Repression of drought-induced cysteine-protease genes alters barley leaf structure and responses to abiotic and biotic stresses

Andrea Gomez-Sanchez, Pablo Gonzalez-Melendi, M. Estrella Santamaria, Vicente Arbona, Angeles Lopez-Gonzalez, Antonia Garcia, Goetz Hensel, Jochen Kumlehn, Manuel Martinez and Isabel Diaz

Abstract

To survive under water deficiency, plants alter gene expression patterns, make structural and physiological adjustments, and optimize the use of water. Rapid degradation and turnover of proteins is required for effective nutrient recycling. Here, we examined the transcriptional responses of the C1A cysteine protease family to drought in barley and found that four genes were up-regulated in stressed plants. Knock-down lines for the protease-encoding genes *HvPap-1* and *HvPap-19* showed unexpected changes in leaf cuticle thickness and stomatal pore area. The efficiency of photosystem II and the total amount of proteins were almost unaltered in stressed transgenic plants while both parameters decreased in stressed wild-type plants. Although the patterns of proteolytic activities in the knock-down lines did not change, the amino acid accumulation increased in response to drought, concomitant with a higher ABA content. Whilst jasmonic acid (JA) and JA-Ile concentrations increased in stressed leaves of the wild-type and the *HvPap-1* knock-down lines, their levels were lower in the *HvPap-19* knock-down lines, suggesting the involvement of a specific hormone interaction in the process. Our data indicate that the changes in leaf cuticle thickness and stomatal pore area had advantageous effects on leaf defense against fungal infection and mite feeding mediated by *Magnaporthe oryzae* and *Tetranychus urticae*, respectively.

Keywords: Abiotic/biotic stress, cysteine-protease, drought, leaf cuticles, stomata, water deprivation.

Introduction

Drought is considered to be one of the most critical threats to agriculture worldwide, and is responsible for the largest losses of crops. Plants defend themselves against adverse conditions, and in particular to drought, by activating an orchestrated and highly regulated molecular network that triggers biochemical, physiological, and morphological changes to minimize damage. Under drought stress, stomata are closed to prevent transpiration, which limits CO₂ uptake, reduces photosynthesis, and subsequently impairs plant growth. Osmotically active compounds and protective proteins accumulate and sink/source allocation is adjusted. Cuticles become thicker due to an increase of both cutin monomers and waxes to avoid water loss, the shoot-to-root ratio decreases, and a deeper root system develops. These events are regulated by changes in the hormone content, mainly by altering the abscisic acid (ABA) levels, which act as key regulator of the global process (Osakabe *et al.*, 2014; Shi *et al.*, 2016; Bi *et al.*, 2017; Xue *et al.*, 2017). Drought stress brings about premature plant senescence, which usually leads to a reduction in the canopy size and to a drop in yield in annual crops. The impact is even more drastic when water deficit occurs during the reproductive or grain filling stages (Barnabás *et al.*, 2008; Mahalingam, 2017).

High protease activities are closely associated with plant responses to different stresses, particularly water deficit, and they enhance the protein turnover required for metabolic processes and nutrient recycling (Khanna-Chopra *et al.*, 1999; Beyene *et al.*, 2006; Kidric *et al.*, 2014; Botha *et al.*, 2017). Drought-sensitive plants have a higher proteolytic activity than resistant ones (Beyene *et al.*, 2006; Simova-Stoilova *et al.*, 2010). Plant proteases are key players involved in these proteolytic events that are linked to the responses to stress. The C1A cysteine-protease (CysProt) family, also known as papain-like CysProt, is one of the most abundant (Rawlings *et al.*, 2018). Martínez and Diaz (2008) grouped the C1A CysProt family as cathepsin L-, B-, H-, and F-like based on their gene structure and relationships. Although most of their members still have unknown functions, some reports have demonstrated the participation of the C1A family in senescence, abscission, autophagy, programmed cell death, protein mobilization in seeds and tubers, and response to different stresses (van der Hoorn, 2008; Martínez *et al.*, 2012; Diaz-Mendoza *et al.*, 2016a, 2016b; Velasco-Arroyo *et al.*, 2016; Sueldo and van der Hoorn, 2017; Bárány *et al.*, 2018). The up-regulation of C1A CysProt genes is essential for protein breakdown throughout stress responses by triggering reorganization of metabolism, remodelling of cell protein compounds, degradation of damaged or unnecessary proteins, and remobilization of nutrients (Rabbani *et al.*, 2003; Parrott *et al.*, 2010; Guo and Gan, 2012; Roberts *et al.*, 2012; Díaz-Mendoza *et al.*, 2014; Wang *et al.*, 2018). Papain-like CysProts not only respond to abiotic stresses but also to biotic ones mediated by pathogens and pests (Kempema *et al.*, 2007; Shindo and van der Hoorn, 2008; Diaz-Mendoza *et al.*, 2017; Thomas and van der Hoorn, 2018).

Most research carried out so far on the relationship between CysProt and drought has examined the function of individual members. Thus, Zang *et al.* (2010) demonstrated that the

overexpression of the *Triticum aestivum* CysProt (*TaCP*) gene confers greater tolerance to water deficit than in wild-type plants. Likewise, the ectopic expression of the sweet potato CysProt (*SPCP2*) gene in *Arabidopsis* alters plant growth and enhances the tolerance to drought and/or salt stress (Chen *et al.*, 2010). Conversely, CysProt may play the opposite role, as demonstrated by increased sensitivity to drought stress and altered phenotypic traits observed in transgenic *Arabidopsis* plants constitutively expressing the *SPCP3* gene (Chen *et al.*, 2013). C1A CysProt activities can be inhibited by a specific group of molecules termed cystatins (Benchabane *et al.*, 2010; Martínez *et al.*, 2012, 2016). Accumulating evidence suggests that cystatin over-expressing plants with less CysProt activity present more tolerance to drought (Zhang *et al.*, 2008; Quain *et al.*, 2014; Tan *et al.*, 2017). Recently, Velasco-Arroyo *et al.* (2018) have examined the effects of cystatins on plant behaviour under a deficit watering regime by independently silencing two barley drought-induced cystatin genes, *Icy-2* and *Icy-4*.

