

# Identification of a homolog of *Arabidopsis* DSP4 (SEX4) in chestnut: its induction and accumulation in stem amyloplasts during winter or in response to the cold

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## ABSTRACT

Oligosaccharide synthesis is an important cryoprotection strategy used by woody plants during winter dormancy. At the onset of autumn, starch stored in the stem and buds is broken down in response to the shorter days and lower temperatures resulting in the buildup of oligosaccharides. Given that the enzyme DSP4 is necessary for diurnal starch degradation in *Arabidopsis* leaves, this study was designed to address the role of DSP4 in this seasonal process in *Castanea sativa* Mill. The expression pattern of the *CsDSP4* gene in cells of the chestnut stem was found to parallel starch catabolism. In this organ, DSP4 protein levels started to rise at the start of autumn and elevated levels persisted until the onset of spring. In addition, exposure of chestnut plantlets to 4 °C induced the expression of the *CsDSP4* gene. In dormant trees or cold-stressed plantlets, the CsDSP4 protein was immunolocalized both in the amyloplast stroma and nucleus of stem cells, whereas in the conditions of vegetative growth, immunofluorescence was only detected in the nucleus. The studies indicate a potential role for DSP4 in starch degradation and cold acclimation following low temperature exposure during activity–dormancy transition.

*Key-words:* protein-phosphatase; storage carbohydrates; winter dormancy.

## INTRODUCTION

Dormancy is an adaptive mechanism that enables woody plants to survive the freezing temperatures of winter (Lang 1987). The process is complex and characterized by growth cessation, bud set, extensive metabolic remodelling, an acquired high tolerance to cold and, in deciduous trees, by

leaf senescence and abscission. The induction of dormancy occurs in response to seasonal environmental signals. In most woody plants, shortening of the photoperiod induces growth cessation, bud set and some degree of cold acclimation (Wareing 1956; Nitsch 1957). The subsequent drop in temperatures then leads to a greater tolerance to cold and leaf fall (Weiser 1970; Howe *et al.* 1999; Arora, Rowland & Tanino 2003). The phytochrome system plays an important role as a day length sensor (Olsen *et al.* 1997; Olsen 2006), and it has been recently reported that in poplar, the photo-periodic control of dormancy induction is driven by a molecular mechanism that shares components with the mechanism that controls flowering time in *Arabidopsis* (Böhlenius *et al.* 2006). The molecular features of the role played by low temperatures (LTs) in the establishment of winter dormancy are less well understood (Arora *et al.* 2003; Ramos *et al.* 2005; Benedict *et al.* 2006; Welling & Palva 2006; Rohde & Bhalerao 2007; Horvath 2009).

The protective role suggested for oligosaccharides in the cold acclimation of herbaceous plants (Thomashow 1999) may also be important for the cryoprotection of woody plants during winter dormancy. Correlation between oligosaccharide accumulation and increased freezing tolerance has been shown in trees (Sakai & Larcher 1987; Sauter & van Cleve 1994; Cox & Stushnoff 2001). Changes have also been observed in tree carbohydrate metabolism in response to short days (SDs) or LT, or during endodormancy. At the start of autumn, starch stored in the stem and buds is broken down, whereas at the same time, the build-up of several oligosaccharides has been described, primarily of sucrose, trehalose, raffinose and stachyose (Sauter 1988; Rinne, Saarelainen & Junttila 1994; Witt & Sauter 1995; Renaut *et al.* 2004). Sucrose levels rise in direct response to SD whereas raffinose and stachyose levels increase later in autumn, in response to the temperature drop (Hinesley *et al.* 1992; Ashworth, Stirm & Volenec 1993; Rinne *et al.* 1994; Sauter & van Cleve 1994; Cox & Stushnoff 2001). Under chilling conditions, sucrose and trehalose have been found to rapidly accumulate, whereas the increase in raffinose is delayed occurring after 1 week at 4 °C (Renaut *et al.* 2004). Most of these results have been confirmed by transcript and metabolite profiling in the aspen dormancy

cycle and indicate that starch breakdown plays an important role in this process by generating cryoprotectants and energy (Druart *et al.* 2007). Thus, the initiation of starch degradation and its control must be a key factor for the freezing tolerance acquired in the dormancy state.

In the chloroplasts of *Arabidopsis* leaves, a dual specificity protein phosphatase (DSP) has been identified and incriminated in starch metabolism. This enzyme has been designated by the different authors as SEX4 (encoded at the Starch Excess 4 locus, Niittylä *et al.* 2006), PTPKIS1 (Fordham-Skelton *et al.* 2002) or DSP4 (Kerk *et al.* 2006; Sokolov *et al.* 2006). DSP4 is required for normal starch degradation and a role has been proposed for this enzyme in the protein phosphorylation/dephosphorylation mechanisms that modulate the activity of starch degrading enzymes (Kerk *et al.* 2006; Niittylä *et al.* 2006). Interestingly, DSP4 resembles laforin, the dual specificity phosphatase that regulates glycogen accumulation in animal cells and whose inactivity causes Lafora's disease, a neurodegenerative disorder characterized by excessive glycogen build-up (Minassian 2001). Recently, it has been shown that laforin is able to dephosphorylate glycogen or amylopectin *in vitro* and it has been proposed that this enzyme acts as a glycogen phosphatase *in vivo* (Worby, Gentry & Dixon 2006; Gentry *et al.* 2007; Tagliabracci *et al.* 2007). A lack of laforin leads to hyperphosphorylated glycogen, which, over time, becomes a poorly branched, insoluble polysaccharide that tends to aggregate, paralleling the formation of the Lafora bodies typical of Lafora's disease (Tagliabracci *et al.* 2008). Kötting *et al.* (2009) have similarly noted in *Arabidopsis* that DSP4 is a phosphoglucan phosphatase *in vivo* and have attributed it a role in starch catabolism. Moreover, the structural basis for the glucan phosphatase activity of *Arabidopsis* enzyme has been recently described (Vander Kooi *et al.* 2010). In addition, human laforin complements the *Arabidopsis dsp4 (sex4)* phenotype and laforin and DSP4 have been described as functional equivalents (Gentry *et al.* 2007). These findings indicate that starch dephosphorylation is probably the main role of DSP4 in plants.

Herein we report the induction of an enzyme homolog of *Arabidopsis* DSP4 in chestnut stem tissues in response to LTs or during winter dormancy. In both these situations, starch degradation takes place in these tissues. The timing of expression and intracellular localization of the enzyme support a role for this enzyme in this process.

