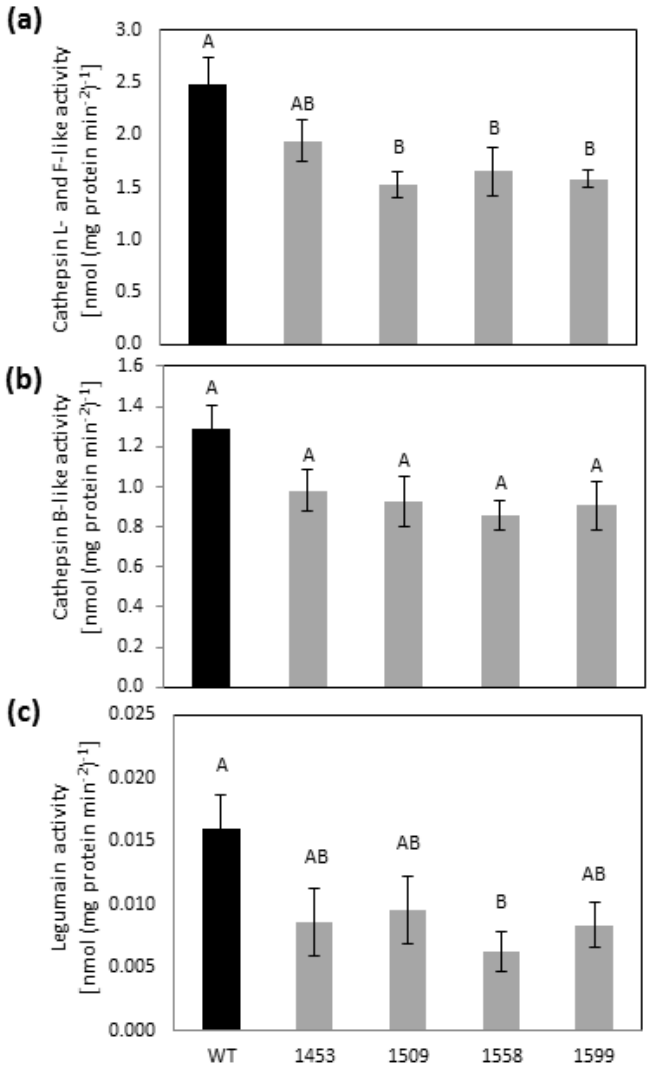


Figure S 6.4. Phenotype of barley plants at (a, e) 4, (b, f) 6, (c, g) 8 and (d, h) 10 weeks of development. Wild-type (WT), KD Icy2 (1318, 1322, 1390 and 1399 lines) and KD Icy4 (1453, 1509, 1558 and 1599 lines) silencing plants.

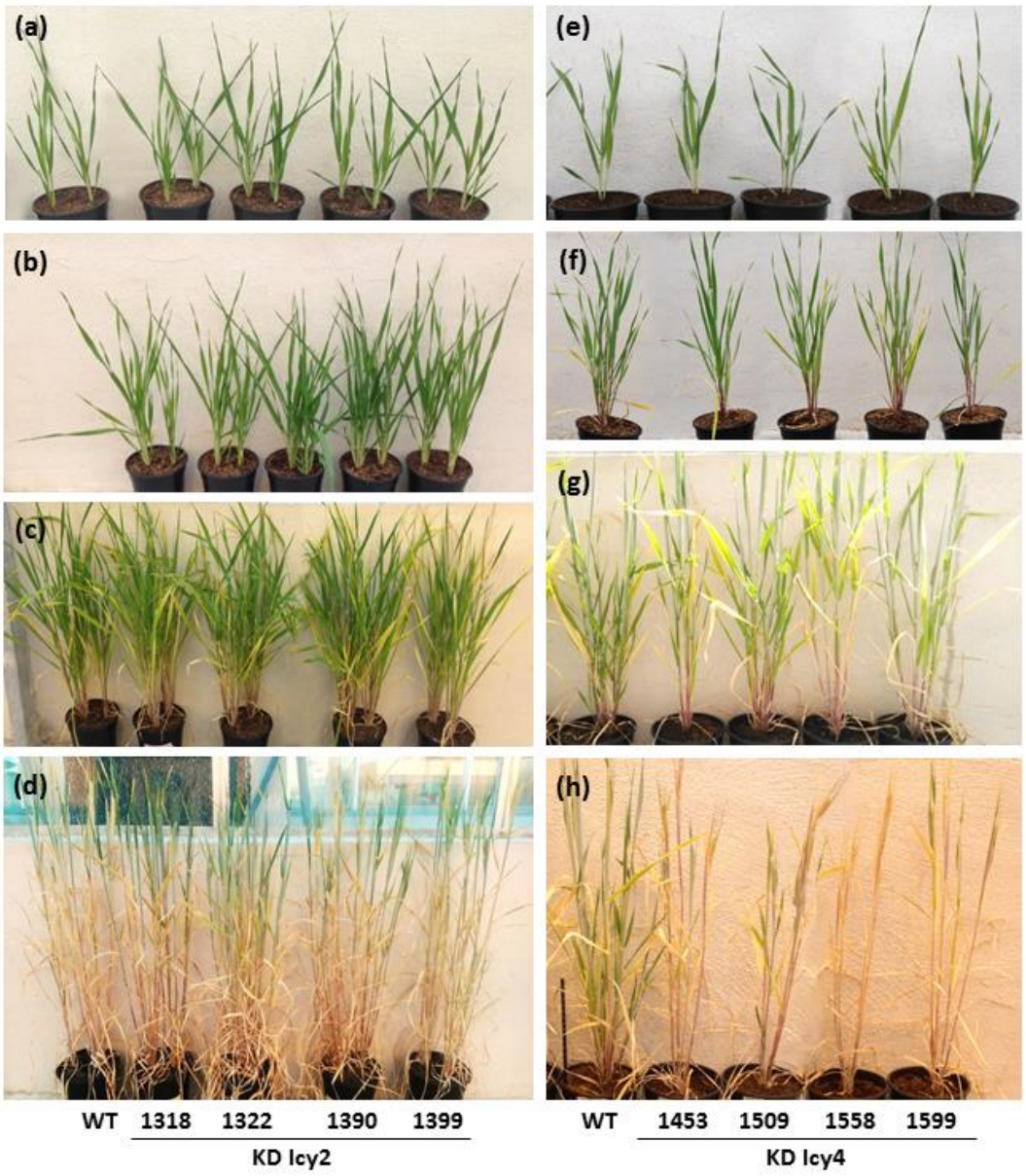


Figure S 6.5. Physiological and biochemical parameters for wild-type (WT), KD Icy2 (1318 and 1399 lines) and KD Icy4 (1453 and 1599 lines) silencing barley plants at 14 days of water deprivation (drought, black bars) or under optimal watering regime (control, grey bars) treatments. (a) Stomatal conductance (*gs*); (b) plant biomass in grams of fresh weight (gfw); (c) plant water content (PWC%) and (d) soil water content (SWC%). (e) Chlorophylls and (f) carotenoids contents in milligrams per grams of dry weight (gdw). Data represent the mean ±SE of at least 6 technical replicates from three independent experiments. Different letters indicate significant differences between plant lines and treatment, as determined by a Two-way ANOVA test (Tukey´s, HSD, at $p<0.05$).