Barley (*Hordeum vulgare*) is an excellent model to study drought-associated events in plants due to its high capability to adapt to severe conditions (Dawson *et al.*, 2015). The participation of the barley C1A CysProt family members in different physiological processes and their roles in plant growth under stress conditions have already been established, for example in N starvation, darkness, high carbohydrate levels, and pathogen and pest attack (Martínez *et al.*, 2009, 2012; Parrott *et al.*, 2010; Diaz-Mendoza *et al.*, 2016b; Velasco-Arroyo *et al.*, 2016; Santamaria *et al.*, 2018a, 2018b), and a recent study has highlighted the function of specific barley cystatins related to responses to drought (Velasco-Arroyo *et al.*, 2018).

This work examines the responses of the entire C1A CysProt family in barley leaves to drought, and characterizes two of the genes that are up-regulated, namely *HvPap-1* (*HORVU5Hr1G082810*) and *HvPap-19* (*HORVU4Hr1G010390*). Unexpected changes in leaf cuticles and stomata were found when the expression of these drought-induced genes was reduced, and the consequences for plant defense responses of these structural alterations are examined and discussed.

Materials and methods

Plant material and growth conditions

Grains of barley (*Hordeum vulgare* L. cv. 'Golden Promise') were germinated in individual pots (8 × 8 × 8 cm) with a mixture of soil and vermiculite (3:1), at 22 °C under a 16/8 h light/dark photoperiod, for 7 d in Sanyo MLR-350-H chambers. They were then subjected to drought stress by continuous water deprivation for 14 d, following the method of Velasco-Arroyo *et al.* (2018). Control plants were grown under the same conditions but were watered to maintain soil moisture at 70%, which Velasco-Arroyo *et al.* (2018) reported as optimal watering. At 14 d old, plants were transferred to bigger pots (15 cm diameter × 13 cm high) and grown in a controlled greenhouse to allow grain production.

Knock-down lines for the *HvPap-1* and *HvPap-19* genes (KD Pap1 and KD Pap19, respectively) were generated using artificial microRNA (amiRNA) technology according to Hensel *et al.* (2009). The generation and molecular characterization of the homozygous KD Pap1 plants (lines 1128, 1130, 1175, and 1176) was previously reported by Diaz-Mendoza

et al. (2016a). The amiRNA construct for the KD Pap19 lines was engineered from the pNW55 vector by replacing the 21 bases of the natural Osa-202 MIR528 miRNA to specifically silence the *HvPap-19* gene (5'-TTTATTAACGGGCACATCCA-3'). Homozygous barley transgenic plants were obtained by double-haploid technology (Marthe *et al.*, 2015). Molecular analysis (gene copy number, mRNA expression levels) of the KD Pap19 plants (lines 1770, 1776, 1779, 1782) was performed as described by Diaz-Mendoza *et al.* (2016a). Grains of transgenic barley lines were germinated under the same conditions described above for the wild-type (WT) Golden Promise plants. At 7 d old, transgenic and WT plants were subjected to drought stress imposed by continuous water deprivation for 14 d, with controls being watered on alternate days. Pots were placed over plastic plates to individualize watering. After the drought treatment, the oldest leaf (leaf 1) of each plant was harvested, frozen by plunging into liquid nitrogen, and stored at -80 °C until further analysis.

At 7 d old, leaves of plants of KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1776), and the WT were infected with a strain of the fungus *Magnaporthe oryzae* expressing the pCAMP-Guy11 protein fused to GFP (green fluorescent protein; Leung *et al.*, 1988), kindly provided by Dr Sesma (CBGP, UPM-INIA), following the method of Diaz-Mendoza *et al.* (2017). The plants were incubated at 25 °C, 65% relative humidity (RH), under a 16/8 h light/dark photoperiod. Disease symptoms were monitored at different time points, and leaves were harvested after 3 and 7 d post-inoculation (dpi). Samples were frozen in liquid nitrogen and stored at -80 °C until analysis.

Independently, the same transgenic and WT lines were infested with 20 barley-adapted females per plant of the two-spotted spider mite *Tetranychus urticae* London strain (Acari: Tetranychidae), following the method of Santamaria *et al.* (2018b). After incubation at 25 °C, 65% RH, under a 16/8 h light/dark photoperiod, leaf damage was monitored after 7 d of mite feeding. Samples were frozen in liquid nitrogen and stored at -80 °C until analysis.

Real-time quantitative PCR analyses (RT-qPCR)

Leaves of transgenic KD Pap19 and WT lines were used to analyse transgene copy number and to study gene expression levels. Copy number was calculated by the $2^{-\Delta\Delta CT}$ method according to Velasco-Arroyo *et al.* (2016). Leaves of KD Pap1, KD Pap19, and WT lines harvested after 14 d of drought or control conditions were used for RT-qPCR analysis, with four replicates. RNA extraction, cDNA synthesis, and RT-qPCR conditions were performed as described by Velasco-Arroyo *et al.* (2016). Quantification was standardized to the mRNA levels of barley *cyclophilin* (*HvCyc1*). Expression levels of the *M. oryzae* small subunit of ribosomal RNA (*Mo28S-rRNA*) and the *T. urticae* ribosomal protein 49 (*TuRp49*) were also quantified by RT-qPCR according to Diaz-Mendoza *et al.* (2017). Fold-change values were expressed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) relative to the normalized expression of the same genes in non-treated plants. Primer sequences are listed in Supplementary Table S1 at JXB online.