## MATERIALS AND METHODS

### Plant material and growth conditions

For the field experiments, stem samples (2-year-old branch internodes) and leaves were harvested from adult European chestnut trees (*Castanea sativa* Mill.) in Zarzalejo, Madrid (4°11' W, 40°35' N). For the year-long experiments, samples were collected every month at around midday. For the diurnal rhythm experiments in natural conditions, samples were collected in the months of June (22.8 °C average temperature; 15 h, 5 min average day length) and December

(4.9 °C average temperature; 9 h, 16 min average day length). Controlled-environment experiments were performed using 16- to 24-week-old chestnut seedlings in growth chambers under the conditions described in Ramos *et al.* (2005). For the long-day (LD) trials, seedlings were grown at 22 °C and subjected to a 16 h light/8 h dark (16:8) photoperiod. Exposure to cold (4 °C) was undertaken for up to 7 weeks under the same light regime. SD experiments were performed for up to 6 weeks on plants that had been grown under LD conditions at 22 °C and thereafter subjected to a 8 h light/16 h dark (8:16) photoperiod from dawn. Continuous light (LL) experiments were performed on plants that had been grown under LD conditions at 22 °C and thereafter subjected to LL from dawn. After subjecting the plants to the different treatments, tissue specimens were collected at 3 h intervals. Each experiment was performed at least twice. After their collection, samples were immediately frozen in liquid nitrogen and stored at -80 °C. Plant material was ground using an IKA-A11basic Analytical mill (IKA® Werke GmbH & Co. KG, Staufen, Germany).

### Isolation of the cDNA clone

The chestnut *CsDSP4* cDNA clone was isolated from a  $\lambda$ Uni-ZAP XR cDNA library following standard procedures (Strauss 1987). The library was constructed using chestnut stem poly (A)<sup>+</sup> RNA isolated from plants in winter (Ramos *et al.* 2005). The probe used for the *CsDSP4* clone was a cDNA fragment obtained by enriching for winter-specific transcripts using the PCR-Select cDNA subtraction kit (Clontech, Mountain View, CA, USA). To obtain the full-length cDNA *CsDSP4* clone, the corresponding fragment was used as probe under high-stringency hybridization and washing conditions. Probes were labeled with [ $\alpha$ -<sup>32</sup>P] dATP using a random-primed DNA labeling kit (Roche Applied Science, Indianapolis, IN, USA).

### Real time qRT-PCR expression analysis

Total RNA was obtained from chestnut stems and leaves as described by Chang, Puryear & Cairney (1993) with a modification introduced after LiCl precipitation when the RNeasy Plus Mini Kit columns from Qiagen (Hilden, Germany) were used. This kit includes a step to eliminate any contaminating genomic DNA in the total RNA sample. To check for the lack of degradation, RNA was separated by electrophoresis on a formamide-formaldehyde denaturing agarose gel. RNA purity and quantity were checked with a Nanodrop Spectrophotometer (Thermo Scientific, Wilminton, DE, USA). For each sample, single stranded cDNA was synthesized from 1  $\mu$ g of total RNA using the Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA). This mix includes oligo (dT)<sub>20</sub> and random hexamers (Life Technologies/Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized in a 2720 thermocycler (Life Technologies/Applied Biosystems, Carlsbad, CA, USA). The gene *CsPRR5* was used as an experimental control (Ibañez *et al.* 2008; *CsPRR5* accession

number: ABV53464). Gene-specific primers were designed using Primer Express 2.0 (Life Technologies/Applied Biosystems) for the genes as follows:

- 1 *CsDSP4* forward 5'-GCTCATGCTTCCCTAAATTGG-3' and
- 2 *CsDSP4* reverse 5'-GCCTGTAAGAATATCAGCGGC-3';
- 3 *CsPRR5* forward 5'-GGCAAATCGTTTCCAAGTGA-3' and
- 4 *CsPRR5* reverse 5'-TAGAAGAGTTGACAAGGACA-TA-3';
- 5 *Cs18S* forward 5'-TCAACTTTCGATGGTAGGATAG-TG-3' and
- 6 *Cs18S* reverse 5'-CCGTGTCAGGATTGGGTAATTT-3'.

Real-time polymerase chain reactions were performed in an optical 96 well plate in an ABI PRISM 7300 Sequence Detector System (Life Technologies/Applied Biosystems) using SYBR Green to monitor dscDNA synthesis (Bookout *et al.* 2006) with the modification introduced by Ibañez *et al.* (2008). Reaction mixtures contained 1× Power SYBR Green Master Mix reagent (Life Technologies/Applied Biosystems), 250 nM gene-specific primers (except *Cs18S*, for which 25 nM gene specific-primers were used) and 0.8 μL of the previously synthesized cDNA (except *Cs18S* for which 0.8 μL of a 25-fold fresh dilution of cDNA was used) in a final volume of 20 μL. The following standard conditions were used in all PCRs: 40 cycles of 95 °C for 30 s and 60 °C for 1 min. A dissociation step was performed after amplification to confirm the presence of a single amplicon. To estimate variation in the technique, three technical replicates were run for each biological replicate. Data were analysed using 7300 System SDS 1.4 software (Life Technologies/Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal ( $\Delta R_n$ ) versus cycle number, baseline data and the  $R_n$  threshold were detected automatically to obtain Ct (threshold cycle) values. Amplification efficiency for each gene was calculated based on four template dilutions from 500 to 0.5 ng and the equation  $E = 10^{-1/\text{slope}} - 1$ , with slopes in the range  $\text{slope} = -3.3 \pm 0.1$  and  $E = 2$ . mRNA abundances for each candidate gene were calculated as: relative transcript abundance =  $2^{(-\Delta\Delta Ct)}$ . For the sample chosen as calibrator,  $\Delta\Delta Ct = 0$  and therefore the fold change = 1. Quantified data are shown in the graphs as relative amounts of mRNA. The sample showing maximum expression (lower  $\Delta Ct$ ) was used as calibrator and 18S ribosomal RNA was used as the reference gene to normalize data. The absence of genomic DNA contamination was checked using non-retrotranscriptase controls (RT-) and the absence of environmental contamination using non-template controls (NTCs).

### Western blot protein expression analysis

Total protein was obtained from stem material ground in liquid nitrogen to the consistency of flour as described in Garcia-Casado *et al.* (2000). One hundred milligrams were

extracted (4:1, v/w) with Laemmli sample buffer, 5% mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 1 mM phenylmethylsulfonyl fluoride and 1× of protease inhibitor cocktail for plant cell and tissue extracts, DMSO solution (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature (RT), with 2 min ultrasonic pulses and vortexing every 10 min. After centrifugation at 15 000 g for 15 min at RT, proteins in the supernatants were 4 h precipitated with 16% (w/v) TCA, washed twice in cold acetone and then dissolved in Laemmli buffer for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The gel was blotted to a polyvinylidene fluoride (PVDF) membrane in a 10% methanol SDS-PAGE running buffer. PVDF-adsorbed proteins (Hybond-P, GE Healthcare, Piscataway, NJ, USA) were probed with purified rabbit polyclonal antibodies to CsDSP4 prepared by Eurogentech S. A. (Herstal, Belgium) against a synthetic peptide (LRKRLTGDDP-DLSMD) located in the C terminal region of the protein. Protein blots were blocked in 5% non-fat dry milk, 0.1% Tween 20 TBS (20 mM Tris-HCl, pH 7.4, 137 mM NaCl) for 1 h at RT under mild shaking. After three 10 min rinses in TBST, blots were incubated overnight with the primary antibody diluted 1:400 in the blocking buffer at 4 °C under mild shaking. After three 10 min rinses in TBST, blots were incubated with a goat anti-rabbit antibody conjugated to horseradish peroxidase (diluted 1:400 000 in the same blocking buffer for 1 h at RT with mild shaking). After three 10 min rinses in TBST, proteins were chemiluminescence detected (Hyperfilm ECL, GE Healthcare, Piscataway, NJ, USA) with ECL plus Western blotting detection reagents from GE Healthcare following the manufacturer's instructions.