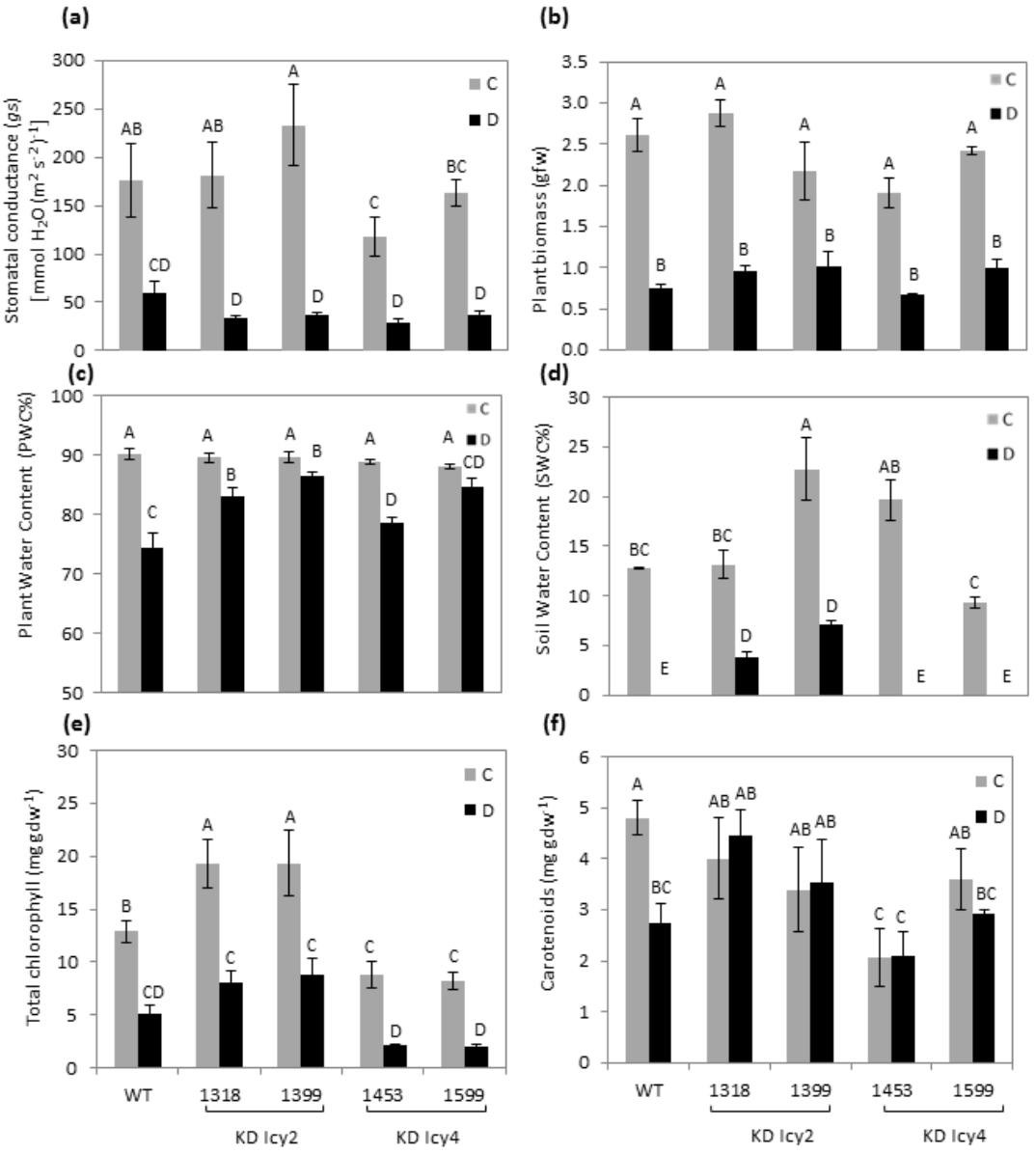


Figure S 6.6. Expression of barley CysProt-coding genes *HvPap-1*, *-4*, *-12*, *-16* and *-19*, in wild-type (WT), KD Icy2 (1318 and 1399) and KD Icy4 (1453 and 1599) silencing barley leaves after 14 days of water deprivation (drought, black bars) or under optimal watering regime (control, grey bars) treatments. Transcript levels of (a) cathepsin F-, (b, d) L-, (c) H- and (e) B-like members. Data were determined by RT-qPCR and are expressed as relative mRNA levels of cysteine proteases genes (*HvPap-1*, *-4*, *-12*, *-16* and *-19*), normalized to barley *cyclophilin* mRNA content. Data represent the mean ±SE of at least 6 technical replicates from three independent experiments. Different letters indicate significant differences between plant lines and treatment, as determined by a Two-way ANOVA test (Tukey´s, HSD, at $p<0.05$).

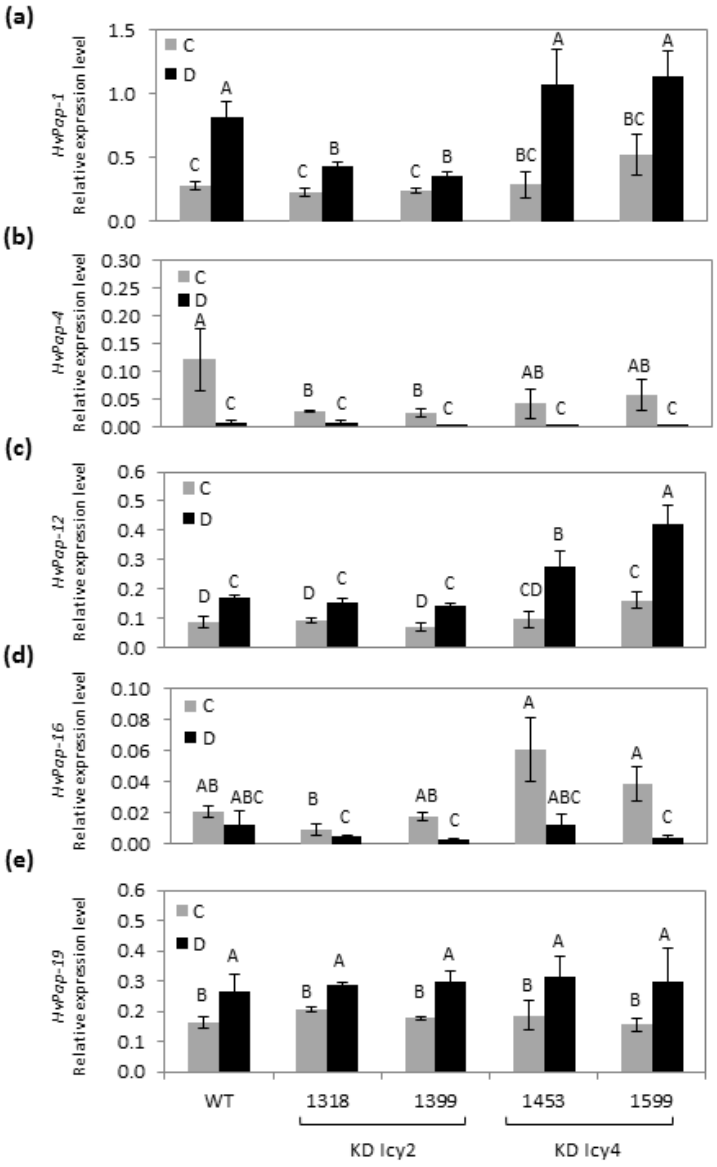


Table S 6.1. Primer sequences used for RT-qPCR amplification to analyze the copy number in transgenic barley plants. *HvCycl* gene (cyclophilin), *Hv4Hppd* gene (4-hydroxyphenyl-pyruvate dioxygenase), *Icy-2* gene and *Icy-4* gene (cystatins), and *miR* (Osa-MIR528 miRNA gene).

