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RESEARCH PAPER

The gene geranylgeranyl reductase of peach (*Prunus persica* [L.] Batsch) is regulated during leaf development and responds differentially to distinct stress factors

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Abstract

Plant geranylgeranyl hydrogenase (CHL P) reduces free geranylgeranyl diphosphate to phytil diphosphate, which provides the side chain to chlorophylls, tocopherols, and plastoquinones. In peach, the single copy gene (*PpCHL P*) encodes a deduced product of 51.68 kDa, which harbours a transit peptide for cytoplasm-to-chloroplast transport and a nicotinamide binding domain. The *PpCHL P* message was abundant in chlorophyll-containing tissues and flower organs, but barely detected in the roots and mesocarp of ripening fruits, suggesting that transcription was related to plastid types and maturation. The message was not revealed in shoot apical meristems, but spread thoroughly in leaf cells during the early stages and was located mainly in the palisade of mature leaves, which exhibited higher transcript levels than young ones. Hence, the transcription of *PpCHL P* was likely to be regulated during leaf development. Gene expression was monitored in leaves responding to natural dark, cold, wounding, stress by imposed darkening, and during the curl disease. Transcription was stimulated by light, but repressed by dark and cold stress. In darkened leaves, the *PpCHL P* message was augmented concomitantly with that of *CATALASE*. In wounded leaves, the message decreased, but recovered rapidly, whereas in curled leaves, a reduction in gene expression was related to leaf damage intensity. However, transcript signals increased locally both in cells mechanically wounded by a needle and in those naturally injured by the pathogenic fungus *Taphrina deformans*. These data suggest that *PpCHL P* expression was regulated by photosynthetic activity and was possibly involved in the defence response.

Key words: Geranylgeranyl reductase, leaf development, peach, *Prunus persica* L., regulation, stress response.

Introduction

In plants, phytol (Phy) represents the side chain of chlorophyll (Chl), tocopherols (TP), and phylloquinones (PQ) and is necessary for their integration into plastid membranes (Soll *et al.*, 1980, 1983; Soll, 1987; Bollivar *et al.*, 1994). In both Chl and TP synthesis, the Phy chain is provided by geranylgeranyl pyrophosphate (GGPP), a plastidial isoprenoid, formed by four molecules of isopentenyl pyrophosphate (IPP), which are derived from the cytosolic (Rohmer *et al.*, 1993) and chloroplastidic (Lichtentaler *et al.*, 1997) pathways. More precisely, in Chl synthesis, GGPP can either be reduced to phytyl pyrophosphate (PhyPP) and esterified with the chlorophyllide to generate phytyl chlorophyll (Chl_{PHY}), or first prenylated to chlorophyllide and then hydrogenated to form geranylgeranylated chlorophyll (Chl_{GG}). In the TP pathway, GGPP is channelled via condensation of PhyPP to homogentisate,

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which is a precursor of the aromatic ring of tocopherols (Schoch *et al.*, 1977; Benz *et al.*, 1980; Soll *et al.*, 1980). The three-step hydrogenation of GGPP into PhyPP is catalysed by the NADPH-dependent geranylgeranyl reductase (EC 1.3.1). This enzyme is conserved in photosynthetic organisms (bacteria, algae, and plants) and commonly named CHL P, referring to the unit P of prokaryote chlorophyll synthase (Bollivar *et al.*, 1994; Keller *et al.*, 1998). *In vitro*, the *Arabidopsis thaliana* CHL P reduces both Chl_{GG} and free GGPP into Chl_{PHY} and PhyPP, respectively. Hence, the same CHL P may be recruited into the CP, TP, and PQ pathways (Keller *et al.*, 1998). Subsequently, CHL P was demonstrated to provide Phy for both Chl and TP synthesis; *CHL P* antisense expression in tobacco resulted in the reduced content of both Chl, mostly present as Chl_{GG} forms, and TP (Tanaka *et al.*, 1999; Havaux *et al.*, 2003). However, the prevailing Chl_{GG} forms did not affect the harvesting and transfer of light energy and so the high sensitivity to photo-oxidative stress of the transgenic plants was mainly attributed to the diminished content of TP (Grasses *et al.*, 2001). In tocopherol-deficient antisense *CHL P* leaves exposed to high light and low temperature, the inhibition of photosystem II occurred more rapidly and lipid peroxidation was exacerbated compared with controls. Hence, TP were proposed to protect plant membrane lipids against photodestruction (Havaux *et al.*, 2003).

TP and PQ are antioxidant compounds which shield plants from ageing and photo-oxidative processes (Niyogi, 1999; Munnè-Bosch and Alegre, 2002). They also constitute vitamins E and K, respectively, and affect the nutritional value of fruits (Valk and Hornstra, 2000), their stability in the post-harvesting cold chain, and shelf life (Goffman and Bohme, 2001). Therefore, the genes affecting the synthesis of these compounds, such as *CHLP* (Havaux *et al.*, 2003), may be useful tools as expressed markers in breeding programmes or genetically manipulated to improve traits of fruit quality (Shintani and Della Penna, 1998).

Genes coding for CHL P have been characterized in prokaryotes (Bollivar *et al.*, 1994) and a few herbaceous species such as *Arabidopsis* (Keller *et al.*, 1998) and tobacco (Tanaka *et al.*, 1999), but not from any fruit tree species so far. Patterns of gene expression were previously compared in peach leaves at distinct developmental stages (Giannino *et al.*, 2000) and a cDNA fragment was selected as more abundant in mature than young leaves and the deduced product shared a high homology with plant geranylgeranyl reductases. Some structural features of *PpCHL P* are described here, and the leaf message during development and in response to light is monitored. Moreover, to test a possible *PpCHL P* involvement in antioxidant mechanisms of defence, the transcript abundance and localization were determined in leaves subjected to abiotic stresses and attack by the pathogenic fungus *Taphrina deformans*.

Materials and methods

Plant material and growth conditions

The adult plant OP16 (open pollinator 16, *Prunus persica* cultivar Chiripa) was seed-derived and chosen as the starting material. OP16 seeds were germinated *in vitro* and two clonal lines were micropropagated according to Gentile *et al.* (2002). Fully regenerated plants were transferred into peat/mould and sieved soil (4:2, v:v) and kept in growth chambers until environmental adaptation occurred (25 °C, 16 h of white light for 2 weeks). Clones were subsequently transferred into a greenhouse (minimal temperature, 16 °C) under a natural photoperiod and light intensity. One-year-old plants were moved to an open field and the first flowering occurred 1 or 2 years later. All tissues sampled were collected from plants grown in the open field and immediately frozen in −80 °C when necessary.