### Fixation and sectioning of plant tissue

Immunolocalization experiments were performed on stem samples (2-year-old branch internodes from adult trees and stem plantlets collected at 0900 h). Fresh material was cut into 0.5 to 1.0 cm pieces and fixed in 4% formaldehyde phosphate-buffered saline (PBS: 10 mM, pH 7.2) freshly prepared from powdered paraformaldehyde (Sigma-Aldrich). Penetration of the fixative into the tissues was achieved under vacuum and samples were then kept in the formaldehyde solution for 2 d at 4 °C. Fixed tissues were stored at 4 °C in 0.1% formaldehyde PBS. Before their use, specimens were washed with soft shaking in PBS 3 times for 10 min each. Thirty to 50 μm cross-sections were cut under deionized water on a vibratome (1000 plus-Starter-CE). Intact sections were obtained at low speeds and wide amplitudes at a sectioning angle of 20°. To avoid breakage during freezing, the sections were cut into four using a razor blade and stored at -20 °C until further use.

### Immunolocalization of CsDSP4

It was made sure that sections did not dry out during the immunolocalization procedure. Manipulations were

conducted on multiwell slides (Fisher Scientific Inc., Pittsburgh, PA, USA) by floating the tissue sections on drops of different solutions. To improve penetration of the antibodies into the tissues, the sections were permeabilized by dehydration/rehydration in a methanol series (30, 50, 70, 100, 70, 50, 30 for 5 min each) and then digested with 2% cellulase (Onozuka R-10) in PBS for 60 min at RT. After three washes in PBS, a 5% bovine serum albumin (BSA) blocking solution and 10% goat serum in PBS were added for 3 h at RT in a humid chamber. The purified rabbit polyclonal antibodies to CsDSP4 were applied at a dilution of 1/50 in 1% BSA in PBS from a fresh stock ( $5 \mu\text{g } \mu\text{L}^{-1}$ ). Incubation with the primary antibody was carried out at 4 °C overnight in a humid chamber. In parallel, a permeability control was run using a monoclonal antibody to total RNA, applied undiluted (BWR4, Eilat & Fischel 1991). As a negative control, the anti-CsDSP4 antibody was blocked with its corresponding antigen by incubating the antibody and peptide at a ratio 1/2 (v/v) at 4 °C overnight and then used as primary antibody under the above-described conditions. As a control for the secondary antibody, the primary antibody was replaced with PBS. After three washes of 10 min each in PBS, a secondary antibody was applied at a dilution 1/25 in PBS for 45 min in the dark. Alexa 488 anti-rabbit and anti-mouse antibodies (Life Technologies/Invitrogen, Eugene, OR, USA) were used to detect DSP4 and RNA, respectively. Secondary antibodies were washed three times in PBS for 10 min each time. Nuclei were stained with a  $10 \mu\text{g mL}^{-1}$  solution of 4',6-diamidino-2-phenylindole (DAPI; SERVA, Heidelberg, Germany) in 2% Triton X-100/PBS, applied overnight at RT in the dark. Finally, the sections were washed in PBS and mounted onto multiwell slides with Prolong™ Antifade (Life Technologies/Invitrogen) and kept in the dark at RT overnight. Series of sections spanning complete cells were captured by a Leica SP2-AOBS confocal microscope (Buffalo Grove, IL, USA) under laser-line excitation wavelengths of 365 nm (for DAPI) and 488 nm (for DSP4 and total RNA). Confocal images were analysed using the program Image J (<http://rsbweb.nih.gov/ij/>).

### Starch staining

Starch was detected in the same specimens by I<sub>2</sub>KI staining (Ruzin 1999) followed by observation using a Zeiss Axio-phot microscope under bright field. Images were captured by a Leica DFC 300FX CCD color camera equipped with Leica Application Suite 2.8.1 build 1554 acquisition software.

## RESULTS

### Rhythmic expression of the *DSP4* gene in chestnut leaves

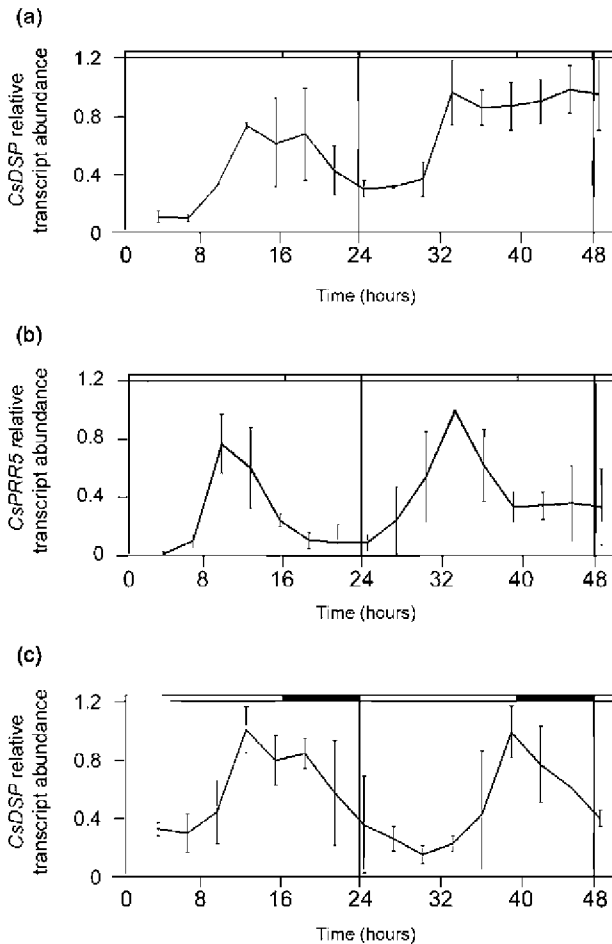
To identify the genes specifically expressed during winter dormancy in a deciduous tree, cDNA subtraction was conducted on stem tissue (2-year-old branch internodes)

collected around midday in June and December from adult European chestnut. Among the clones preferentially expressed in winter, a cDNA fragment with high similarity to the *Arabidopsis* gene *DSP4* was identified. The corresponding full-length cDNA clone (*CsDSP4*) was then isolated from a *C. sativa* winter stem library. A phylogenetic analysis comparing CsDSP4 with *Arabidopsis* DSP proteins (Kerk, Templeton & Moorhead 2008) and DSP4 homologs from tomato (LeDSP4) and potato (StDSP4) (Fordham-Skelton *et al.* 2002) has shown that the closest relative of CsDSP4, LeDSP4 and StDSP4 among the *Arabidopsis* DSP enzymes is AtDSP4 (Supporting Information Fig. S1). Alignment of the deduced amino acid sequence of CsDSP4 with known DSP4 sequences revealed a high degree of similarity among these enzymes (Supporting Information Fig. S2). Thus, the chestnut protein shows the basic characteristics of previously described DSP4 enzymes: a putative chloroplast transit peptide, a catalytic domain including the active site motive 'HCX<sub>3</sub>R', and a carbohydrate-binding domain (CBD) featuring conserved amino acids essential for starch binding (Kerk *et al.* 2006; Niittylä *et al.* 2006; Sokolov *et al.* 2006). The chloroplast transit signal peptide was predicted by the methods of Bannai *et al.* (2002; PSORT) and Schein, Kissinger & Ungar (2001; PCLR). Percent identities of the catalytic domain and CBD between AtDSP4 and CsDSP4 are 86.0 and 63.7, respectively.