Barley genes	**Primers**
HvCycl	forward: 5'- CCTGTCGTGTCGTCGGTCTAAA -3' reverse: 5'- ACGCAGATCCAGCAGCCTAAAG -3'
Hv4Hppd	forward: 5´- GCTCCAAATCTTCACCAAGC -3' reverse: 5´- CTCTTCCCCTCTCTCGTCCT -3'
miR	forward: 5´- AGTTATGCGGCATTGATACCGGTCAGGAGATTCAGTTTGA -3´ reverse: 5´- AATTATGCGGCATAGATTCCGGTAGAGAGGCAAAAGTGAA -3´
Icy-2	forward: 5´- TCCTGGAGTCGATCTTGGTTTC -3´ reverse: 5´- CAAGCATACTGTTGCGGCTTC -3´
Icy-4	forward: 5´- TTGTTGAATGCGGCACGAT -3´ reverse: 5´- GCAGCCAACAGTACCCTGAGTT -3´

Table S 6.2. Primer sequences used for RT-qPCR assays. *HvPap-4* and *HvPap-16* genes (cathepsin L-like proteases), *HvPap-1* gene (cathepsin F-like protease), *HvPap-19* gene (cathepsin B-like protease) and *Icy-1* to *Icy-13* genes (cystatins).

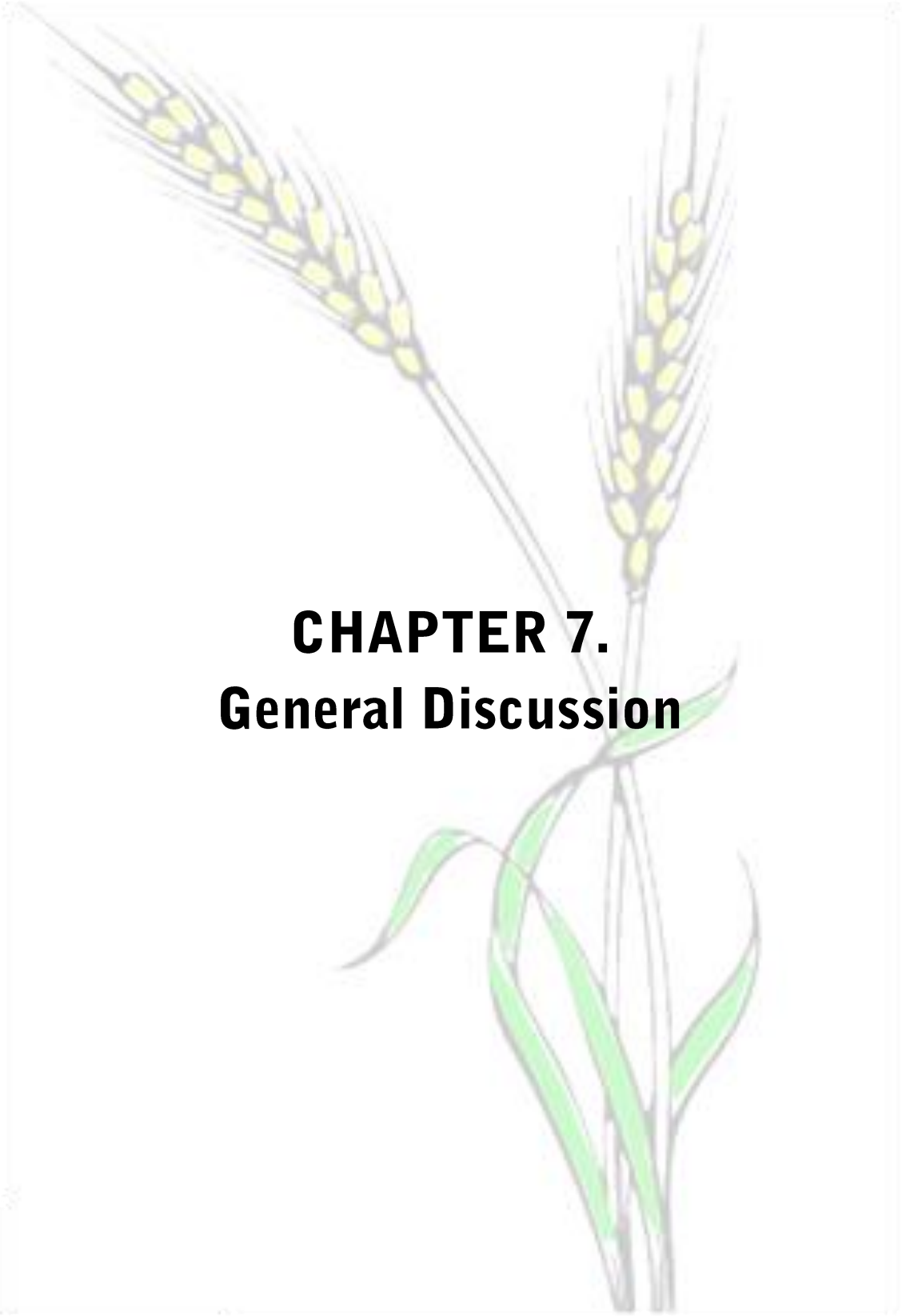
Barley genes	Primers
HvPap-1	forward: 5'- TCCTGGAGTCGATCTTTGGTTTC -3' reverse: 5'- CAAGCATACTGTTGCGGCTTC -3'
HvPap-4	forward: 5´- CCTTGAGAGTCCTTGTTCCCGA -3' reverse: 5´- CCATGTTCGTCGTTTTAACCGA -3'
HvPap-12	forward: 5´- ATGTGCGCTATTGCTACCTGC -3´ reverse: 5´- CACCTTATTCATGTCTGGCGAA -3´
HvPap-16	forward: 5´- CTGGATCGGTAAGAACTCGTGG -3´ reverse: 5´- TGATGGAGGTGCCATCATATGA -3´
HvPap-19	forward: 5´- CACCTTATTCATGTCTGGCGAA -3´ reverse: 5´- TGCCCGCTTAATTTGACAGG -3´
Icy-1	forward: 5'- CGGCCGCCTAATGAAAAGA -3' reverse: 5'- CATATTACGCCAGTATCCAGCG -3'
Icy-3	forward: 5´- ATGGCACCCAAGATGCAC -3' reverse: 5´- TTCAGAACCTCATGTTCCGACC -3'
Icy-5	forward: 5´- CTGCGTGTCTTTTTTGCGC -3´ reverse: 5´- CCTGCCTCATATCGTACAGCCT -3´
Icy-6	forward: 5´- TGCCGGTTAAATTCATCTGCTC -3´ reverse: 5´- CAGGAACTGAATGCCTGACGA -3´
Icy-7	forward: 5´- TTGGTGTCAAGGTTGGCATGT -3´ reverse: 5´- CCAAATCGATCGCTTGGTTG -3´
Icy-8	forward: 5'- CTACGTCGCCGAGGACTACGAG -3' reverse: 5'- TCAAAGGGGCGAGCGGACGAG -3'
Icy-9	forward: 5´- CGTCTCCCAAATAATCGACAGG -3' reverse: 5´- CGAGAAAAAGAGTGCAACCAGG -3'
Icy-10+13	forward: 5´- AACCAGTTGTGGAGCACAACA -3´ reverse: 5´- TGAACTCCAACACTGCCCAA -3´
Icy-11	forward: 5´- TATCAGCTCGTCATTGACGCC -3´ reverse: 5´- TGAACTTTCGTGTCTTCGCG -3´
Icy-12	forward: 5´- TCTGTGTCATCGTTGTTGCGA -3´ reverse: 5´- CCCCTGGATATGTTGGTCGTT -3´