Stress assay conditions

In light-response experiments, 6-month-old seedlings, grown at 25 °C, 16/8 h of light/dark, and a light intensity of 100 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation (*PAR*) were tested. Batches of fully expanded leaves (FEL) were sampled 1, 3 or 7 h after dark induction and 1 h after light restoration.

The outdoor plants were pruned in the vase shape, consisting of three branches each 120° distant from the next. After the vegetative burst (from March onwards) the main branches got prolonged and bore secondary shoots on both the mature (woody) segment and the herbaceous extension. With regard to imposed dark assays, FEL of secondary shoots borne at the mid and distal positions (7th and 15th nodes, respectively) of primary branches were wrapped with aluminium foil so as to form an air chamber around them. This was done at 09.00 h on sunny days in May, subsequently leaves were sampled at 21.00 h, after 36 h of treatment. FEL from distinct shoots of one mother plant were collected and the experiment was repeated with two other mother plants. The aluminium foil was chosen so as to allow a partial transpiration of leaves and enhance light reflection (instead of black mittens or cloth) and mitigate heat stress phenomena. The leaf temperature of four wrapped and untreated leaves was measured using a leaf clip holder placed at the centre of the lamina and connected to a MINI-PAM photosynthesis yield analyser (Meinn Walz, Germany). Measures were carried out at 13.00 h and 21.00 h (28 h and 36 h after treatment, respectively). Regardless of their position, the wrapped leaves (WL) exhibited an average increase of *c.* 1.4 °C compared with controls (CL). At 13.00 h, air temperature was 23±0.2 °C, basal WL was 20.9±0.3 °C (standard error), basal CL was19.5±0.1 °C; apical WL was 21.8±0.4 °C; apical CL was 20.4 ±0.1 °C. At 21.00 h, air temperature was 17±0.2 °C, basal WL was 17.4 ±0.2 °C, basal CL was 15.8±0.4 °C; apical WL was 17.5±0.1°C; apical CL was 15.7±0.3 °C. Sampling was done at 21.00 h (36 h after treatment) as it was considered that *CHLP* was down-regulated, and such a pattern of expression was used as the control. WL did not appear chlorotic, wilting or flabby, although partially folded symmetrically with respect to the central vein, and just as green as CL.

In wounding assays, FEL of secondary shoots were injured by tweezers in the morning of a sunny day (June). FEL from distinct shoots of one mother plant were pooled and collected 1, 2 or 4 h after injury. Controls were untreated leaves collected at the comparable time after wounding. As for *in situ* experiments, leaves borne at the shoot apex were gently scarified with a thin needle and sampled after 24 h.

In cold-treatment experiments (May), 1-year-old potted plants (no flower buds) were moved from outdoors and acclimated in growth chambers for 1 week at 22 °C, 16/8 h of light/dark with light intensity as indicated above. Subsequently, the temperature was rapidly lowered to 4 °C and FEL were sampled from primary and secondary shoots after 24 h and 48 h. Leaves of acclimated plants before the treatment were collected as control samples.

Southern blot analysis

The technique was performed as previously described (Giannino *et al.*, 2000). Filters were hybridized at 62 °C, washed twice (2×, 1×, SSC/0.1%SDS) at 60 °C for 10 min and exposed to Biomax films (Kodak) for 4–12 h at −80 °C. The probe spanned the 1166–1578 cDNA nucleotide stretch.

Northern blot analysis

RNA isolation was reported in Giannino *et al.* (2000), and 7 μg was loaded in northern blots according to Sambrook *et al.* (1989), using formamide/formaldehyde gel electrophoresis. Filters were hybridized at 42 °C in formaldehyde containing buffer o/n (Ultrahyb, Ambion) and washed twice (2×, 1×, SSC/0.1%SDS) at 42 °C for 10 min, then washed once (0.1× SSC/0.1%SDS) for 50 min at 50 °C, then exposed to Biomax films (Kodak) for 4 h at −80 °C. The *CHL P* probe was the same as for Southern blots. The *CATALASE* probe spanned the stretch 223-829 (accession no. AJ496418.1) and in some cases two signal bands were detected; the 1.8 kb signal was attributed to *CATALASE 1* (Bagnoli *et al.*, 2004). The *18S RNA* probe spanned the stretch 485–1211 (accession no. L28749.1). The optical density of signal bands was determined according to the ID Image Analysis Software (Kodak Digital Science™) and relative gene expression was represented in histograms (Ms Excel program) as the optical density ratio between the tested gene and *18S r-RNA*.

Isolation and sequence analysis of cDNA and genomic clones

A 445 base pairs (bp) fragment was cloned after DDRT-PCR experiments performed on RNA of adult and young leaves using a decamere FW3 (5′-GGAGAAGTGGG-3′) and an oligo dT (Giannino *et al.*, 2000). To achieve the full length *PpCHL P*: a 940 bp fragment was cloned using FW1 (5′-AAGATGGACAACTGTAAGCC-3′), designed on conserved stretches of soybean and arabidopsis, and BW1 (5′-CCTTCTGCAACACATCCAACAC-3′), located within the 3′ region; then a 523 bp transcript was cloned, using BW5 (5′-CCCATTCTCACTCGCCCTGTTCC-3′) to reverse transcribe RNA from leaves in 5′ RACE experiments (In Vitrogen). The primer combinations FW0 (5′-GCTCCGACCCAAAAAAAAATGGC-TTCC-3′)/BW5 and FW1/BW3 (5′-CCATCTCCCTCCTAA-GTGC-3′) were used to screen for introns. PCR components were: genomic DNA (200 ng) and/or cDNA (2 μl), 1 μM of each primer, 0.5 mM dNTPs, *Taq* DNA polymerase (TaqQUIA, Quiagen) 2.5 U, 1/10 of 10× *Taq* buffer, 2.5 mM $MgCl_2$, in a final volume of 50 μl. PCR conditions: starting cycle at 95 °C for 3 min; 35 cycles at 95 °C for 1 min, either 58 °C (in cDNA based and RACE-PCR experiments) or 62 °C (with genomic DNA) for 90 s and 72 °C for 90 s, final extension at 72 °C for 5 min. All PCR fragments were cloned into pGEM-T vector system (PROMEGA) and sequenced by the ENEA service, Rome.