Phenotypic assessment, and physiological and biochemical parameters

Plant growth and stress symptoms were monitored over 14 d of drought, comparing controls versus treated WT and transgenic lines. Six replicates were sampled. The subsequent growth and development of plants were monitored weekly under controlled greenhouse conditions. Phenotypes and physiological parameters were monitored as the drought stress progressed, and were recorded at the same time of the day to avoid circadian effects. Plant above-ground biomass was determined at the end of each treatment as fresh weight, and then after drying in an oven at 70 °C (dry weight). Soil water content was measured with a soil moisture sensor (SM150 Delta-T-Devices, Cambridge, UK). Stomatal conductance was determined using a leaf porometer (SC-1 Decagon-T, Pullman, USA). Quantum yield, an indicator of the efficiency of PSII, was evaluated using a portable fluorometer (FluorPen FP 100, Photon Systems Instruments, Drasov, Czech Republic). Photosynthetic pigments (total chlorophyll and carotenoids) were quantified from 100 mg of leaves of transgenic and

WT plants after 14 d of drought and control conditions, following the method of Velasco-Arroyo *et al.* (2016). Pigment content was calculated using extinction coefficients and the equations of Lichtenthaler (1987).

Protein quantification and western blot assays

Total soluble proteins were extracted from water-stressed and non-stressed leaves of KD Pap1, KD Pap19, and WT lines as described by Velasco-Arroyo *et al.* (2016) and quantified on a microplate reader (Varioskan Flash, ThermoScientific) according to the method of Bradford (1976), using BSA as a standard. Four of replicates were sampled. Immunoblotting was performed as described by Velasco-Arroyo *et al.* (2016) with an antibody specific to the peptide NH2-CQEKKHFSIDAYQVNSDPHD-COOH of the HvPap19 protein. The optimal dilution for the primary antibody was as previously determined by Diaz-Mendoza *et al.*, (2016a).

Microscope analyses

Leaves were cut from KD Pap1, KD Pap19, and WT barley plants grown with or without drought for 14 d and the cuticles were stained with Auramine O according to Buda *et al.* (2009); 12 replicates were used. The specimens were mounted in a mixture of glycerol:PBS (1:1, v/v) and observed using a Leica TCS SP8 confocal microscope under an Argon laser line of 458 nm, with the emission of the dye collected in the range 490–560 nm. Stacks of *xyz* and *xzy* series were analysed using the programs LAS X and Fiji. For the *xzy* series, *xz* planes (perpendicular to the leaf surface) were taken along the *y*-axis. The distance between the first and the last *xz* planes was 8.12 µm for all *xzy* series. Up to 50 individual measurements were taken along the cuticle of the maximum projections of the *xzy* images using the program Fiji. The stomatal apertures were contoured and their area measured using Fiji.

For leaves infected with the fungus *M. oryzae*, the progress of the infection was monitored using a Leica TCS SP8 confocal microscope. The growth of the fungus was detected by the expression of green fluorescent protein (GFP, excitation 488 nm, emission range 494–564 nm). Chlorophyll autofluorescence was used as a marker of cell viability (excitation 633 nm, emission range 699–741 nm). Random tacks of 21 *xyz* planes were collected at 40× magnification along the apical 2 cm of the leaves.

Quantification of amino acids

Samples were obtained from leaves of plants that had been water-stressed for 7 d and from controls of KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1776), and WT plants. Four replicates were used. Samples of 20 mg were re-suspended in methanol, disrupted by three cycles of freeze/thawing, and processed with a TissueLyser LT small bead mill (Qiagen). After centrifugation at 15 700 rpm for 20 min, the supernatant was transferred to a new tube and dried in a SpeedVac. The metabolite extracts were re-suspended by 1 min vortex mixing in 0.1 mol mL⁻¹ of formic acid containing 0.2 mmol L⁻¹ methionine sulfone (internal standard) and then centrifuged (15 700 rpm, 15 min). The clear solution was analysed by capillary electrophoresis–electrospray ionization–time-of-flight MS (CE-ESI-TOF MS). Every sample was prepared and analysed with three technical repeats.

Nineteen nitrogen metabolites (Ala, Arg, Asn, Asp, betaine, Ile+Leu, Gln, Glu, His, Lys, Met, Phe, Pro, sarcosine, Ser, Trp, Tyr, and Val) were analysed by CE-ESI-TOF MS essentially as described by Moraes *et al.* (2011). The metabolites were previously identified in WT barley samples by comparison of their migration times and spectra with pure standards. A Capillary Electrophoresis System (7100, Agilent Technologies) coupled to a 6224 time-of-flight MS system (Agilent Technologies) 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 of formic acid in 10% methanol (v/v) at 20 °C. The 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 thus higher accuracy 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 200 °C, and flow rate 10.0 l min⁻¹. The capillary voltage was 3500 V. Data were acquired in positive Dual-ESI mode with a full scan from *m/z* 87 to 1000 at a rate of 1.41 scan s⁻¹. The resulting CE-MS data files were cleaned of background noise and unrelated ions by the Targeted Feature Extraction tool with Profinder software (B.08.00, Agilent). Linear regression coefficients were calculated for each compound and metabolites were quantified in the samples.