Table S 6.3. Quantitative analysis of metabolite levels in plant leaves of wild-type (WT), KD Icy-2 (1318 and 1399) and KD Icy4 (1453 and 1599) silencing barley plants after 14 days of water deprivation (drought) or under optimal watering regime (control) treatments. Values of amino acids are represented in nanomoles and values of carbohydrates and others components are represented in micrograms per milligrams of dry weight. Results of quantitative analyses for identified barley metabolites are normalized to an internal standard, averaged over technical replicates. Data represent the mean ±SE of three technical replicates from two independent experiments. White background represents control condition and grey background represents drought treatment. Numbers in blue (Part-I) indicate the comparison of each transgenic line with the WT, both under control and drought treatments. Differences are indicated as: -1: significant down-regulation, 0: no significant difference, 1: significant upregulation (One-way ANOVA, Dunnet´s test, $p<0.05$). Numbers in red (Part-II) indicate the comparison between control and drought treatments for each individual line. Differences are indicated as: -1: significant down-regulation, 0: no significant difference, 1: significant upregulation (Two-way ANOVA, Tukey´s test, HSD, $p<0.05$).

Metabolite	Mean Values WT	Std. Error WT	Mean Values 1318	Std. Error 1318	Direction of change vs WT	Mean Values 1399	Std. Error 1399	Direction of change vs WT	Mean Values 1453	Std. Error 1453	Direction of change vs WT	Mean Values 1599	Std. Error 1599	Direction of change vs WT
Asp	6.01	0.32	6.06	0.08	0	5.05	0.07	-1	6.27	0.09	0	9.41	0.17	1
Thr	3.23	0.13	3.29	0.10	0	2.46	0.07	-1	3.28	0.03	0	5.20	0.10	1
Ser	3.22	0.16	3.20	0.06	0	3.31	0.05	0	3.47	0.18	0	5.12	0.24	1
Glu	4.95	0.29	5.10	0.07	0	4.75	0.12	0	5.50	0.10	0	8.00	0.24	1
Pro	3.41	0.18	3.34	0.10	0	2.81	0.01	0	3.58	0.06	0	5.15	0.10	1
Gly	6.32	0.35	6.41	0.07	0	5.96	0.04	0	6.78	0.25	0	9.66	0.24	1
Ala	6.23	0.30	6.38	0.04	0	5.29	0.06	-1	6.54	0.20	0	9.75	0.19	1
Cys	0.29	0.01	0.29	0.00	0	0.26	0.01	0	0.29	0.01	0	0.44	0.02	0
Val	3.28	0.15	3.30	0.05	0	2.89	0.04	0	3.46	0.06	0	5.21	0.09	1
Met	1.37	0.08	1.37	0.00	0	0.87	0.01	0	1.42	0.01	0	2.22	0.05	1
Ile	1.75	0.08	1.76	0.02	0	1.44	0.02	0	1.88	0.18	0	3.06	0.06	1
Leu	4.63	0.21	4.70	0.09	0	3.54	0.06	-1	4.81	0.20	0	7.65	0.19	1
Tyr	1.70	0.09	1.66	0.04	0	1.13	0.02	0	1.71	0.17	0	2.75	0.04	1
Phe	2.54	0.13	2.50	0.02	0	1.76	0.03	-1	2.57	0.07	0	4.02	0.08	1
His	1.30	0.05	1.36	0.03	0	0.74	0.02	0	1.31	0.04	0	2.03	0.02	1
Lys	3.36	0.14	3.45	0.09	0	2.64	0.03	-1	3.53	0.06	0	5.41	0.07	1
Arg	2.64	0.14	2.68	0.04	0	1.58	0.03	-1	2.76	0.00	0	3.55	0.07	1
Glucosa	4.30	0.36	4.60	0.32	0	4.58	0.29	0	5.04	0.06	1	4.89	0.21	0
Arabinosa	2.08	0.16	2.20	0.09	0	1.94	0.11	0	2.46	0.02	0	2.34	0.00	0
Galactosa	1.56	0.07	1.68	0.26	0	1.67	0.02	0	2.05	0.06	0	1.57	0.02	0
Xilosa	5.21	0.27	3.29	1.06	-1	5.14	0.18	0	4.86	0.22	0	5.60	0.07	0
Butanedioic Ac.	4.45	0.03	8.42	0.70	1	5.24	0.24	1	9.05	0.77	1	7.62	0.27	1

Table S 6.3. Part-II

Metabolite	Mean Values WT	Std. Error WT	Direction of change vs WT	Mean Values 1318	Std. Error 1318	Direction of change vs WT	Direction of change vs 1318	Mean Values 1399	Std. Error 1399	Direction of change vs WT	Direction of change vs 1399	Mean Values 1453	Std. Error 1453	Direction of change vs WT	Direction of change vs 1453	Mean Values 1599	Std. Error 1599	Direction of change vs WT	Direction of change vs 1599
Asp	2.99	0.12	-1	8.96	0.18	1	1	5.90	0.11	1	1	6.10	0.15	1	0	10.47	1.00	1	0
Thr	1.31	0.05	-1	4.35	0.06	1	1	2.60	0.08	1	0	2.59	0.02	1	0	5.34	0.47	1	0
Ser	1.44	0.08	-1	4.51	0.05	1	1	2.84	0.02	1	-1	3.19	0.06	1	0	6.11	0.64	1	0
Glu	2.03	0.09	-1	5.65	0.11	1	1	3.73	0.05	1	-1	3.77	0.06	1	-1	9.44	1.06	1	0
Pro	1.55	0.07	-1	4.71	0.02	1	1	2.93	0.06	1	0	2.87	0.04	1	0	5.83	0.58	1	0
Gly	2.76	0.15	-1	8.43	0.20	1	1	5.81	0.02	1	0	6.04	0.20	1	0	11.22	1.18	1	0
Ala	2.97	0.13	-1	9.14	0.19	1	1	5.95	0.09	1	1	6.09	0.16	1	0	11.42	1.17	1	0
Cys	0.15	0.00	0	0.28	0.03	0	0	0.25	0.00	0	0	0.23	0.01	0	0	0.41	0.04	0	0
Val	1.52	0.02	-1	4.29	0.09	1	1	2.81	0.11	1	0	2.76	0.03	1	0	5.75	0.52	1	0
Met	0.61	0.02	0	2.09	0.04	1	0	1.23	0.13	0	0	1.37	0.03	0	0	2.29	0.23	1	0
Ile	0.71	0.01	-1	2.23	0.05	1	1	1.41	0.04	0	0	1.40	0.01	0	0	3.20	0.29	1	0
Leu	2.17	0.04	-1	6.97	0.11	1	1	4.17	0.05	1	1	4.23	0.02	1	0	8.19	0.76	1	0
Tyr	0.60	0.02	-1	2.24	0.03	1	1	1.25	0.08	0	0	1.23	0.00	0	0	2.80	0.32	1	0
Phe	1.18	0.04	-1	3.94	0.05	1	1	2.49	0.05	1	1	2.51	0.05	1	0	4.19	0.43	1	0
His	0.53	0.02	-1	1.89	0.03	1	0	1.10	0.07	0	0	1.11	0.03	0	0	2.16	0.20	1	0
Lys	1.66	0.03	-1	5.45	0.06	1	1	3.15	0.03	1	1	3.22	0.02	1	0	6.17	0.54	1	0
Arg	1.09	0.03	-1	1.87	0.04	0	-1	2.19	0.04	0	1	1.53	0.03	0	-1	4.06	0.44	1	0
Glucosa	3.60	0.12	0	4.91	0.30	1	0	4.40	0.02	0	0	4.62	0.12	0	0	4.74	0.55	0	0
Arabinosa	1.72	0.03	0	2.23	0.18	0	0	1.92	0.05	0	0	2.73	0.40	0	0	2.39	0.26	0	0
Galactosa	1.41	0.04	0	1.52	0.03	0	0	1.78	0.02	0	0	1.25	0.09	0	0	1.51	0.14	0	0
Xilosa	4.32	0.41	-1	4.65	0.58	0	0	4.46	0.28	0	-1	4.59	1.35	0	0	6.00	0.72	1	0
Butanedioic Ac.	3.00	0.39	-1	5.60	0.80	1	-1	2.35	0.18	0	-1	6.75	0.32	1	-1	6.01	0.01	1	0