Given that DSP4 is known to control starch degradation in *Arabidopsis* leaves at night (Niittylä *et al.* 2006; Kötting *et al.* 2009), we examined the possibility that the *CsDSP4* gene was rhythmically expressed in the chestnut leaves all day long. Through RT-PCR, we analysed its expression in leaves collected from 16- to 24-week-old seedlings grown under LL conditions at 22 °C over a 48 h period. Although mRNA levels of the *CsDSP4* gene were found to cycle throughout the first day, its behaviour in LL conditions could not be described as strictly circadian. Hence, on the second day the *CsDSP4* mRNA increase was not followed by a corresponding drop and levels remained high until the end of the experiment (Fig. 1a). As a positive control of the assay, the standard cyclic behaviour of the clock circadian gene *CsPRR5* was confirmed (Ibañez *et al.* 2008) (Fig. 1b). However, when we examined leaves of adult chestnuts grown under natural light and temperature conditions (in Zarzalejo, Madrid; 4°11' W, 40°35' N) during the month of June when vegetative growth takes place, a strong diurnal rhythm of mRNA levels of the *CsDSP4* gene was observed (Fig. 1c). The transcript peaked at dusk and this level of expression persisted for several hours in line with the role assigned by Niittylä *et al.* 2006 and Kötting *et al.* 2009 to AtDSP4 in night starch degradation in *Arabidopsis* leaves.

### Seasonal expression of the *CsDSP4* gene in chestnut stems

Next, we assessed the expression pattern of *CsDSP4* over the year in chestnut 2-year-old branches collected around midday under natural conditions. In this experiment, *CsDSP4* mRNA levels started to rise in September and



**Figure 1.** Quantitative RT-PCR analysis of *CsDSP4* gene expression levels in chestnut leaves under continuous light (LL) and vegetative growth conditions. (a) *CsDSP4* gene expression patterns observed in leaves taken from 16- to 24-week-old chestnut seedlings grown under standard conditions (LD, 22 °C) and then transferred to conditions of LL and 22 °C. Samples were collected at 3 h intervals. Open and shaded bars above the graph represent subjective day and night lengths, respectively. (b) Circadian clock *CsPRR5* gene expression patterns observed in leaves taken from 16- to 24-week-old chestnut seedlings grown under standard conditions (LD, 22 °C) and then transferred to conditions of LL and 22 °C. Samples were collected at 3 h intervals. Open and shaded bars above the graph represent subjective day and night lengths, respectively. (c) *CsDSP4* gene expression patterns observed in leaves taken from adult chestnuts collected in June. Samples were collected at 3 h intervals. Open and filled bars above each graph represent natural day and night lengths, respectively, as provided by the National Institute of Meteorology, Madrid. Relative transcript abundances are indicated in the graphs.

remained elevated until March (Fig. 2). From May to August expression of the transcript was low. Using an antibody raised against an oligopeptide selected from the sequence between the CB-domain and C-terminal residue (this oligopeptide is indicated in Supporting Information Fig. S2), these mRNA were noted to quantitatively correlate with the expression of the protein. Our Western blots

indicated the high presence of the DSP4 enzyme from November to March (Fig. 2). The only protein detected in branch material using the purified polyclonal antibody has a molecular mass estimated at around 40 kDa by SDS-PAGE, and is in good agreement with that inferred from the nucleotide sequence of the *CsDSP4* clone.

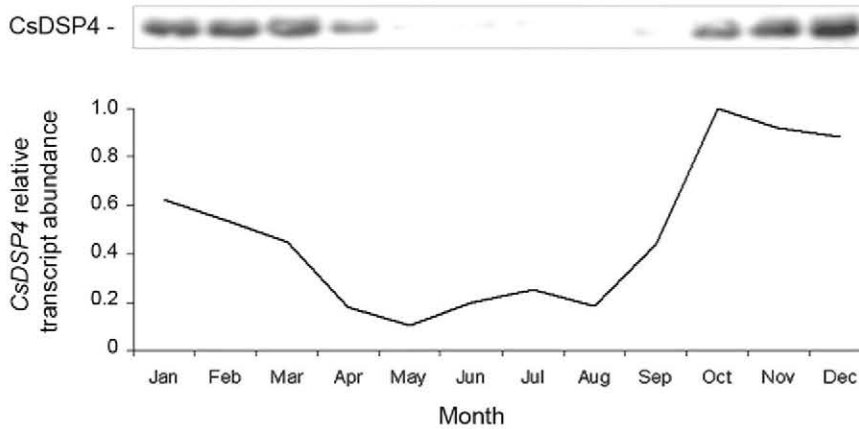
The expression of the *CsDSP4* gene in branches of adult chestnuts grown under natural conditions in June also follows a diurnal rhythm. Patterns in stems were essentially similar to those observed in leaves, although mRNA expression levels were lower. However, through Western blotting we observed that the amount of CsDSP4 protein, which was also low, did not follow a clear rhythmic pattern throughout the day/night cycle (Fig. 3a). In view of our previous finding that circadian clock performance in the chestnut tree is disrupted in winter (Ramos *et al.* 2005; Ibañez *et al.* 2008), we examined the expression behaviour of the *CsDSP4* gene in adult tree branches in December. Our results indicated relatively high *CsDSP4* mRNA levels but did not reveal the diurnal rhythm observed during the vegetative growth period. Besides, the permanent presence of mRNA was associated with the presence of the protein (Fig. 3b).

### ***CsDSP4* gene responses to LTs in chestnut seedling stems**

We then went on to investigate *CsDSP4* gene expression in response to factors that determine the onset of tree dormancy. Thus, the behaviour of this gene was examined in plantlets independently exposed to LT or SD photoperiod. When 16- to 24-week-old plantlets grown under normal conditions [16 h light, 8 h dark (16:8 LD) and 22 °C] were subjected to cold stress (16:8 LD and 4 °C) for up to 7 weeks, *CsDSP4* transcript and CsDSP4 protein levels increased (Fig. 4). Each set of samples was collected 3 h after the start of the light period, which is when under normal conditions *CsDSP4* expression levels are low. The time course of induction was slow, with no significant increases in mRNA and protein levels noted until 9 d. The behaviour of the *CsDSP4* gene in response to a SD photoperiod was also assessed. Thus, plantlets grown under standard conditions were transferred to conditions of 8 h light, 16 h dark (8:16 SD) and 22 °C for up to 6 weeks. These results revealed no rise in *CsDSP4* gene expression in the chestnut seedling stems throughout the experiment (data not shown).