In situ *hybridization*

Excised tissues were fixed, dehydrated, embedded in paraffin, cut into 8 μm sections and hybridized (55 °C) to a digoxygenin-labelled antisense RNA probe as described by Cañas *et al.* (1994). A cDNA clone spanning the 3′ *PpCHL P* cDNA stretch (1166–1578) was linearized by the endonuclease *Spe*I and the digoxygenin-labelled RNA probe was generated by T7-polymerase driven *in vitro* transcription (Giannino *et al.*, 2000).

Results

The peach CHLP *full-length cDNA and its deduced product*

The full-length cDNA (AY230212) of *PpCHL P* consisted of 1621 bp with an ORF of 1401 bp encoding a deduced polypeptide of 466 amino acids. A stretch of eight adenines (32–39), a pyrimidine rich tract (8–31), and a stop codon (5–7) in frame with the ATG start were scored within the 5′ UTR, whereas a canonical polyadenylation signal spanned the 1579–1584 stretch in the 3′ UTR.

The deduced protein *Pp*CHL P (Fig. 1A) was 51.68 kDa (BioEdit Alignment Sequence Editor) and exhibited the maximal identity with *Glycine max* (81%), followed by *Nicotiana tabacum* (79%), *Arabidopsis thaliana* (76%), *Mesembryanthemum cristallinum* (76%), and *Oryza sativa* (53%). The transit peptide (tp) for cytoplasm-to-chloroplast transport (1–56) was assigned by using the Chlorop 1.1 Server (http://www.cbs.dtu.dk/services/) and the mature product of 410 amino acids was estimated as 45.57 kDa. Within the highly variable region of tp, the GLRQ group (11–14) and a few other residues (Fig. 1A) were tightly conserved. The GXGXXG motif which is associated with the binding of nicotinamide nucleotides was assigned within the stretch 53–85 (Atta-Asafo-Adjei *et al.*, 1993). From the group LRVAV onwards (61–69), peach CHL P was highly homologous to these species (ClustalW and Genedoc programs) and a phylogram (Fig. 1B) was constructed by clustering regions devoid of tp. Plant CHLPs fell into a unique and highly supported monophyletic group, branch length of peach CHL P (0.07) was closest to that of *A. thaliana*, whereas the enzyme of rice diverged from dicotyledonous species.

Genomic organization

The leaf genomic DNA of peach, almond (*Prunus amygdala*), sweet cherry (*Prunus avium*), and plum (*Prunus dulcis*) was endonuclease-restricted, size-fractionated, transferred onto a nylon membrane and hybridized with a radiolabelled probe spanning the 3′ UTR of *PpCHL P* cDNA (Fig. 2). The full-length cDNA (and the probe) did not contain sequences recognized by the endonucleases (Fig. 2A). As for peach, one band was signalled in all restrictions, suggesting the occurrence of one gene copy. A one band pattern was also observed in plum, whereas in almond and cherry the signal number varied from one to two bands. The latter profile could be due to the occurrence of either multiple *CHL P* copies or enzyme recognition sites which were absent in the peach gene. The neat bands suggested that *CHL P* genes of these species (subfamily: Drupoideae) shared a high degree of homology at the nucleotide level.

In order to search for introns, PCR experiments were performed on genomic DNA of peach, plum, and cherry with primer couples designed along the *PpCHL P* cDNA (Fig. 2A). As for peach, an intron of 633 bp was located within the ORF (Fig. 2A), exhibited a GT/AG editing motif, and was rich in A/T bases. As for the other species, the intron position was also tightly conserved, though the length varied accordingly (data not shown).