CHAPTER 7.
General Discussion

7.1. GENERAL DISCUSSION

Previous research on CysProt and PhyCys shed light about the implications of these counterparts during a key physiological process in barley, grain germination (Martinez *et al*., 2009; Cambra *et al*., 2012). Specifically, it was demonstrated that the barley HvPap-1 cathepsin F-like peptidase participated as an active component in the mobilization of grain stored proteins, mainly hordeins, during last stages of grain germination. Its strictly controlled regulation was exerted, at least in part, by the action of endogenous cystatins. On the other hand, HvPap-1 was also expressed in vegetative tissues such as leaves and roots, presuming its participation in additional physiological roles. The molecular and functional characterization of the entire cystatin family along with their potential CysProt targets through *in vitro* and *in vivo* approaches in distinct barley tissues also demonstrated the importance of this complex proteolytic network (Martinez *et al*., 2009). Interestingly, one barley cystatin, HvCPI-4, was reported as the only member able to inhibit both C1A and C13 CysProt families (Martinez *et al*., 2007). Formerly information encouraged us to complete data on germination through *in vivo* analyses and, importantly, to initiate the research on a key proteolytic-based process, leaf senescence specifically induced by abiotic stresses.

Although developmental and stress-induced senescence share commonalities and diverge in certain molecular mechanisms, specific information is still quite fragmentary. Regarding the main goal of the process, what is clear is that any type of senescence implies intense proteolysis, different subcellular compartments, several types of proteases and regulators as well as a complex traffic of proteins, aminoacids and nutrients that ultimately impact grain filling, thus influencing quality, yield and compromising offspring success. In the last stages of a leaf´s lifespan, organ-level senescence and, eventually cell death, represent the ruling processes (Woo *et al*., 2016). Intriguingly, senescing leaves show more coordinated temporal changes in transcriptomes than do growing leaves, with sophisticated regulatory networks comprising distinct interorganellar coordination of various transcriptomes (Kim *et al*., 2016). So far, transcriptomic and proteomic data have consistently assigned a major role to members from the C1A CysProt family in several plants species facing abiotic

stress (Parrott *et al*., 2007; Gregersen *et al.,* 2008; Martinez *et al.,* 2012; Hollmann *et al.,* 2014; Diaz-Mendoza *et al*., 2014; Velasco-Arroyo *et al*., 2016). Cystatins play a key role in leaves by controlling the action of CysProt during protein recycling along senescence and through the activation of protective metabolic cascades under abiotic stress (Prins *et al*., 2008; Quain *et al*., 2014; Je *et al*., 2014). Massive analyses of gene expression have facilitated the unveiling of the molecular events and pathways associated to leaf senescence. Particularly, microarray barley experiments highlight the importance of transcriptomic approaches to find out the members of a particular protein family (Parrott *et al*., 2007; Gregersen *et al*., 2008).

The whole C1A family from barley, which comprises 41 members, as well as the whole cystatin family, made up by 13 genes, were analyzed in the current study under different abiotic stresses able to trigger leaf senescence. Several C1A members belonging to different subgroups as well as various cystatin genes were upregulated at the transcriptional level upon the exposure to darkness and drought. Interestingly, several C1A-encoding genes exhibited significant increased relative expression under both stress conditions, such as *HvPap 1*, *HvPap-12*, *HvPap-19* and *HvPap-22,* whereas the expression of *HvPap-4* was induced by darkness and repressed by drought. In the case of the inhibitors, while *Icy-4* was upregulated under both tested stresses, other cystatins were altered after a specific treatment or did not respond to it. These results suggest certain degree of specificity depending upon the activated senescence-related proteolytic mechanism triggered by a particular stress, although several proteases and inhibitors may be acting coordinately along the course of a particular, or various environmental cues.

We initially tested two different stressors in whole 7-day-old WT barley plants grown under severe conditions: nitrogen starvation, since it represents an essential nutrient that in small-grain cereals such as barley, wheat and rice, is greatly (up to 90%) mobilized from vegetative tissues to grains (Gregersen *et al*., 2008), apart from being a major constituent of Calvin cycle enzymes, like Rubisco; and darkness, which apparently does not pose a threat in nature, but it represents a simple way to directly impact on the photosynthetic machinery and to rapidly alter associated metabolism,

since the prolonged lack of light obviously implies a cellular collapse. In fact, when we studied all biochemical and morphological parameters, darkness appeared to cause the greatest effects. Chlorophyll abundance, useful indicator of the chloroplast status, tends to remain constant in photosynthetically active leaves (Sorin *et al.,* 2015). Chlorophyll reduction was one of the first symptoms perceived in stressed plants, and carotenoids accumulation seemed to increase in all cases since the ratio chlorophyll/carotenoids diminished, consistently with its presumed protective role towards specific abiotic stresses. After darkness, chloroplasts formed aggregates and cells were close to collapse. All these data clearly indicated that, under these conditions, whole barley stressed plants had already activated their intrinsic senescence programs. Consequently, we further analyzed other molecular and biochemical parameters. The significant decrease in protein levels in darkness and N-starved leaves in comparison to the controls suggested either the inhibition of protein synthesis or/and the activation of protease activities associated to nutrient recycling. Both stresses induced cathepsin L-/F- and B-like activities, suggesting a functional redundancy in protein turnover. Data were supported by the up-regulation of genes encoding barley C1A CysProt, particularly *HvPap-1, HvPap-12* and *HvPap-19*, later confirmed by protein accumulation as shown on immunoblot assays. Rubisco, the most abundant protein in plants, is likely the major target for proteases when proteolytic processes are activated (van der Hoorn, 2008; Theonen *et al.,* 2007; Krupinska *et al.,* 2012; Martinez *et al.,* 2012) and its accumulation showed the greatest degradation after darkness in this study. The subcellular location of HvPap-1 and HvPap-19 CysProts revealed a dynamic trafficking, probably involving specific organelles developed under senescence like SAVs or similar vesicles, which contain proteins derived from chloroplasts and which are enriched on CysProts (Otegui *et al.,* 2005; Ishida *et al.,* 2008; Carrion *et al.,* 2013, 2014). Interestingly, HvPap-16, another C1A member which accordingly to previous studies was only expressed in leaves (Martinez et al., 2009), exhibited a steady pattern in the epidermal cells both under control and stress conditions, suggesting that this particular member may not be intervening on plastidial degradation.