Hormonal analysis

Freeze-dried plant material was ground to fine powder, weighed in 2-ml microtubes and spiked with 25 µl of an internal standard mixture (containing ABA-*d*₆, DHJA and ¹³C₆-SA) to correct for analyte losses. Extraction was carried out in 1 ml ultrapure water for 10 min in a ball mill at room temperature using 2-mm glass beads. After extraction, the homogenates were centrifuged at 10 000 rpm for 10 min at 4 °C to remove debris. Supernatants were recovered, pH adjusted to 3.0 with 30% (v/v) acetic acid, and partitioned twice against an equal volume of di-ethyl ether. The combined organic layers were evaporated under vacuum in a centrifuge concentrator (Jouan, Saint Germaine Cedex, France). The dry residues were subsequently reconstituted in 0.5 ml of 10% (v/v) aqueous methanol and the resulting solutions filtered through 0.20-µm PTFE syringe membrane filters. Filtered extracts were analysed by tandem LC/MS in an Acquity SDS UPLC system (Waters Corp., USA) coupled to a TQS triple quadrupole mass spectrometer (Micromass Ltd., UK) through an electrospray ionization source. Separations were carried out on a C18 column (Gravity C18, 50 × 2.1 mm, 1.8 µm particle size, Macherey-Nagel, Germany) using a linear gradient of ultrapure methanol and water, both supplemented with acetic acid to a 0.1% (v/v) concentration, at a constant flow rate of 0.3 ml min⁻¹. During analyses, the column was maintained at 40 °C and the samples at 10 °C to slow down degradation. Plant hormones were detected in negative electrospray mode by their specific precursor-to-product ion transitions (ABA, 263/153; JA, 209/59; SA, 137/93; SAGE, 299/93; OPDA, 291/165; JA-Ile, 322/130) and quantified using external calibration curves with standards of known amounts.

Magnaporthe oryzae and Tetranychus urticae bioassays

For plants infected with the fungus *M. oryzae*, disease symptoms were monitored at 3 d and 7 d post-inoculation (dpi). Lesions observed on infected leaves were scanned with a Scanjet 5590 Digital Flatbed Scanner (HP). The area of damaged leaf surface was measured using the Assess 2.0: Image Analysis Software for Plant Disease Quantification. Six replicates per line were obtained and three independent experiments were performed.

For plants infested with the spider mite *T. urticae*, the area of damaged leaf surface was monitored after 7 d of mite feeding and quantified using Adobe Photoshop CS software, as described by Santamaría *et al.* (2017). The number of leaves per plant was counted before and after spider mite infestation. Six replicates per line were analysed and three independent experiments were performed.

Statistical analysis

Student's *t*-test was used to compare protease expression in control and drought conditions, and to compare the effects of drought on the cuticle thickness of every genotype. To compare the effects of the genotype under control or drought conditions, one-way ANOVA was used for *HvPap-1* and *HvPap-19* expression, cuticle thickness, stomatal pore area, metabolite contents, and physiological parameters, and two-way ANOVA was used to compare accumulation of hormones. For the bioassays, one-way ANOVA was used to compare foliar damage and pathogen/pest performance, and two-way ANOVA was used to compare the number of leaves. ANOVA analyses were followed by the Student–Newman–Keuls multiple comparison tests.

Results

Response of barley C1A CysProt genes to drought

The expression profiles of the 41 C1A CysProt genes in barley leaves after 14 d of drought were assessed by RT-qPCR assays and were expressed as mRNA levels normalized to the constitutive active *cyclophilin* gene. The results showed that two B-like, one F-like, the only H-like, and eight out of the 34 barley L-like cathepsin genes were expressed in leaves in control and/or drought conditions (Fig. 1). However, only four protease gene members were significantly induced in stressed leaves in comparison to control plants, namely *HvPap-8* (L-like), *HvPap-12* (H-like), *HvPap-1* (F-like), and *HvPap-19* (B-like). To obtain further insights into the role of barley CysProt during drought stress, *HvPap-1* and *HvPap-19* knock-down plants were used. In addition to the KD Pap1 lines (1128, 1130, 1175, and 1176) previously characterized by Diaz-Mendoza *et al.* (2016a), transgenic plants for the *HvPap-19* gene (KD Pap19 lines) were generated using the same artificial amiRNA approach via *Agrobacterium*-mediated transformation. Homozygous KD Pap19 lines were selected for further characterization based on transgene copy number, and transcript and protein contents. Following these criteria, the KD Pap19 lines 1170, 1176, 1179, and 1182 were selected (Supplementary Fig. S1). Lines 1170 and 1176 contained a unique copy of the transgene whereas lines 1179 and 1182 had two gene insertions, as estimated by RT-qPCR assays and the 2^{-ΔΔCT} method. The mRNA expression levels in all of them were reduced compared with the WT control. Immunoblot assays using a specific antibody against a peptide of HvPap-19 (previously checked to avoid cross-reactivity; Diaz-Mendoza *et al.*, 2016a) showed a reduction in the accumulation of the protein content in comparison with the WT (Supplementary Fig. S1). However, neither mRNA nor protein content was completely knocked-out, indicating the knock-down character of these lines.

Fig. 1. Expression of CysProt genes in barley leaves after 14 d of drought or standard watering (control). All 41 C1A CysProt genes in barley were assessed but only those that were expressed under drought and/or control conditions are shown. Data were determined by RT-qPCR and are expressed as relative mRNA levels normalized to that of barley *cyclophilin*. The data are means (±SE) of three independent biological replicates. Significant differences between means were determined using unpaired *t*-tests: **P*<0.05.