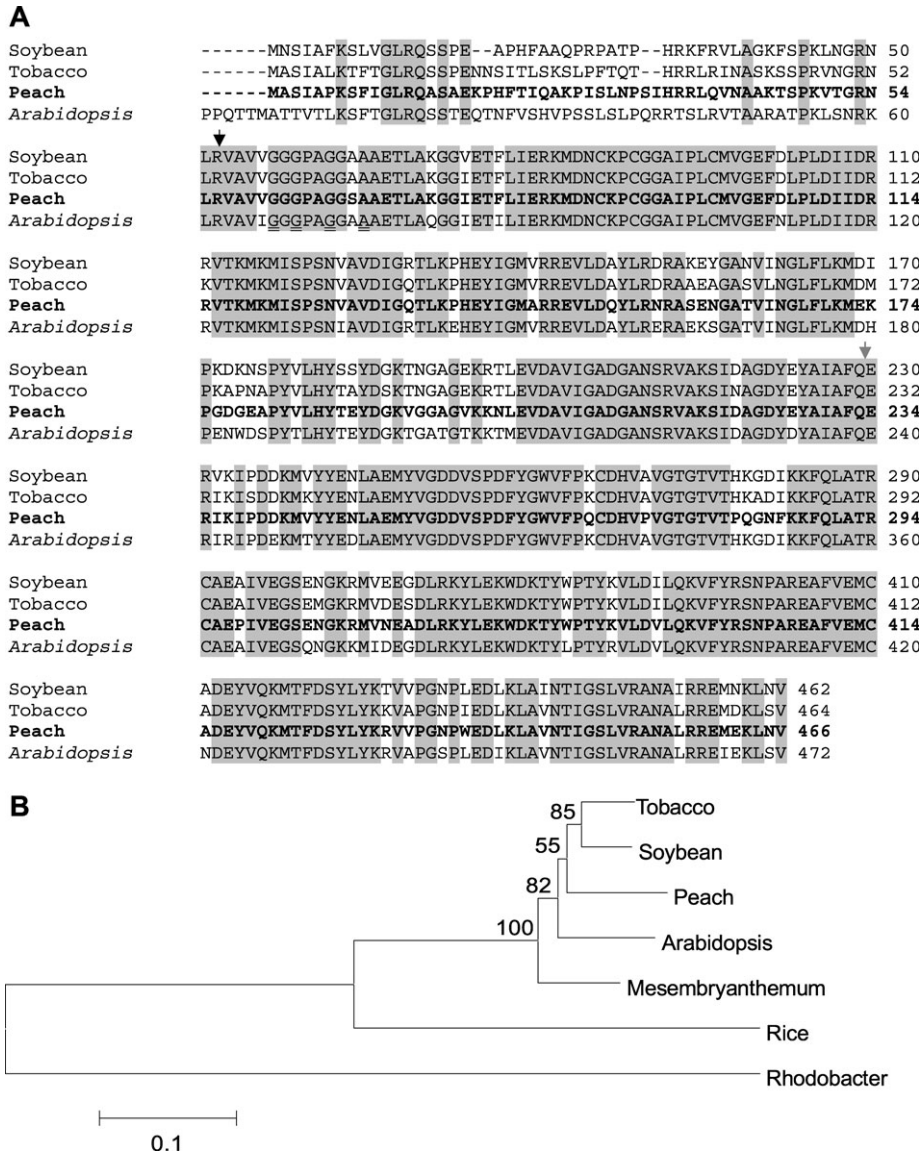

Fig. 1. Features of the protein encoded by *PpCHL P*. (A) Amino acidic alignment of deduced protein *Pp*CHL P in bold (accession no. AY230212) with homologues from other plant species. Strictly conserved amino acids are boxed in grey. The residues of the motif necessary to bind NADPH are double underlined. The black arrow indicates the editing site for the transit peptide. The grey arrow points to the intron position, which is identical to *A. thaliana* and soybean. (B) A phylogram was constructed by the MEGA2 program and clustering analysis performed with the minimum evolution criterion (http://www.megasoftware.net/). Accession numbers of proteins examined: *Arabidopsis thaliana* (AY059860.1); tobacco, *Nicotiana tabacum* (AJ007789.1); soybean, *Gliyne max* (AF068686.3); *Mesembryanthemum crystallinum* (AF069318.1); rice, *Oryza sativa* (AP001080.1); *Rodobacter capsulatus* (Z11165.1). Numbers on the tree indicate bootstraps (1000 replicates), which assign proteins to a clade; the genetic distances are measured by horizontal bars.

Tissue-specific expression

Total RNA was isolated from several tissues of adult plants and hybridized with the probe mentioned above (Fig. 3). At the end of dormancy period (February), a faint signal was revealed in resting vegetative buds, whereas message became abundant in the apical tips of growing shoots (Fig. 3A). No signal band was found in roots (Fig. 3A), though the message could be revealed by RT-PCR (data not shown), indicating a very low level of expression.

The *PpCHL P* transcript was abundant in floral buds before disclosure (February), in sepals, petals, and stamens of open flowers (March) and in green fruits with a diameter of *c.* 3 cm (May), nearly 40 d after flowering (DAF). Mesocarp (pulp) and pericarp (skin) were sampled during fruit maturation (10 cm average diameter) at 110, 118, and 125 DAF. The message (Fig. 3B) was more abundant in fruits at 110 DAF (green pericarp) than in the ripening stages.

PpCHL P *is regulated during leaf development*

Northern analysis was performed on leaves collected from distinct shoots exhibiting a similar length and the same number of internodes. Sampled leaves were then grouped

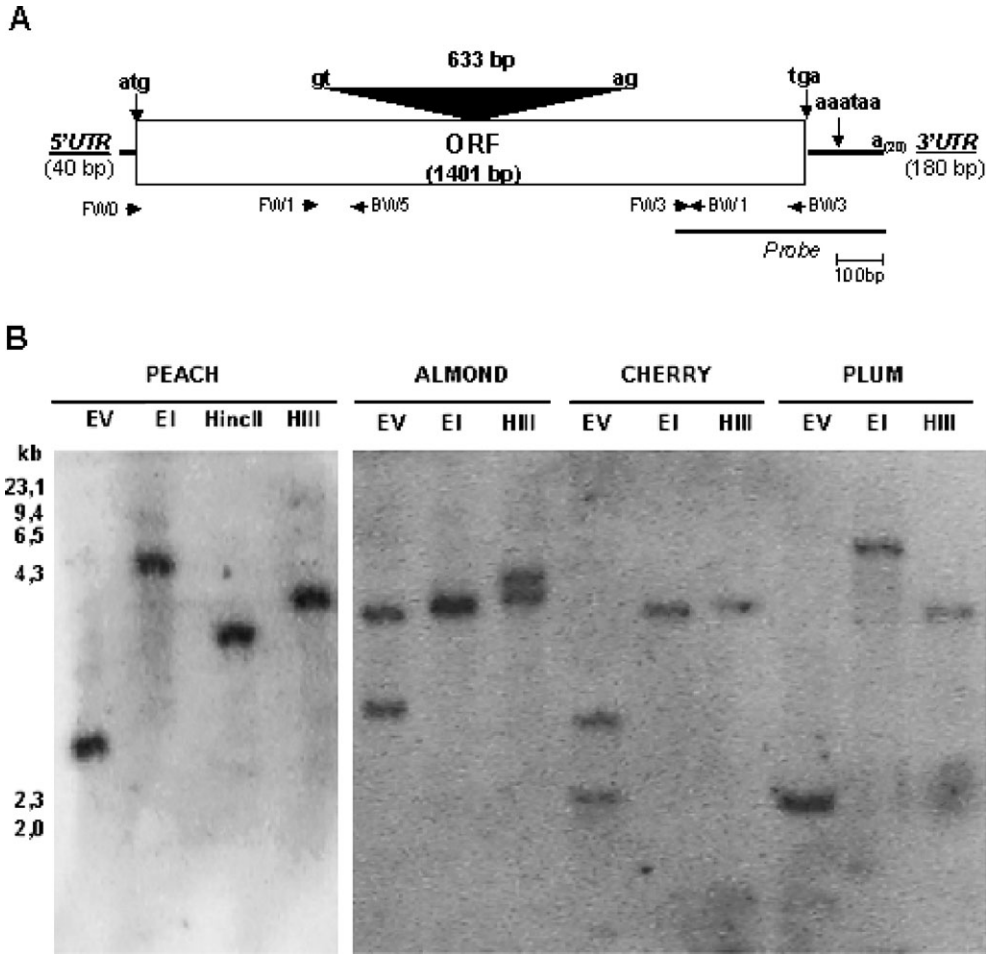

Fig. 2. *CHL P* genomic organization in Drupoideae species. (A) Scheme of the *PpCHL P* gene. Intron length and position are indicated by the black triangle. Start and stop codons and polyadenylation signals are typed. The probe fragment is represented by a black bar. Forward (FW) and backward (BW) directed primers are shown by arrows. UTR, untranslated regions. ORF, open reading frame; bp, base pairs. (B) Genomic DNA was digested with *Eco*RI (EI), *Eco*RV (EV), *Hin*dIII (HIII), and *Hin*cII endonucleases, electrophoresed on 0.8% agarose gel, blotted, and hybridized with the radiolabelled probe indicated above. The molecular weights of a co-migrating DNA marker are in kilo base pairs (kb).