### **Tissue and cellular localization of CsDSP4 in chestnut stems. Confocal microscopy analysis of CsDSP4 expression**

The CsDSP4 enzyme was immunolocalized in stems obtained from 16- to 24-week-old plantlets grown under normal conditions (16:8 LD and 22 °C) (Fig. 5a). We consistently detected the weak presence of the protein in phloem and xylem zones close to the cambium. High resolution observations indicated the occurrence of the CsDSP4

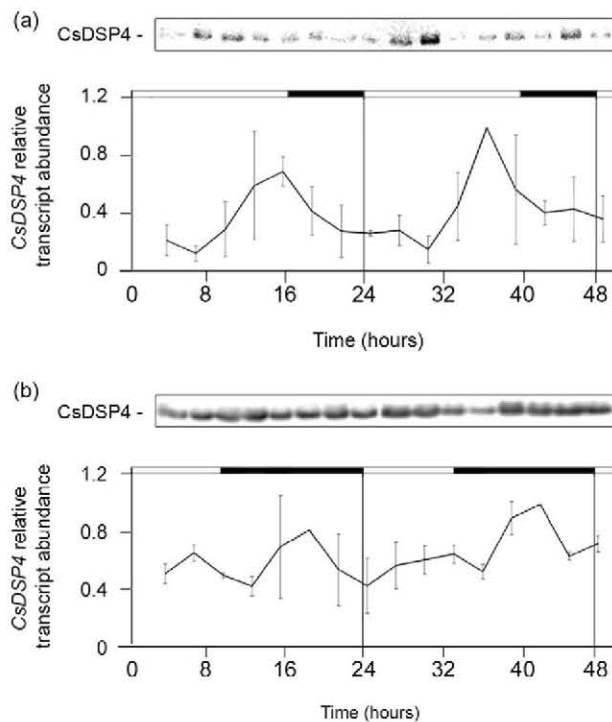


**Figure 2.** *CsDSP4* gene expression in the 2-year-old branches of adult chestnuts throughout the year. Samples were collected around midday at monthly intervals. *CsDSP4* relative transcript abundances as measured by quantitative RT-PCR indicated in the graph. The upper box shows a Western blot revealing the expression of *CsDSP4* protein.

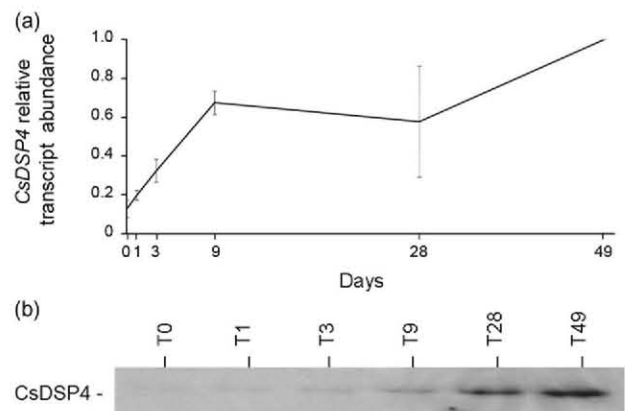
protein inside the cell nucleus revealed by matching of the antibody fluorescence signal and corresponding DAPI control signal (Fig. 6a, b). A movie showing three-dimensional reconstruction of Fig. 6g obtained from confocal images is provided online (Supporting Information Video Clip S1). No differences were detected in protein localization among tissue samples collected at different hours of the day. Similar results were also obtained when we

analysed adult chestnut stem tissue (2-year-old branch internodes) grown under natural conditions over the month of June (Figs 5d & 7a, b).

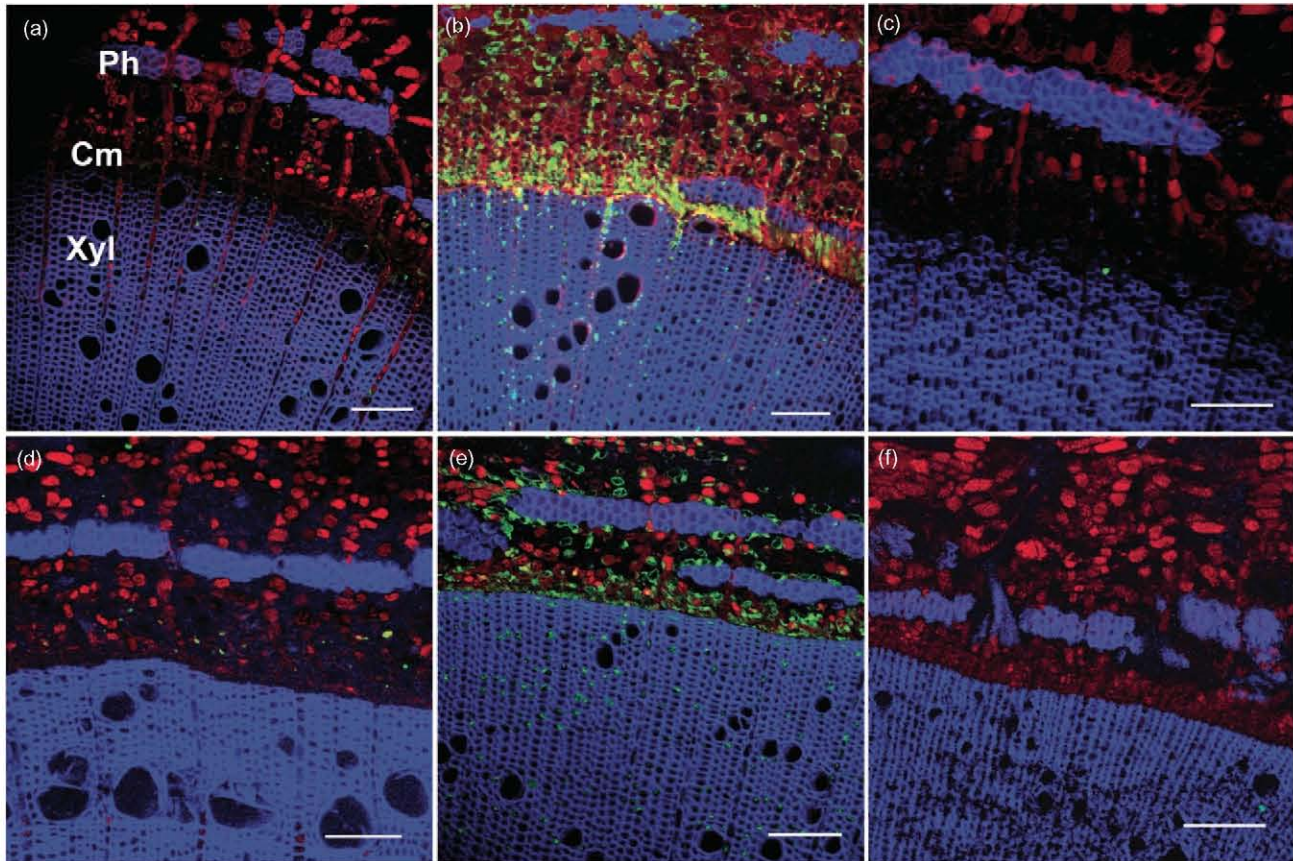
In view of our finding of *CsDSP4* gene expression induced by cold or winter (Figs 2 & 4), we examined the localization of *CsDSP4* protein under these circumstances. Both in the stems of chestnut plantlets kept at 4 °C for 1 week and in the branches of adult chestnuts collected in December, the protein signal was appreciably increased and mostly restricted to phloem parenchyma cells (Fig. 5b, e). Intracellular observations indicated that in these conditions, though still present in the nucleus, *CsDSP4* also appears within amyloplasts (Figs 6d, e & 7d, e). This second site is consistent with a predicted N-terminal chloroplast transit peptide in *CsDSP4* (Supporting Information Fig. S2). The images show that the protein occurs in the amyloplast stroma around the starch grains (Figs 6g–i & 7g–i). In the cold-stressed plantlets, amyloplasts may often be observed to aggregate at the cell peripheries, whereas in