Since *HvPap-1* and *HvPap-19* mRNAs were up-regulated after drought stress in WT plants, the expression patterns for both genes in the knock-down lines were also examined after drought. In comparison to WT plants, a significant reduction of both *HvPap-1* and *HvPap-19* expression was observed not only in the corresponding KD line but also in the line for the other transgene, either grown under watered or drought-stressed conditions (Supplementary Fig. S2). However, the mRNA levels of *HvPap-1* in the drought-stressed KD Pap19 lines were higher than those in the KD Pap1 lines. In contrast, the mRNA levels of *HvPap-19* were similar in the KD Pap1 and KD Pap19 lines both before and after drought stress.

Barley HvPap-1 and HvPap-19 knock-down lines show phenotypic structural alterations

The phenotypes of the three barley genotypes were compared with controls after 14 d of drought. Watered WT and KD Pap19 plants looked more robust and tended to be taller than KD Pap1 plants (Supplementary Fig. S3). Under water stress, WT plants seemed to be more affected with less leaf turgor, while the leaves of both the KD lines stayed upright. In addition, leaf growth of the KD Pap19 lines tended to be greater than that of KD Pap1.

The full growth cycle of the KD Pap1 and KD Pap19 lines was compared to that of WT plants, and no relevant phenotypic alterations were observed during the first 8 weeks of development (Supplementary Fig. S4). By the end of their lifespan, a slight delay in natural senescence was observed in the KD Pap1 lines compared with the non-transformed plants. A delay in senescence in KD Pap1 lines has been previously reported by Diaz-Mendoza *et al.* (2016a). In contrast, the KD Pap19 phenotypes were quite similar to the WT plants through the complete life cycle.

Interestingly, leaves from transgenic and WT plants showed apparent differences in surface texture and hardness. Further examination of leaf cuticle structure and thickness were therefore conducted by confocal microscopy following staining. The thickness of the cuticle on the adaxial side of the leaf was significantly increased in the transgenic KD Pap1 lines relative to the WT (Fig. 2). Under drought, leaf cuticles become thicker in all three barley genotypes, but the KD Pap1 lines presented significantly thicker cuticles than KD Pap19 and WT plants. Moreover, the outer shape of epidermal cells of drought-stressed leaves looked more irregular than the controls (Fig. 2). Staining with Auramine O also showed changes in the stomata, and although the mean number per unit leaf surface area was not altered either among lines or between stressed versus non-stressed leaves (data not shown), some modifications in position, size, and aperture were observed. Generally, the stomata tended to be located in a lower position in relation to the general epidermal cells, particularly in transgenic leaves after drought (Fig. 2). The stomatal pore area of watered-WT leaves was larger than in the transgenic leaves under the same conditions. While leaves of the WT and KD Pap1 plants maintained their stomata open under watered conditions, in the KD Pap19 lines the stomata were almost closed (Fig. 3). Under drought stress, the stomatal pore area

Fig. 2. Cuticles of barley leaf epidermis stained with Auramine O (A–J). Stacks of xz planes across a fixed width (8.12 μm) along the y-axis were collected using a confocal microscope. The leaves are from drought and control treatments for KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1176) and wild-type (WT) plants. Asterisks indicate the positions of stomata in the epidermal layers. (K) Quantification of cuticle thickness was performed by taking up to 50 measurements per image using the program Fiji. Data are means (±SE) of measurements from three independent biological replicates. Different letters indicate significant differences as determined using one-way ANOVA followed by a Student–Newman–Keuls test ($P < 0.05$). Significant differences between pairs of means were determined using unpaired *t*-tests: * $P < 0.05$.

of the KD Pap19 lines was larger than that of KD Pap1 and much larger than in WT plants, which correlated with the stomatal aperture (Fig. 3).

To place the changes in cuticle/stomata features in the context of the drought stress imposed, some physiological parameters associated with the stress were analysed. Soil water content was severely reduced after 14 d of drought treatment independent of the barley genotype (Supplementary Fig. S5A). Dry and fresh weights did not show any differences among the genotypes when the plants were grown under a drought regime (Supplementary Fig. S5B–C). Reductions in other markers of drought stress, such as total chlorophyll and carotenoid contents, were detected in stressed versus control plants but no relevant differences were found between transgenic and WT plants (Supplementary Fig. S5D–F). In contrast, significantly higher quantum efficiency of PSII and greater total protein contents were found in drought-stressed KD Pap1 and KD Pap19 lines in comparison to the WT, whereas no differences were observed under watered conditions (Fig. 4).

Metabolite changes associated with drought stress in HvPap-1 and HvPap-19 knock-down lines and WT plants

To determine whether the differential levels of mRNA detected in the leaves was related to differences in the free amino acid composition, a metabolomics analysis was performed by comparing the amount of free amino acids and related compounds

in transgenic and WT leaves under drought and watering regimes. Significant differences between the concentration of many amino acids were found between the knock-down lines and the WT, both in control and in drought conditions (Supplementary Table S2). In the KD Pap1 lines most compounds had significantly higher or lower concentrations than in WT plants. In spite of these quantitative differences, the contribution of each compound to the total set showed quite similar trends in all the lines, even in the more divergent KD Pap1 1130 line (Fig. 5). The amino acids Glu, Gln, Asp, and Asn, and the compounds betaine and sarcosine were the most abundant metabolites in watered conditions. The contribution to the total pool of metabolites decreased for these compounds in all lines after drought treatment, with the exception of betaine, which increased. A strong increase of Pro in the total pool was found under drought, together with minor increases for several hydrophobic amino acids such as Leu+Ile, Val, Tyr, and Trp.