HvPap-1 is required during grain filling and germination (Cambra *et al*., 2012; Diaz-Mendoza *et al.,* 2016a) and is strongly induced by darkness and N-starvation. Thus, we decided to investigate how alterations on its expression could disturb senescence progress, and thereby nutrient translocation. We used darkness based on previously explained reasoning, under a mild stress, replacing vermiculite by organic soil in order to abolish nutrient limitations. Time required to detect senescence symptoms was obviously longer than in severe experiments. Keech *et al.* (2007) reported a "stand-by mode" in whole darkened Arabidopsis leaves showing low mitochondria activity to preserve active photosynthetic machinery, while individual darkened leaves presented higher mitochondrial activity to deliver energy and speed up degradation. Anyhow, at that time senescence had already started and therefore we fixed 14 days of darkness as the point to perform subsequent experiments with overexpressing and silencing lines (OE Pap1 and KD Pap1, respectively). Under these conditions, barley WT dark-treated plants showed a lower increase in the proteolytic activity as that observed under severe conditions, suggesting a stand-by mode induced by darkness perception. HvPap-1 levels increased, as expected, at mRNA and protein levels in OE Pap1, but this did not result in an increase in the proteolytic activity, probably due to compensating effects among protease activities. In knock-down treated lines both cathepsin L-/F- and B-like protease activities exhibited a clear reduction.

A reduced total protein content in the leaves, which paralleled to that of Rubisco in most of the darkness-treated OE Pap1 lines, together with the reduced autofluorescence emission from the chlorophyll and the low amount of starch in this genotype, led us to assume that leaf senescence was sped up. In contrast, all analyses indicated a delay in the senescence process in KD lines. Interestingly, KD treated plants showed a striking reduction on chlorophyll *b*. The light-harvesting chlorophyll *a*/*b* binding protein complex of LHCII is the main antenna for this photosystem. Plants change their LHCII content depending on the light environment and chlorophyll *b* is indispensable for the accumulation of LHCII. Thus, its degradation induces the catabolism of the complex (Sato *et al*., 2015). Based on these assumptions, it could be

happening that chlorophyll *b* was completely transformed into chlorophyll *a* under a stand-by mode in order to improve and/or protect photosynthesis.

A clear delayed-senescence phenotype of KD Pap1 lines was observed both in barley plants grown either under control or darkness conditions, probably indicating chloroplasts protection from degradation. Another hypothesis assumes a slower growth for these plants, which could be a consequence of alterations from the germination events. Manipulation of the proteolytic machinery is a potential way to enhance grain yield and quality, which lately will influence the mobilization of storage compounds along germination. Barley germination involves the activity of several proteases and amylases that hydrolyze and mobilize storage compounds. The participation of several barley C1A CysProt during germination has been predicted since members of the C1A subgroups L-, B-, H, and F-cathepsins were induced by GA in the barley grain (Holwerda and Rogers, 1992; Martinez *et al*., 2003; Martinez *et al*., 2009; Cambra *et al*., 2012). HvPap-1 was firstly identified in barley grains during germination (Sreenivasulu et al., 2008), it was expressed in grain tissues during this stage and it efficiently degraded stored hordeins *in vitro* (Cambra *et al*., 2012). To demonstrate its *in vivo* involvement along this process, OE Pap1 and KD Pap1 barley transgenic grains were exhaustively analyzed. If HvPap-1 was one of the responsible enzymes to *in vivo* degrade stored proteins, a delay in the germination process should be expected for the KD Pap1 lines as well as an acceleration for overexpressing ones, due to a more efficient hydrolysis over accumulated reserves. Indeed, a decrease in the number of germinated grains over time was observed in KD Pap1 plants. Nevertheless, although OE Pap1 lines consistently exhibited a higher degradationrate for hordeins, the number of germinated grains in these lines was quite similar to that in the WT. HvPap-1 alterations could lead to significant modifications in the grain composition that subsequently affect the normal progression of the germination process, or alternatively the expression of some other hydrolytic enzymes might be enhanced as a compensating effect in these genotypes. Although most of the nitrogen in the grain is incorporated into proteins, free amino acids are crucial during germination, and they are major determinants related to grain processing, quality and food safety (Halford *et al*., 2015). Genetic modifications leading to variations in the

accumulation of storage proteins may alter the amino acid composition in the grain. Differential specificity in the source of nutrients that the embryo can use to develop in a new plant arises from the great variability of results accounting for amino acids, starch and protein contents among compared phenotypes. The lowest accumulation of most amino acids detected in KD Pap1 lines could be related with the slow start of germination exhibited on those lines. OE Pap1 lines strongly accumulated proline and glutamine, in which hordeins are enriched, but this enrichment did not involve an accelerated germination. On the other hand, changes in the in the genetic content of *HvPap-1* provoked modifications in the expression of some other related genes, as evidenced transcriptomic data, which were likewise supported by immunoblots. Immunolocation analyses performed in embryos for proteases HvPap-1, HvPap-6 and HvPap-19 revealed an interesting dotted pattern coincident with protein-enriched bodies. Alterations in the expression for these proteolytic enzymes should be correlated to variations in the enzymatic activity, but results were not as expected, again probably due to enzymatic compensations and, most likely, to the intervention of cystatins. This could explain why in the cases where an increase in the activity of the proteolytic machinery was measured, it was not translated into a higher germination rate, as occurred in OE Pap1 lines.

Cystatins are key members in the regulation of C1A CysProt during barley grain germination (Martinez *et al*., 2009; Cambra *et al*., 2012). According to this, silencing of a cystatin in the grain could lead to an acceleration of the germination process, since inhibition of the target C1A CysProts would be minor. The functional relationship between barley cystatins and cathepsins L- and F-like was inferred from their common implication as counterparts during hordein mobilization upon germination. HvCPI-2 is a good inhibitor of different barley cathepsin L- and F-like CysProts (Martinez *et al*., 2009; Cambra *et al*., 2012) and it also displays a role along this process (Cambra *et al*., 2012; Diaz-Mendoza *et al*., 2016a). Results obtained for KD Icy2 lines reinforce the importance of the complex network modulating mobilization of stored proteins, since the expected acceleration in the germination process was not observed in these lines. Likewise, in OE Pap1 lines, the higher expression of HvPap-1, target for HvCPI-2, may be affecting the expression and late accumulation of this and/or related cystatin

members. These inhibitors would be assuming the goal of finely shelter specific protein fractions from degradation thus regulating germination timing. Again, compensating effects implying proteases and inhibitors that led to perturbed proteolytic patterns would explain the unexpected germination processes observed in KD Icy2 grains.