**Figure 3.** *CsDSP4* expression throughout the day in 2-year-old branches of adult chestnuts under different seasonal conditions. Samples collected at 3 h intervals, in June (a) and December (b). *CsDSP4* relative transcript abundances, as measured by quantitative RT-PCR, indicated in the graphs. Open and filled bars above each graph represent natural day and night lengths, respectively, as provided by the National Institute of Meteorology, Madrid. Western blots showing *CsDSP4* protein expression are provided in the upper boxes.



**Figure 4.** *CsDSP4* gene expression in the stems of chestnut seedlings exposed to low temperatures at indicated times. Stems from seedlings grown under standard conditions [long day (LD), 22 °C] were subjected to 7 weeks of LD at 4 °C. Samples were collected at 0900 h each time. (a) *CsDSP4* relative transcript abundances, as measured by quantitative RT-PCR, indicated in the graph. (b) The Western blot shows *CsDSP4* protein expression at the different time points.



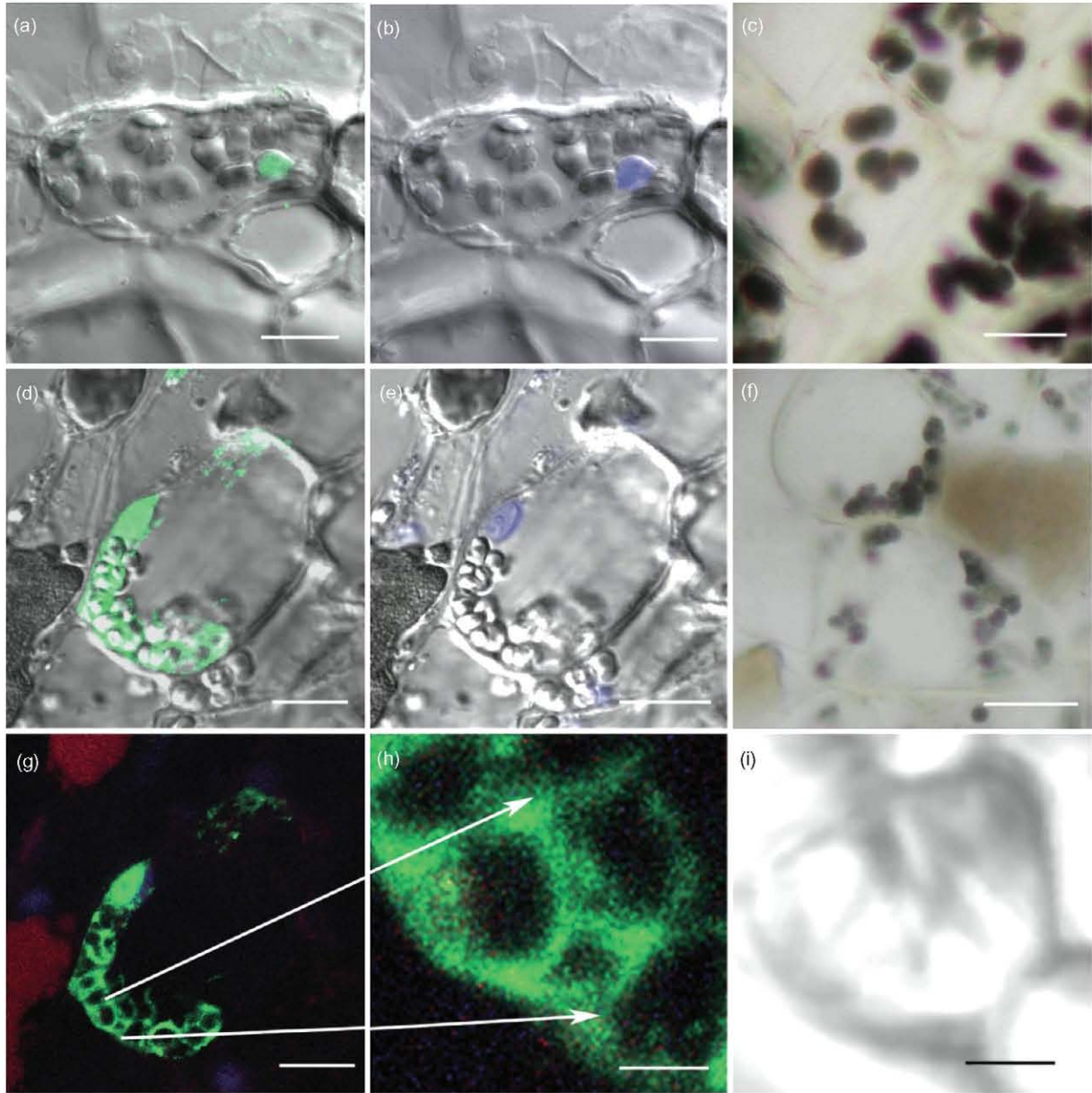
**Figure 5.** Tissue immunolocalization of CsDSP4 by confocal laser scanning microscopy. A weak signal of CsDSP4 protein is detected in phloem and xylem zones close to the cambium in plantlet stems grown under standard conditions (a) and in branches of adult chestnut trees grown under natural conditions over the month of June (d). Appreciable accumulation of CsDSP4 is detected preferentially in phloem parenchyma cells in plantlets kept at 4 °C for 1 week (b) and in adult trees grown under natural conditions over the month of December (e). Negative controls obtained using anti-CsDSP4 antibody blocked with a synthetic specific peptide; images corresponds to (c) plantlet stems kept at 4 °C for 1 week and (f) branches of adult chestnut trees grown under natural conditions over the month of December. In merged images, the green color (Alexa 488) corresponds to the CsDSP4 localization; natural fluorescence in blue corresponds preferentially to lignin; and natural fluorescence in red preferentially indicates cellulose and phenolic compounds. Images obtained in 30  $\mu\text{M}$  cross-sections. Scale bar = 100  $\mu\text{M}$ . Ph, phloem; Xy, xylem; Cm; cambium.

the non-treated plants these appear dispersed in the cytoplasm. These observations were confirmed by IK starch staining of treated and control plant tissues (Fig. 6c, f). Observations of a reduction in size of the starch granules and a lighter blue staining in treated plants are consistent with starch degradation in response to cold stress. Reduced starch contents were also observed in winter amyloplasts detected in the perinuclear region (Fig. 7c, f). Other authors have also described the perinuclear localization of stem cell amyloplasts in winter dormancy (Sauter, Wisniewski & Witt 1996).