Differential accumulation of hormones among KD and WT lines in response to drought

The content of various hormones was determined in leaves grown under drought or watered conditions in order to determine whether the responses to drought stress were regulated similarly between the three barley genotypes (Fig. 6, Supplementary Table 3). The results showed that levels of OPDA, the precursor of the JA pathway, were elevated in the leaves of the KD Pap1 lines grown under control conditions, but in response to drought they fell to levels similar to the other two genotypes (Fig. 6A). On the other hand, JA and its bioactive form JA-Ile accumulated dramatically in response to drought in KD Pap1 and WT leaves. Whilst the results for JA and JA-Ile were inconsistent in the two KD Pap19 lines under control conditions, their levels were significantly lower than those of the WT and KD Pap1 after drought stress (Fig. 6B, C). As expected, the ABA concentration was greatly increased under water deficit in all the leaf samples, but the amounts were significantly higher in the two KD lines under stress than in WT plants (Fig. 6D). Finally, the amount of SA did not differ either among the lines or between the treatments, and

Fig. 3. Stomata of barley leaf epidermis stained with Auramine O, and stacks of xyz series (parallel to the leaf surface) collected using a confocal microscope (A–J). The leaves are from drought and control treatments for KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1176) and wild-type (WT) plants. (K) The stomatal areas were manually contoured and measured using the program Fiji. Data are means ($\pm$ SE) of at least 12 replicates from three independent biological experiments. Different letters indicate significant differences as determined using one-way ANOVA followed by Student–Newman–Keuls tests ($P < 0.05$).

Fig. 4. Physiological parameters of barley KD Pap1, KD Pap19, and wild-type (WT) plants after 14 d of drought or control treatments. (A) Quantum yield, and (B) protein content. Data are means ($\pm$ SE) of at least six replicates from three independent biological experiments. Different letters indicate significant differences as determined using one-way ANOVA followed by Student–Newman–Keuls tests ($P < 0.05$).

Fig. 5. Heatmap of the composition in free amino acids and related compounds before and after 14 d of drought (D) in barley leaves of KD Pap1 (lines 1130 and 1175), KD Pap19 (lines 1770 and 1776), and wild-type (WT) plants compared with well-watered controls (C). The colour coding shows the level of abundance quantified as the proportion (% of total) of each metabolite in the different samples. The original values have been $\ln(x+1)$ -transformed. No scaling is applied to the rows. Both rows and columns are clustered using correlation distance and average linkage.

its glycosylated form, the salicylic acid glucosyl ester (SAGE), only showed significant differences in the KD Pap19 lines, with higher levels after drought treatment (Fig. 6E, F).

HvPap-1 and HvPap-19 knock-down lines differentially respond to biotic stresses

Since the leaf cuticles and stomata were differentially altered in the KD Pap1 and KD Pap19 lines relative to the WT plants, the effects of these structural changes on defense responses to pathogens and pests examined after *M. oryzae* infection and *T. urticae* feeding, respectively. Initially, the *M. oryzae* fungal infection produced spots with necrotic borders that could enlarge and coalesce to colonize the entire leaf (Fig. 7). The highest level of injury was detected in leaves from the KD Pap19 lines, particularly in the oldest leaf (L1) and in the apex of leaf 2 (L2), with the damaged area being very high at 7 d post-inoculation (dpi). In contrast, the damaged area in the KD Pap1 lines was significantly lower, whilst WT leaves showed intermediate levels of damage at both 3 dpi and 7 dpi (Fig. 7). To enable examination of the cellular events taking place during the early stages of the barley–fungus interaction, fluorescent GFP-labelled *Magnaporthe* isolates were used to inoculate the plants. Confocal fluorescence microscopy revealed that invasive hyphae were highly developed at 3 dpi in the KD Pap19 lines, which inversely correlated with leaf autofluorescence due to the chlorophyll, a marker of cell viability (Fig. 8D, E). This

result was consistent with differences in leaf damage. An intermediate balance between fungus-GFP and plant chlorophyll fluorescence was detected in infected WT leaves while autofluorescence was more prominent in infected KD Pap1 lines (Fig. 8A–C).

To study the impact of the *HvPap-1* and *HvPap-19* genes on the performance of *M. oryzae*, RT-qPCR assays were carried out to measure the mRNA levels corresponding to the small subunit of ribosomal RNA (*Mo28S-rRNA*). The results showed a significantly higher quantity of fungal mRNA in leaves of KD Pap19 compared with KD Pap1 and WT plants at 3 dpi (Supplementary Fig. S6). At 7 dpi, the low levels of fungal mRNA found in the KD Pap19 lines was due to the death of most leaf tissue in these lines.

Transgenic and WT plants were also subjected to pest attack by mites of *T. urticae*, and the effects were observed after 7 d of feeding. Small chlorotic spots appeared due to the penetration of mite stylets and subsequent plant cell-sucking (Fig. 9D); greater levels of injury were observed following longer infestation times (data not shown). Infested KD Pap1 lines showed less leaf damage than infested WT plants, and the number of leaves per plant was not affected in comparison with non-infested plants (Fig. 9A, B). In contrast, the impact of mites on the KD Pap19 lines was severe with the leaf injury being much higher than in infested WT plants and the number of leaves per plant was reduced. The performance of *T. urticae* was monitored by quantifying the mRNA levels of ribosomal

Fig. 6. Quantification of hormones in barley KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1176), and wild-type (WT) plants after 14 d of drought or control treatments. (A) OPDA, 12-oxo-phytodienoic acid; (B) JA, jasmonic acid; (C) JA-Ile: JA-isoleucine; (D) ABA, abscisic acid; SA, (E) salicylic acid; (F) SAGE, salicylic acid glucosyl ester. Data are means ($\pm$ SE) from three independent biological replicates. Different letters indicate significant differences as determined by two-way ANOVA followed by Student–Newman–Keuls tests ($P < 0.05$).

protein 49 (*TuRP49*). A significant increase in *TuRP49* gene expression was found when mites fed on the KD Pap19 lines (Fig. 9C), which correlated with the greater leaf damage. Low *TuRP49* mRNA levels in KD Pap1 were associated with the low leaf damage that was observed. Intermediate levels of mite gene expression and leaf damage were observed in infested WT plants when compared to the KD lines.