into three classes based on both the length of the mid-vein and blade folding: small and unfolded (up to 5 cm), medium and expanded (between 6 and 12 cm), large and fully expanded (over 12 cm). The *PpCHL P* message was most abundant in large FEL, followed by medium and small ones (Fig. 4A). The message was also localized by *in situ* hybridization in both apical tips of growing shoots, unfolded and fully expanded leaves. As for apical tips, the message was detected in leaves surrounding the apex, but not in the meristematic dome (Fig. 4B). In unfolded leaves, the transcript was uniformly spread in mesophyll cells (Fig. 4C), whereas they were abundant and mainly confined to the palisade cell layer in FEL (Fig. 4D). In both leaf types, message was undetected either in vascular bundles or along the epidermis (Fig. 4C, D).

Response to light and forced dark

The levels of *PpCHL P* transcript were first monitored in leaves of plants kept under a cycle of 16/8 h light/dark (Fig. 5A) and compared with those of large FEL (Fig. 5B) collected from 06.00 h to 21.00 h from plants growing in the open field in June. The expression of the *ribulose biphosphate carboxylase/oxygenase activase* gene of peach (named *Rubisco activase* or *RUBCA*) was also monitored in order to mark photosynthetic activity. *CHL P* transcript decreased within 1 h from the start of darkness, maintaining a steady-state during the 7 h of darkness, but the message abundance was rapidly restored within 1 h of light (Fig. 5A). Outdoors, the message increased from early to late morning and decreased during the evening (Fig. 5B).

Gene expression was also tested in FEL which were obscured by aluminium foil wrapping (see details in the Materials and methods) for 36 h (Fig. 5C). The *CHL P* transcript appeared more abundant in treated than in control leaves, regardless of their position on the shoot. A similar pattern was observed when RNA was probed with a fragment spanning the ORF of peach *CATALASE* genes (*CAT*), used to mark a burst of oxidative stress.

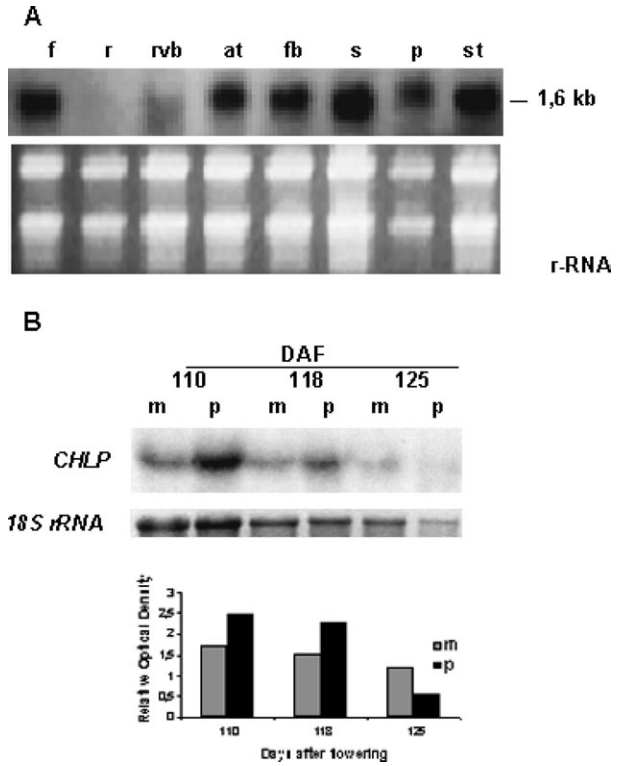

Fig. 3. *PpCHL P* abundance in distinct tissues (A) Northern analysis was performed with the same probe as Southern blots. 1.6 kb (kilobases) indicates the *CHLP* transcript size, estimated by comparison with a co-migrating RNA marker. r-RNA, ethidium-bromide-stained ribosomal RNA was photographed to check for equal loading. f, green fruit (diameter 3 cm); r, root; rvb, resting vegetative bud; at, apical tips (shoot apical meristems and surrounding leaflets); fb, floral buds; s, sepals; p, petals; st, stamens. (B) Top panel: *CHL P* expression during fruit growth. *18S rRNA*, blots were stripped and hybridized with a cDNA probe recognizing 18S ribosomal RNA to check for equal loading. DAF, days after flowering; p, pericarp; m, mesocarp. Bottom: relative optical density to estimate *CHL P* message abundance with respect to *18S rRNA*.