## DISCUSSION

The findings of this study indicate the expression in chestnut of a gene encoding a dual protein phosphatase homologous to the *Arabidopsis* leaf DSP4 enzyme that breaks down starch through its phosphoglucan phosphatase activity (Niittylä *et al.* 2006; Kötting *et al.* 2009; Hejazi *et al.*

2010). Expression of the *CsDSP4* gene was detected in both the chestnut leaves and stems. In conditions of growth during the month of June, adult chestnut stem levels of both mRNA and the protein product were low yet at autumn onset the levels of both increased and remained high until the arrival of spring. This expression pattern suggests that CsDSP4 could play a role in the process of starch degradation occurring in the tree branches. Over the annual growth period, starch builds up and is deposited in the amyloplasts of stem tissue cells, and from the start of autumn and during winter dormancy, this stored starch is broken down to produce oligosaccharides, mainly sucrose, trehalose, raffinose and stachyose, which could afford protection against the cold (Sauter 1988; Rinne *et al.* 1994; Renaut *et al.* 2004). This catabolic process would also serve to satisfy the energy requirements of the plant in the absence of photosynthesis. Interestingly, the *CsDSP4* gene responded differently to factors that determine the onset of tree dormancy. Thus, SD treatment over a period of up to 6 weeks failed to produce

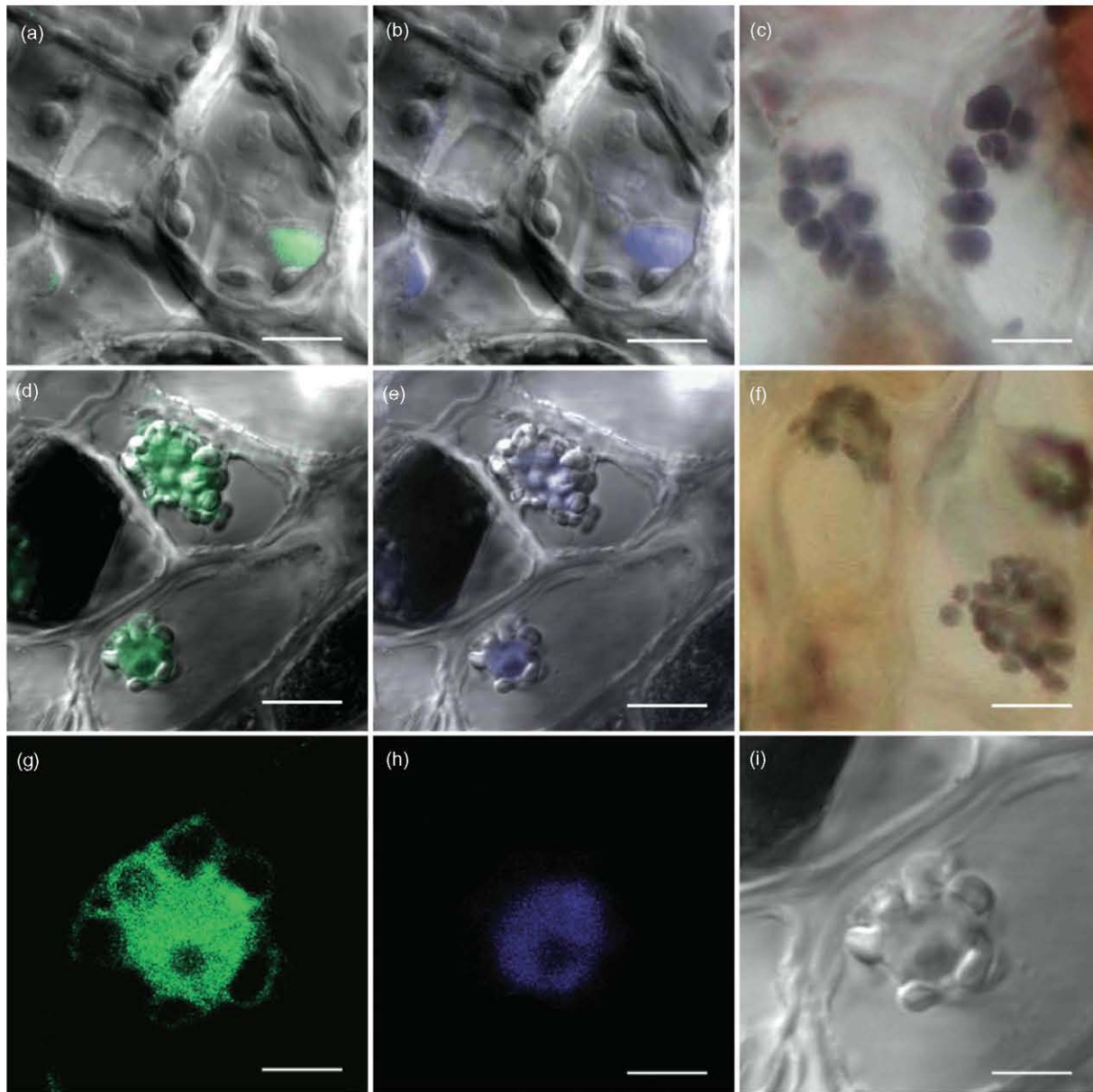


**Figure 6.** Sub-cellular immunolocalization of CsDSP4 and accumulation after cold treatment in the phloem parenchyma cells of plantlet stems revealed by confocal laser scanning microscopy. (a) Immunolocalization of CsDSP4 in plantlet stems grown under standard conditions [long day (LD) and 22 °C]. (b) Fluorescence micrograph after DAPI staining of the same specimen as shown in (a). (d) CsDSP4 accumulation in the nucleus and in the amyloplast stroma in plantlets kept at 4 °C for 1 week. (e) Fluorescence micrograph after DAPI staining of the same specimen as shown in (d). (g, h) Detail of CsDSP4 localization in the amyloplast stroma and (i) bright field image of the amyloplast. Bright field microscope images of (c) plantlets grown under normal conditions and (f) plantlets kept at 4 °C for 1 week obtained after starch staining with iodine-potassium iodide (I<sub>2</sub>KI). Images obtained in 30 μm cross-sections. Scale bar = 10 μm (a–g), 1.5 μm (h–i).

any significant changes in the expression of *CsDSP4*, whereas temperatures of 4 °C induced an expression increase that was notable from the first week of cold exposure. These observations would be consistent with a pronounced increase in *CsDSP4* expression occurring in October when nights start to get cold in Zarzalejo and not in September when the onset of winter dormancy is induced by the shortening photoperiod.