Importantly, a positive correlation between length of the active photosynthetic period and cereal grain yield and quality has been reported (Distelfeld *et al.,* 2014). Accordingly, a delay in leaf senescence, leading to 'stay green' phenotypes (Hörtensteiner, 2007; Gregersen *et al.,* 2013), would determine a higher grain yield mainly due to increased CH accumulation, as observed in the grains of KD Pap1 lines, but inefficient nitrogen remobilization and lower protein content. Conversely, acceleration of senescence confers efficient nitrogen remobilization and higher protein content, such as is the case of OE Pap1 grains, but instead renders a lower total grain yield (Gregersen *et al.,* 2008) since starch and related CH are less abundant. In this context, protein accumulation patterns in the barley grains are important depending on the end-product use of the harvested crops. For instance, the production of alcoholic beverages is based upon fermentation and thus enrichment on CH would be ideal, as well as a faster germination rate. In contrast, to produce feed for animals, higher protein content would be an added value. Interestingly, the total amount of hordeins was higher in OE Pap1 dry grains than in the rest of analyzed seeds. This particular result perfectly links senescence and germination: hordeins, the main storage proteins in the barley grain that accumulate along barley maturation, were more abundant in OE Pap1 lines, which exhibit an accelerated leaf senescence. Furthermore, given that KD Pap1 lines senesced and germinated later, we could also speculate that the senescence timing is determined by the germination speed. However, OE Pap1 plants did not germinate faster but did senesce earlier, therefore indicating that this physiological function was effectively based upon alterations over HvPap-1 levels. From these data, it can be concluded that HvPap-1 is a functional stress-associated gene and alterations in its expression bring about changes in barley abiotic stress responses through modulation of senescence related pathways. Previous reports have shown similar effects in plant behavior by down-regulating the

expression of senescence-related CysProt, in particular *BoCP5* and *CaCP* genes from broccoli and pepper, respectively (Eason *et al.,* 2005; Xiao *et al.,* 2014).

Back to abiotic stress, genetically modified plants silencing those cystatin-encoding genes which were induced under drought treatment, *Icy-2* and *Icy-4*, were further characterized, presuming a putative role for these PhyCys in the control of specific senescence-related proteolytic mechanisms. Fourteen days of soil water deficit was chosen by measuring specific physiological parameters indicators of the plant and environmental (soil) status, guiding us towards the precise point at which drought had induced leaf senescence but plants were still healthy enough to perform all required analyses. Altered phytocystatin expression has been postulated as a way to palliate negative effects facing climate change (Kunert *et al.,* 2015), under which severe drought episodes are expected to increase. Proteolysis is a crucial event involved in the response to drought, and modifications of PhyCys expression patterns should be correlated to a modulation of CysProt activity. Under drought, plants reprogram their metabolism in order to overcome this negative episode and importantly, senescence pathways that fulfill degradation of compounds for recycling and survival are activated during this, in principle, reversible stage.

Surprisingly, the phenotypes of barley knock-down plants for *Icy-2* and *Icy-4* (KD Icy2 and KD Icy4, respectively) were different. The natural phenotype of KD Icy2 plants was characterized by a higher biomass when comparing to WT and a delayed senescence, resembling a kind of functional stay-green, in which the entire senescence syndrome was delayed, slowed down, or both (Thomas and Howarth, 2000). Conversely, KD Icy4 plants had lesser biomass than WT and exhibited an earlier chlorophyll disappearance, aside from differences in size and tiller number. In several crops, drought stress resistance coincides with a stay-green trait (Gregersen *et al*., 2013). Under stress, previously observed phenotypes were indeed confirmed. Furthermore, KD Icy2 plants exhibited increased drought tolerance, apparently through a delay in leaf senescence, an hypothesis previously reported (Rivero et al., 2007).

Since C1A CysProt have been largely associated to the cellular dismantling occurring during natural or stress-induced senescence processes (Diaz-Mendoza *et al.,* 2014, 2016b), phenotypes observed for KD Icy2 and KD Icy4 plants should be related to the differential inhibition of drought-associated C1A CysProt. Three out of the five C1A proteases analyzed (intriguingly *HvPap-1*, confirmed as a functional senescence-associated protease) were up-regulated after drought either in WT or modified genotypes. Interestingly, whereas these three members and the cathepsin L-like protease HvPap-4 were induced after darkness and low nitrogen treatments (Velasco-Arroyo *et al.,* 2016), HvPap-4 was repressed after drought. Differential induction/repression of C1A CysProt and their inhibitors may be subjacent to the general variability on cathepsin activities, which decreased with drought in WT and KD Icy2 lines, whereas increased in KD Icy4, consistently with the higher protein degradation observed for these lines. Therefore, the distinguishing element between KD Icy2 and KD Icy4 could rely on the inhibitory properties of the silenced inhibitors. HvCPI-2 is able to efficiently inhibit HvPap-1, as well as several cathepsin L-like barley proteases (Martinez *et al.,* 2009; Cambra *et al.,* 2012b). HvCPI-4, although a worst inhibitor of these proteases, represents the only barley PhyCys able to additionally inhibit C13 legumain proteases (Martinez *et al.,* 2007; Julian *et al.,* 2013). Two barley legumains were detected in both vegetative and germinative tissues and responded to biotic and abiotic stimuli, and HvCPI-4 was able to *in vitro* inhibit them and *in vivo* interact with HvLeg-2 (Julián *et al*., 2013) With these premises, an increase in concomitant CysProt activity was expected when downregulating *Icy-4* in response to stress, data confirmed in the current research.

Conversely, when *Icy-2* gene is silenced, an unexpected decrease in the protease activity was observed. The barley plant responds by reducing the expression of *HvPap-1* and by increasing the expression of the broad range inhibitor *Icy-4* gene after drought treatment. These alterations, probably together with additional modifications in the expression of other proteases and/or inhibitors provokes a lower protein degradation, a conservation of amino acid levels and a lower alteration of the membrane integrity. The senescence process is delayed, KD Icy2 plants remain greener after the treatment, and the soil is able to retain some water as the plants

reduce water losses, probably due to a modulation of stomata closure at that time. These results confirm an active protective role for some cystatins *in planta*, as previously reported (Quain *et al*., 2014; Kunert *et al*., 2015). On the contrary, the reduced stomata conductance displayed by KD Icy4 plants might be a consequence of activated senescence programs in response to drought, reflecting an already initiated degradation instead of a leaf protection function.

The increased in specific proteolytic activities concomitant with higher protein degradation rates observed in treated KD Icy4 plants did not lead to stronger senescence phenotypes when comparing to WT, probably because the high basal accumulation of amino acids in these plants allowed a quick replacement of degraded proteins. Differential levels of metabolites, mainly amino acids, could be linked to the capacity to maintain cell turgor and viability. On the other hand, the weaker phenotypes detected on these plants (fewer and thinner tillers and leaves) could be the result of an altered germination. In line with this assumption, when KD Icy2 barley germination was assessed, rates were slower as expected, with an initial growth parallel to WT. Hereafter, senescence is delayed and photosynthesis is extended under natural conditions. Paradoxically, KD Icy2 grains contained more protein. However, the regular amount of starch, either detected with lugol staining or biochemically quantified, in combination with the reduced hordein fraction allowed us to hypothesize that this extended photosynthetic period drives the increased accumulation of other proteins fractions other than hordeins, globulins and albumins. Likewise, the lowest accumulation of most amino acids, especially proline and glutamine, in the grains of KD Icy2 lines could be related with the lower amount of storaged hordeins. These last data suggest that pleiotropic effects associated to the silencing of any of these inhibitors lead to a quicker/slower response to the abiotic treatment. Whether differential responses to silencing PhyCys indeed have consequences over grain yield and composition is a question that remains to be elucidated.