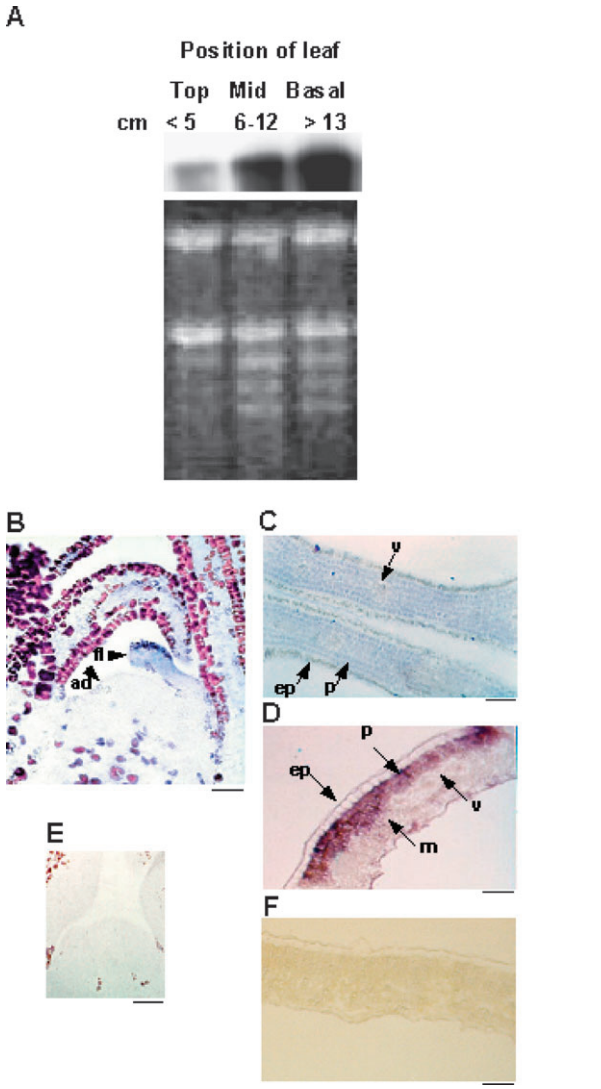

Fig. 4. *PpCHL P* expression during leaf development. (A) Northern analysis was carried out with RNA derived from leaves of diverse sizes borne at the top, middle and distal positions of shoots in June. Leaves were divided into three classes, indicated in centimetres, based on the length of central veins. (B–F) Message localization by *in situ* hybridization in shoot apical meristems and in leaves. A digoxygenin-labelled antisense probe (Fig. 1A) was used. The blue signal is a result of complete tissue dehydration, while the purple signal is after a partial dehydration. (B) Shoot apical meristem during the vegetative season (May); ad, apical dome; fl, forming leaf. (C) Unfolded leaf. (D) Fully expanded leaf; ep, epidermis; m, mesophyll; p, palisade layer; v, vases. (E, F) Control experiments were performed with a probe in the sense orientation on apices and leaves, respectively. Size bars: 40 μm in (B), 80 μm in (C), 65 μm in (D, F), 50 μm in (E).

Response to cold treatment and wounding

One-year-old plants were exposed to 4 °C and the message of *PpCHL P* and *RUBCA* was observed to decrease within 48 h in FEL borne on primary shoots (Fig. 6A).

The blades of FEL were injured by tweezers and sampled after 1, 2 or 4 h (Fig. 6B). The *CHL P* transcript decreased abruptly after the first hour, but it was fully restored within 4 h. The *CAT* message, used as stress marker, followed an analogue trend as *CHL P*, although the level at 4 h was greater than that of non-treated leaves. Small leaves were also gently punctured and *in situ* experiments (Fig. 6C, D) revealed a large abundance of message in the damaged cell areas 24 h after the treatment.

Response to leaf curl disease

The expression of *PpCHL P* was tested in leaves naturally infected by the fungus *Taphrina deformans* and showing distinct degrees of damage (Fig. 7A–C). In severely affected leaves, the gene was poorly expressed compared with healthy leaves (Fig. 7D). Similarly, transcript appeared less abundant in infected than healthy leaf sectors (Fig. 7D). *In situ* experiments were performed on leaf portions which included green and red curly sectors (black box in Fig. 7C). The signal featured in the palisade of intact sectors, it was quite intense in cell areas with lesions and absent in chlorotic sectors (Fig. 7E, F).

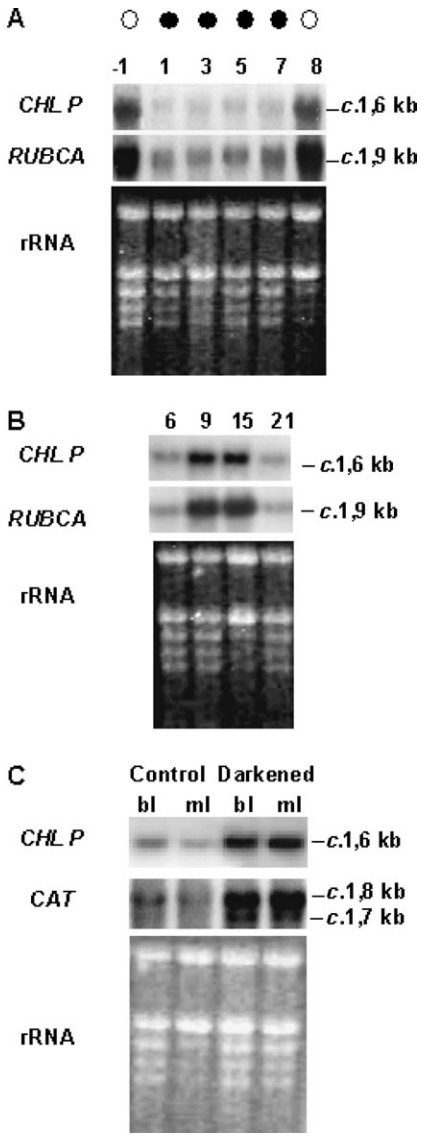

Fig. 5. Leaf response to light and imposed dark. (A) Time-course of *PpCHL P* expression after lights were extinguished. 1–7, hours during the dark (black circles). −1, hour before the dark started; 8, hour after the light restoration (white circles indicate lights on). The expression of *Rubisco activase* (*RUBCA*) was used as the marker for photosynthesis activity. The total RNA loaded was stained by ethidium bromide and transcript sizes are in kilobases (kb). (B) Daily variation of *PpCHL P* message abundance in fully expanded leaves. 6–21, hours of the day when leaves were collected. (C) *PpCHL P* response after 36 h of leaf darkening; *CAT*, transcripts of the *CATALASE* genes used to mark stress response; bl, leaves at basal, ml, at mid-positions of shoots. Control, non-wrapped leaves at 21.00 h.

Discussion

The nuclear *CHL P* of peach harbours an ORF of 1401 bp and encodes a chloroplast targeted polypeptide of 51.68 kDa, while the chloroplastic form is estimated at 45.5 kDa. In the 5′ UTR, a pyrimidine-rich tract (PRT) featured, recalling that of animal 5′ TOP genes (Terminal OligoPyrimidine), which plays a regulatory role during translation (Amaldi and Pierandrei-Amaldi, 1997; Crosio *et al.*, 2000).