In natural conditions, *CsDSP4* showed a diurnal rhythmic expression pattern both in the leaves and branches of the chestnuts, with mRNA levels peaking during the last daylight hours and initial nighttime hours. As mentioned in the Results, the cyclic expression of *CsDSP4* cannot be described as strictly circadian and neither did it exclusively depend on light. In conditions of constant light, levels of mRNA for this gene cycled during the first day





**Figure 7.** Sub-cellular immunolocalization of CsDSP4 and accumulation during the winter dormancy period in phloem parenchyma cells of adult tree branches, as revealed by confocal laser scanning microscopy. (a) Immunolocalization of CsDSP4 in chestnut under natural conditions of growth over the month of June. (b) Fluorescence micrograph after 4',6-diamidino-2-phenylindole (DAPI) staining of the same specimen as shown in (a). (d) CsDSP4 accumulation in the nucleus and in the amyloplast stroma under natural conditions of growth over the month of December. (e) Fluorescence micrograph after DAPI staining of the same specimen as shown in (d). (g) Detail of CsDSP4 localization. (h) Fluorescence micrograph after DAPI staining and (i) bright field image of the same specimen as shown in (g). Bright field microscope images of (c) chestnut trees under natural conditions of growth over the month of June and (f) under natural conditions of growth over the month of December obtained after starch staining (I<sub>2</sub>KI). Images obtained in 30  $\mu\text{M}$  cross-sections. Scale bar = 10  $\mu\text{M}$  (a–f), 5  $\mu\text{M}$  (g–i).

but the expression increase produced was not followed on the second day by a corresponding expression decline with levels remaining high. However, the cyclic expression of *CsDSP4* in stem tissue was interrupted in winter when the circadian clock is disrupted (Ramos *et al.* 2005; Ibañez *et al.* 2008) despite some chestnut genes continuing to show diurnal cyclic behaviour in these conditions (unpublished results). Further, the behaviour shown by this

gene – whereby mRNA displays a diurnal rhythmic expression pattern but the protein shows no such pattern – resembles that described in the *Arabidopsis* model for the genes that code for several enzymes involved in both starch and maltose metabolism (Smith *et al.* 2004; Lu, Gehan & Sharkey 2005; Graf *et al.* 2010). The lack of a cyclic pattern in the levels of CsDSP4 protein observed here has also been described for these enzymes.

The persistence of high CsDSP4 levels detected here could be an effect of the elevated expression of the genes encoding elements of the circadian oscillator, which occurs in winter or in response to LTs (Ramos *et al.* 2005; Ibañez *et al.* 2008). Thus, the disruption of the clock could help to protect the tree from the cold. Recently, Ibañez *et al.* (2010) reported that the down-regulation of *Populus LHY* genes compromises freezing tolerance and the expression of *Populus CBF1*.

As does its homolog in *Arabidopsis*, the CsDSP4 enzyme features a catalytic domain, a CBD, and a chloroplast transit peptide (cTP). In *Arabidopsis* leaves, DSP4 has been localized in chloroplasts, as its cTP component would suggest (Niittylä *et al.* 2006; Sokolov *et al.* 2006). However, in chestnut stems, our observations indicate the dual localization of the enzyme. CsDSP4 was immunolocalized mainly in the cell nuclei of stems of adult trees examined during vegetative growth (in June) or of plantlets grown in standard conditions (16:8 LD and 22 °C), although the protein lacks a nuclear localization sequence according to nucpred (<http://www.sbc.su.se/~maccallr/nucpred>). In contrast, in dormant trees (in December) or in cold-stressed plantlets the protein was detected both in the nucleus and amyloplast stroma. Although there have been reports of dual targeting of proteins to distinct intracellular locations (Silva-Filho 2003; Schwacke *et al.* 2007; Grabowski *et al.* 2008), we could consider other possibilities. In effect, the human laforin gene that complements the *dsp4* (*sex4*) *Arabidopsis* phenotype (Gentry *et al.* 2007) and encodes a cytoplasmic protein bears an alternative splicing transcript encoding a laforin isoform which is targeted to the nucleus (Ganesh, Suzuki & Yamakawa 2002). The role of chestnut DSP4 in the nucleus clearly differs from its function in plastids and could be related to its capacity to interact with plant protein kinases (Fordham-Skelton *et al.* 2002).

Over the past few years, much has been added to the existing body of knowledge of starch degradation metabolism. It is clear that degradation pathways differ between plant organs and that distinct pathways may operate within the same organ (Zeeman, Kossmann & Smith 2010). Starch degradation is best known in leaves, where starch is degraded through two mechanisms, a major exoamylolytic/debranching pathway involving the DSP4 enzyme, and a minor endoamylolytic pathway. The latter process has also been widely explored in cereal endosperms where  $\alpha$ -amylase plays a central role in starch hydrolysis (Smith, Zeeman & Simth 2005; Delatte *et al.* 2006; Zeeman *et al.* 2007; Fettke *et al.* 2009). However, there is little information on starch metabolism occurring in tree stem amyloplasts. In poplar,  $\alpha$ -amylase could play an important role in degrading starch in this organ, though the available evidence is indirect (Witt & Sauter 1995, 1996; Elle & Sauter 2000). Our findings point to a possible role of the DSP4 enzyme, suggesting a similar mechanism in the stem as that primarily operating in the chloroplasts of leaves.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Phylogram of *Arabidopsis* DSP proteins (Kerk *et al.* 2008) and DSP4-like proteins of other plant species including CsDSP4. The source of *Arabidopsis* sequences is MIPS. At3g52180 is identified as AtDSP4. Accession numbers are provided for LeDSP4 and StDSP4. The phylogenetic tree was constructed using the neighbour-joining method implemented in CLUSTAL W (Thompson, Higgins & Gibson 1994). Numbers on branches indicate the number of bootstrap replicates (out of 1000) that give rise to the topology.

**Figure S2.** Amino acid sequence and structural motifs of CsDSP4. (a) Schematic representation of CsDSP4 protein structure. Indicated are: the chloroplast transit peptide, the catalytic domain and the C-terminal carbohydrate binding (CB) domain. Amino acid residues in bold correspond to the predicted phosphatase site. (b) Comparison of deduced amino acid sequences from cDNAs encoding DSP4. Species identifiers are: At, *Arabidopsis thaliana* (GenBank accession no. AJ302781); Cs, *Castanea sativa* (GenBank accession no. AY616240); Le, *Lycopersicon esculentum* (GenBank accession no. AJ313509); and St, *Solanum tuberosum* (GenBank accession no. DQ241838). Symbols according to CLUSTALW (Thompson *et al.* 1994). The peptide selected to prepare the antibody used in this work is underlined.

**Video Clip S1.** A movie showing three-dimensional reconstruction of Fig. 6g obtained from confocal images.

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