Discussion

Plant survival under drought stress, which is of particular significance in the current context of global warming, depends on the ability to detect the stress and make adjustments to mitigate the impact. Plants induce molecular mechanisms, modify physiological processes, and adapt their growth patterns to optimize their use of water under drought stress (Osakabe

et al., 2014). Altered expression of members of the cysteine-protease (CysProt) family is often linked to these changes since turnover with rapid degradation and mobilization of proteins is enhanced under stress conditions (Kunert *et al.*, 2015; Velasco-Arroyo *et al.*, 2016). Our examination of expression levels of the 41 barley C1A CysProt genes after 14 d of water deficit revealed that four of them, *HvPap-1*, *HvPap-8*, *HvPap-12*, and *HvPap-19*, were induced (Fig. 1) suggesting a putative role in proteolytic processes specifically triggered by drought stress.

Most efforts to understand the role of CysProts under a drought conditions have been made by overexpressing genes ectopically (Chen *et al.*, 2010, 2013; Zang *et al.*, 2010). In our study, for the first time *CysProt* knock-down lines were generated in order to examine plant responses to drought. The transgenic *HvPap-1* and *HvPap-19* lines showed differences in some biochemical and physiological parameters associated

Fig. 7. Plant phenotypes and leaf damage after *M. oryzae* infection of leaves of barley KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1176), and wild-type (WT) plants. Plant phenotypes after (A) 3 d and (B) 7 d of infection. (C) Quantification of leaf damage measured as mm² of injured foliar area in all leaves of each plant after 3 d and 7 d of infection. Data are means ($\pm$ SE) from four independent plants. Different letters indicate significant differences as determined by one-way ANOVA followed by Student–Newman–Keuls tests ($P < 0.05$).

with drought stress in comparison to WT plants (Fig. 4, Supplementary Fig. S5). While drought caused a significant decline in the maximum quantum yield of PSII in WT plants, the efficiency of PSII was virtually unaltered in the knock-down lines. Likewise, the total amount of proteins, an important parameter associated with drought, was greatly reduced in stressed leaves of the WT but only slightly decreased in the two transgenic lines. These findings demonstrated the participation of the two CysProts in the plant adaptation to drought stress. The patterns of proteolytic activity in the knock-down lines did not show significant differences compared to the WT (data not shown), probably due to compensating effects as a consequence of the functional redundancy of CysProts that has been reported previously (Velasco-Arroyo *et al.*, 2016, 2018). Thus, although the contribution of each metabolite was quite similar

in all lines both before and after drought (Fig. 5), it induced greater increases in many free amino acids in the KD lines than in the WT, consistent with proteolytic compensation effects. Increases in proline and betaine, two osmolytes with roles in the protection of subcellular structures in stressed plants, are known to be related to enhanced tolerance to drought (Ashraf and Foolad, 2007), and thus the increases would protect the plants from dehydration.

As previously reported (Velasco-Arroyo *et al.*, 2016), the KD Pap1 plants presented a slight delay in senescence compared to WT plants (Supplementary Fig. S4). The growth behaviour of the KD Pap19 plants across the whole life cycle did not show any important modifications. So, adjustments in growth seemed to be mainly dependent on the CysProt gene. There are examples of plants expressing CysProt transgenes

Fig. 8. Confocal images of leaves of barley KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1176), and wild-type (WT) plants infected with *M. oryzae* expressing the protein pCAMB-Guy11 fused to green fluorescent protein. The images were taken at 3 d post-inoculation. The green fluorescence of the hyphae and chlorophyll autofluorescence, as a marker of tissue damage, were detected using a confocal microscope. Series of 21 xyz images were collected from random areas along the apical 2-cm of the leaves. Maximum projections are shown.

with different growth patterns. Transgenic *Arabidopsis* plants constitutively overexpressing *PSPC2* and *SPCP3* CysProt from sweet potato present earlier floral transition from vegetative to reproductive growth, in addition to alterations in seed germination in comparison to WT plants (Chen *et al.*, 2010, 2013). On the other hand, *HvPap-1* overexpressing barley lines exhibit a similar growth phenotype to that observed for WT plants (Velasco-Arroyo *et al.*, 2016). Interestingly, the delayed-senescence-phenotype of the KD Pap1 lines was accompanied by the presence of thicker leaf cuticles than WT plants, and alterations in the stomatal position, size and aperture (Figs 2, 3). These unexpected structural modifications, which were even more notably after drought, clearly supported the longer life cycle since cuticles act as physical barriers that protect plants and help their survival (Bi *et al.*, 2017; Xue *et al.*, 2017). It is difficult to find the link between the low expression of a CysProt gene in the KD lines and the alterations in the structure of the leaf cuticle. CysProts participate in multiple physiological processes either directly involved in degrading proteins or sometimes in degradation of pre-proteins to generate active forms, such as enzymes (Grudkowska and Zagdańska, 2004;

Martínez *et al.*, 2012). This led us to hypothesize that the different enzymes required for the synthesis of the cuticular wax components and cutin matrix could somehow be the target of the CysProts. Cutin and wax are synthesized in the ER and exported via ATP-binding cassette (ABC) transporters across the plasma membrane to the polysaccharide cell wall (Yeats and Rose, 2013; Xue *et al.*, 2017). Barley CysProts and cystatins have been localized in the ER and Golgi apparatus (Martínez *et al.*, 2009, 2012); thus, an imbalance in the enzymes in the KD lines could affect the structure and/or composition of the cuticle. Alternatively, pleiotropic effects associated with the silencing of the CysProt genes due to a parallel reprogramming of other genes could have mediated the leaf structural changes that were observed.