As for genomic organization, *CHL P* is a single copy gene in peach and plum just as in *A. thaliana* (TAIR blast no. AT1G74470.1), whereas small member families are not excluded in cherry and almond. The *CHL P* members of the four Drupoideae species share a common high homology and keep the intron position strictly conserved within the group and with respect to *A. thaliana* and soybean (accession no. AAD28640.1). This may have significance in the regulation of gene transcription and suggests a common ancestor for the species. In peach, the occurrence of a single copy is supported by both Southern analysis and the absence of transcript polymorphism of several messages that were sequenced from distinct tissues. Due to its multifunctionality and weak hydrophobicity, a unique CHL P enzyme has been proposed to be recruited in metabolic pathways which occur in distinct plastidial structures (Keller *et al.*, 1998). Thus, subcellular specificity may be conferred by post-translational modification rather than by transcript diversity. In support of this, the transit peptide of the nucleus-encoded chloroplastic LHCP protein is not necessary for intra-organelle positioning, since LHCP itself harbours a signal for membrane-specific localization (Lammpa, 1988).

Gene expression analysis shows that the *PpCHL P* transcript is confined to tissues exposed to light and containing plastids such as chloroplasts and chromoplasts. As for photosynthetic tissues, *PCHL P* appears developmentally regulated since transcripts were detected in forming leaves, but not in vegetative SAMs, and their abundance increased in accordance with leaf size and development. The average content of chlorophyll is related to the size of peach leaves (Merlo and Passera, 1991); therefore, it is likely that the higher level of *PpCHL P* expression in mature as opposed to young leaves could be related to the increase of chlorophyll synthesis and turnover. In addition, message appeared spread thoroughly in mesophyll cells of uncurled leaves, whilst it was abundant and mainly located in the palisade layer of FEL. Overall, these data strongly suggest that *PpCHL P* expression is related to chloroplast maturation and photosynthesis. Consistent with this, *PpCHL P* expression was also triggered by light: message abundance increased from morning to afternoon, diminished with the onset of darkness and was maintained at steady levels during the night. A similar pattern of expression was described for *RUBCA* in apple (Watillon *et al.*, 1993), suggesting that *PpCHL P* expression is regulated by the photosynthetic activity.

In cold-stressed leaves, the *PpCHL P* and *RUBCA* expression diminished. Photosynthetic efficiency is negatively affected in plants exposed to cold, especially if associated with high light (Savitch *et al.*, 2001). However, in this work, plants were not tested under these combined stress conditions, which are also reported to prompt tocopherol synthesis synergistically (Wise and Naylor, 1987).

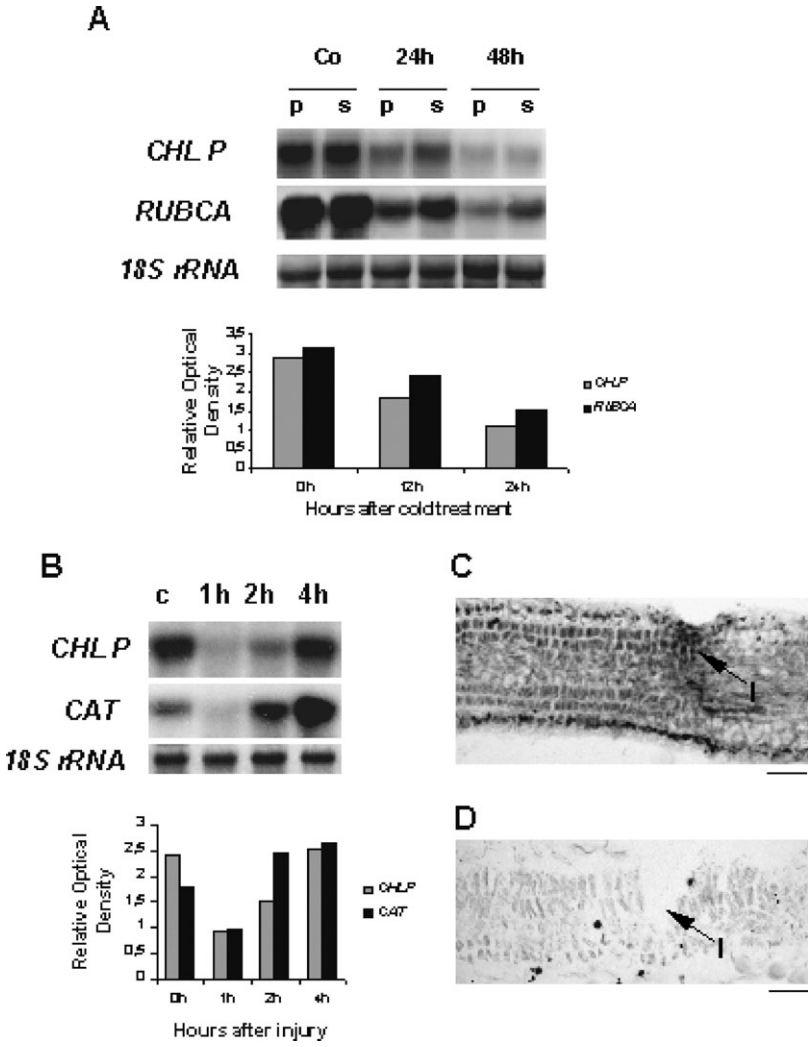

Fig. 6. Leaf response to cold and wounding. (A) Response to low temperature. Top panel: *PpCHL P* expression in fully expanded leaves borne on primary and secondary shoots of young plants after being exposed to 4 °C for 24 h and 48 h. *RUBCA*, *Rubisco activase* expression was used as the control for the photosynthesis trend. Blots were stripped and hybridized with a cDNA probe for 18S rRNA to check for equal loading. Co, non-treated leaves; p, fully expanded leaves of primary and s, secondary shoots. Bottom panel: relative optical density to estimate the *CHL P* and *RUBCA* transcript abundance (referred to 18S rRNA) in leaves borne on primary shoots. (B–D) Wounding response. (B) Top panel: *PpCHL P* expression in fully expanded leaves after 1, 2 or 4 h of tweezer injury. *CAT*, transcripts of *CATALASE* genes; *18S rRNA*, radioactive detection of 18S ribosomal RNA to check for equal loading. Bottom panel: relative optical density of *CHLP* and *CAT* message abundance. (C) Transcript localization by *in situ* hybridization in unfolded leaves which were gently scarified by a needle. l, lesion. (D) Control for the *in situ* experiment with a probe in the sense orientation. Size bars: 20 μm in (C) and 25 μm in (D).