Results from this thesis support previous literature at the same time that broadening information related to C1A-PhyCys complex alterations in barley. This

manipulation has the potential to modulate sensitivity towards specific abiotic stresses, through modifications over established developmental leaf senescence programs. According to presented data, proteolytic reprogramming should be considered since the plant tries to compensate the genetic modifications by modulating the expression of some other peptidases or inhibitors, as occurred for OE Pap1 and KD Icy2 lines. On the other hand, it is corroborated the *in vivo* relevance of this proteolytic network during barley grain remobilization upon germination, in a manner that might be economically important for agriculture. Ongoing experiments related to other specific members, such as HvPap-16 or HvPap-19, are being undertaken through analyses on specific transgenic lines and the obtaining of double mutants. Besides, according to presented results, a research related to traffic and characterization involving specialized vesicles, such as SAVs, is going to be developed. Several RNAseq analyses over selected transgenic lines, in some cases subjected to a combination of abiotic and biotic stresses, have also been performed to find out underlying molecular events which could help to elucidate interconnected pathways between leaf senescence, stress and CysProt-Cystatin complexes. Studies on whole-plant senescence using multi-omics approaches will greatly broaden the information regarding senescence at the whole plant level, to further understand how it is systematically achieved and differentially regulated in response to internal and external factors (Kim *et al*., 2016). Data from agronomic valuable crops would further help to improve productivity.

7.2. REFERENCES

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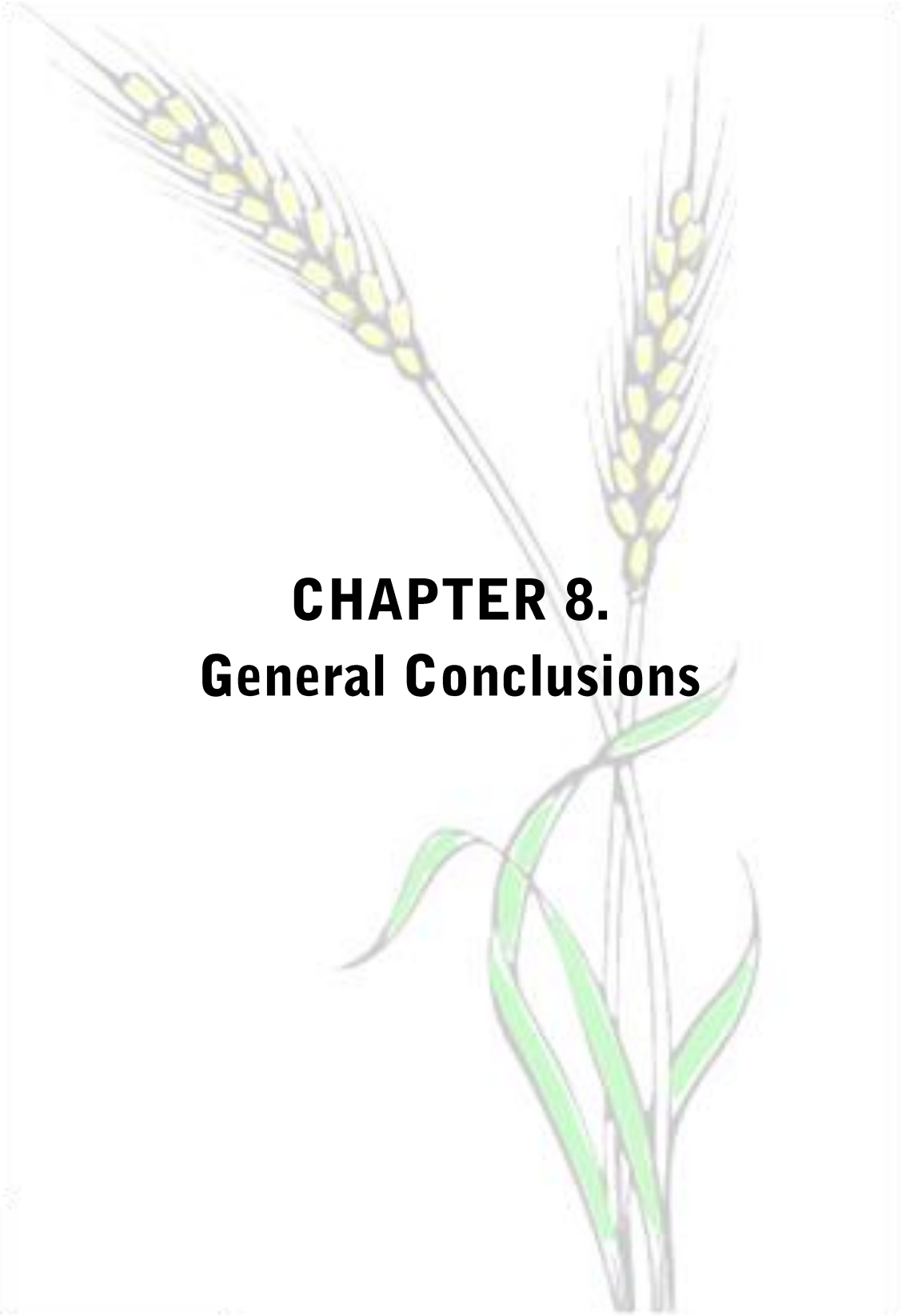

CHAPTER 8.
General Conclusions

The results of this thesis permit the extraction of several conclusions alluding to the role of barley C1A cysteine proteases and their inhibitors throughout leaf senescence and grain germination:

1. In barley, members from all C1A CysProt groups are expressed in response to abiotic stresses able to induce leaf senescence, such as darkness, nitrogen starvation and drought. A certain degree of specificity is likely to rule the process. Some members respond to various environmental cues, such as HvPap-1, HvPap-12 and HvPap-19, while others display opposite patterns, such as HvPap-4 and HvPap-6.

2. The delayed-senescence phenotype displayed by knock-down *HvPap-1* plants, resembling a stay-green phenotype, and the opposite trend exhibited by overexpressing *HvPap-1* lines, either under natural and darkness-induced senescence, designates *HvPap-1* as a functional stress-associated gene. Alterations in its expression bring about changes at the biochemical and molecular levels, indicating a modulation in the responses through alterations over established developmental leaf senescence pathways.

3. The delay in germination observed in silencing *HvPap-1* lines, together with alterations in the grain composition, confirms a role for this protease during degradation of stored proteins. Overexpressing *HvPap-1* barley grains accumulate the highest amount of hordeins and show increased levels of proline and glutamine, in which hordeins are enriched on. However, this observation is not accompanied by an accelerated germination rate.

4. Among cystatins, *Icy-4* appeared as the main upregulated gene both under darkness and drought, while *Icy-2* was specifically altered under drought.

5. Under drought, knock-down *Icy-2* plants exhibited an increased tolerance, apparently through a delay in leaf senescence. Conversely, knock-down *Icy-4*

plants display opposite trends, similar to those observed for overexpressing *HvPap-1* lines under darkness.

6. When a drought-induced cystatin is silenced, the other is overexpressed, suggesting a cooperative role for both members in response to the stress. The broad range of targets for HvCPI-4, along with the high efficiency of HvCPI-2 in the inhibition of particular drought-induced proteases, would allow a tight modulation during protein degradation in response to water deprivation.

7. Either in leaves undergoing senescence or in germinating barley grains, the plant tries to compensate the genetic modifications by modulating the expression of some other peptidases and inhibitors. Therefore, proteolytic reprogramming should be considered when designing biotechnological strategies based on the manipulation of mechanisms involving CysProt-PhyCys.

8. From the data reported on this thesis it can be concluded that senescence timing is influencing grain filling and composition, therefore impacting on germination events. Accordingly, manipulation of lifespan on cereals through biotechnological approaches based on proteolytic mechanisms seems a promising strategy to produce grains with enhanced properties for specific uses.

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