Among the physical adaptations to drought stress, an efficient stomatal structure is essential for an appropriate and rapid response. Usually, plant growth is impaired under water deficit due to a decrease in stomatal opening that limits CO₂ uptake and has negative consequences on photosynthesis (Osakabe *et al.*, 2014). Although stomata tend to be closed under drought, leaves of KD Pap1 and, especially, KD Pap19 plants kept their stomata open (Fig. 3). This could be an adaptation to compensate for the reduction in the transpiration process due to the presence of their thicker cuticles (Kosma *et al.*, 2009; Bi *et al.*, 2017). In addition, drought stress led to an increase in accumulation of ABA (Fig. 6), which is the main driver of stomatal closure to diminish gas exchange and transpiration, and to reduce water loss (Osakabe *et al.*, 2014; Sah *et al.*, 2016). The high ABA concentrations detected in leaves of drought-stressed plants, and particularly in both the KD lines, is in line with its role as a modulator to avoid water losing. Since these high levels did not result in stomatal closure, the KD lines might be deficient in their ability to sense ABA. In parallel, the accumulation of JA and its active form JA-Ile in water-stressed KD Pap1 and WT plants was in agreement with previous reports in other plant species grown under drought (Mahouachi *et al.*, 2007; de Ollas *et al.*, 2015). Recent reports have demonstrated that JA regulates a subset of plant responses to drought by modulating ABA biosynthesis and accumulation (de Ollas and Dodd, 2016; De Ollas *et al.*, 2018). Curiously, levels JA and JA-Ile were significantly lower in leaves of water-stressed KD Pap19 lines than in KD Pap1 and WT plants. This hormone is potentially required for the maximum sensitivity of stomata to drying soil (De Ollas *et al.*, 2018), and the low levels of JA and JA-Ile could have been correlated with the stomatal apertures observed in these plants even under drought stress.

Cuticles are not only barriers to desiccation but also form protective structures to biotic stress. On this basis, we decided to challenge the different genotypes with the fungal pathogen *M. oryzae* and the phytophagous pest *T. urticae*. As expected, KD Pap1 plants, whose leaves presented thicker cuticles, showed less damage after 7 d of fungal infection or mite feeding than WT plants (Figs 7–9). In contrast, the foliar damage detected on KD Pap19 plants after pathogen and pest attack was higher than in WT plants. The leaf damage correlated with the amount of fungus or mite mRNAs determined in each case. The enhanced defense shown by the KD Pap1 lines against *T. urticae* attack was not in line with previous data from

Fig. 9. Plant phenotypes and mite performance after 7 d of infestation with *T. urticae* for barley KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1776), and wild-type (WT) plants. (A) Quantification of leaf damage measured as mm² of injured foliar area in all leaves of each plant. (B) Total number of leaves per plant of mite-infested and non-infested plants. (C) Mite performance as quantified by the relative expression levels of the *T. urticae* ribosomal protein 49 (TuRp49) as determined by RT-qPCR. Data are expressed as mRNA levels normalized to barley *cyclophilin*. All data are means ($\pm$ SE) of either (A, B) four plants or (C) three independent biological replicates. Different letters indicate significant differences as determined by either (A, C) one-way or (B) two-way ANOVA followed by Student–Newman–Keuls tests ($P < 0.05$). (D) Representative examples of mite-infested leaves, where L1 is the oldest leaf and L2 is the next leaf on the plant.

bioassays performed by Diaz-Mendoza *et al.* (2017). It is likely that the different plant response was due to differences in the mite population used to infest the plants: while Diaz-Mendoza *et al.* (2017) employed adult mites that were adapted to barley, here the infestation was performed with a barley-adapted synchronized population of female mites. Only females at the same stage of development were used for the feeding bioassays. The different susceptibility of KD Pap1 and KD Pap19 plants to biotic stresses also probably did not only depend on the cuticle thickness. For example, hormones and regulators actively participate in the responses triggered by biotic stresses and act as important cues for producing greater resistance. Previous reports examining effects of pathogens or insects on leaves with either overexpression or silencing of CysProt have shown that the plant responses and the impact on the pathogen/pest are specific for each plant–organism interaction (Pechan *et al.*, 2002; Gilroy *et al.*, 2007; Shindo *et al.*, 2012; Höwing *et al.*, 2014).

We can conclude that barley *HvPap-1* and *HvPap-19*, which encode cathepsin F- and B-like CysProt, respectively, are functional genes involved in drought stress processes. Their absence, even partial, alters phenotypic traits and unexpectedly produces

leaf structural alterations that may confer differential susceptibility to abiotic and biotic stresses.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Characterization and selection of transgenic lines.

Fig. S2. mRNA content in leaves of the different barley genotypes.

Fig. S3. Barley phenotypes after drought treatment.

Fig. S4. Phenotype of barley plants at 4–12 weeks of development.

Fig. S5. Physiological parameters in the different barley genotypes.

Fig. S6. Expression of a *M. oryzae* constitutive gene after infecting different barley genotypes.

Table S1. List of primers used in this study.

Table S2. Amino acids and related compounds in the leaves of the different barley genotypes.

Table S3. Hormone contents in the leaves of the different barley genotypes.

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