In severely wounded leaves, the *PpCHL P* transcription decreased immediately and drastically, but was fully restored within 4 h. The triggering of the *CAT* transcript also occurred 2 h after treatment, marking the occurrence of oxidative burst phenomena (Guan and Scandalios, 2000). It is speculated that the damage to the photosynthetic apparatus may cause degradation and/or leakage of *Pp*CHL P substrate and activate feedback mechanisms that down-regulate gene expression. However, the restoring of *PpCHL P* expression occurs rapidly within the process of damage repair, which includes *de novo* synthesis of both chlorophyll and side chains of anti-oxidant compounds. In addition, message signals were more intense in injured than unaffected cells after inferring localized scarification to leaves. In plants, the increase in the levels of lipid hydroperoxydes were monitored after wounding up to 120 min and related to cytological events occurring up to 24 h (Fabbri *et al.*, 2000). Hence, the local accumulation of *PpCHL P* transcript may play a role in TP synthesis necessary to protect lipid membranes from peroxidation as observed in photoprotection mechanisms (Havaux *et al.*, 2003).

In aluminium-foil-obscured leaves a slight increase of temperature (*c.* 1.4 °C) was measured, while the *PpCHL P* expression was triggered alongside that of *CAT* compared with controls. In the latter, the *CAT1* message was less

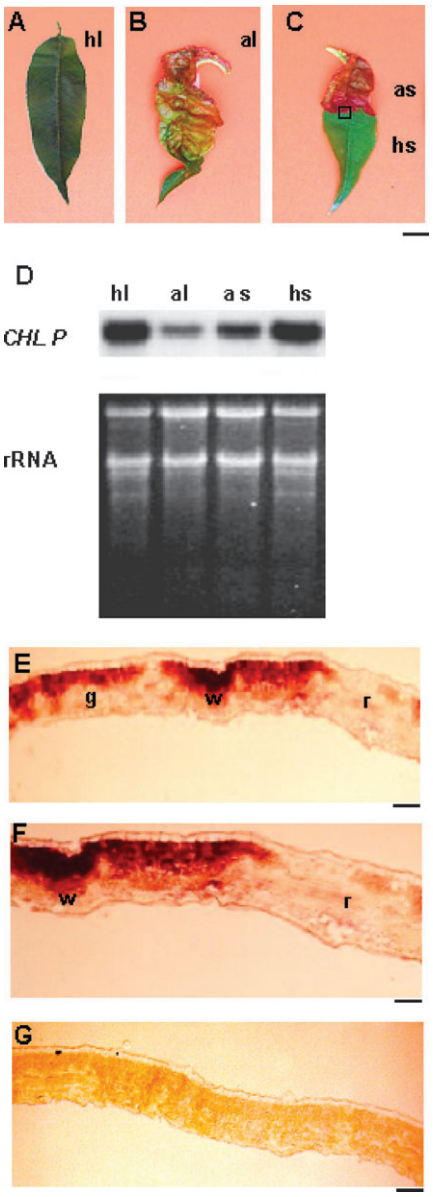

Fig. 7. PpCHL P response in leaves affected by the curl disease. (A) Healthy; (B) severely affected; and (C) partially affected leaf of peach plants; (D) *PpCHL P* transcript abundance by northern analysis. hl, healthy leaf; al, affected leaf; as, necrotic and hs, healthy sectors of partially damaged leaves. (E, F) *In situ* hybridization, the black box in (C) indicates the sectors examined; the partial dehydration of tissues renders *PpCHL P* transcript as a purple signal in (E) and (F), shot at progressive increased magnifications. (G) Control with a dig-labelled probe in the sense orientation. g, green; w, fungus-wounded; and r, red sectors of affected leaves. Size bars: 3 cm in (A), (B), and (C); 65 μm in (E), 55 μm in (F), 65 μm in (G).

abundant at 21.00 h and this was consistent with *CAT1* down-regulation associated with natural dark (Bagnoli *et al.*, 2004). Increased levels of *CAT* were also detected in senescing leaves of *Brassica napus* (Buchanan-Wollaston, 1997), while several stress oxidative enzymes are triggered in mitten-darkened leaves of *Arabidopsis*, which undergo rapid senescence (Weaver and Amasino, 2001). In addition, the oxidative stress associated with plant ageing occurs in chloroplasts, accompanied by decreased levels of chlroplastic β-carotene and α-tocopherol (Munnè-Bosch and Alegre, 2002). In this context, the hypothesis that the *PpCHL P* response may have a role in the synthesis of anti-oxidant compounds during oxidative stress and/or senescence is supported.

In leaves affected by the curl disease, the abundance of the *PpCHL P* message decreases in both chlorotic sectors and in necrotic leaves. This is consistent with the diminishment of chlorophyll *a+b* content (Montalbini and Buonaurio, 1986) and the net reduction of photosynthesis rate documented for infected leaves (Raggi, 1995). However, *in situ* analysis viewing healthy and damaged sectors revealed that transcripts accumulated in damaged as well as in neighbouring cells. These interact with the parasite which is reported to deliver auxin and cytokinin in axenic cultures and to induce the accumulation of IAA and cytokinins in curled leaves (Perley and Stowe, 1966; Johnston and Trione, 1974; Sziràki *et al.*, 1975; Yamada *et al.*, 1990; Bassi *et al.*, 1984). Plant responses to biotic and abiotic stresses exploit overlapping metabolic pathways, in which lipid peroxides, reactive oxygen species, and subsequent metabolites (e.g. jasmonate and traumatic acids) play several roles (Baron and Zambryski, 1995; Ebel and Mithöfer, 1998; Liechti and Farmer, 2002). The *in situ* pattern of the *PpCHL P* transcript at the level of fungus-damaged cells shares many similarities with that observed in punctured cells. These patterns lead to the hypothesis that *PpCHL P* may play a role in the metabolism of antioxidant/defence compounds to protect the membrane lipids of chloroplasts (Grasses *et al.*, 2001; Havaux *et al.*, 2003).

To sustain these hypotheses on *PpCHL P* functions, an antisense technology would be helpful, and virus-induced gene silencing (VIGS) approaches are being tested (Ratcliff *et al.*, 2001). VIGS allows traditional *A. tumefaciens*-mediated transformation to be bypassed, to which peach is more recalcitrant than its related species (Scorza *et al.*, 1990; Gentile *et al.*, 2002). In the authors' laboratories, *PpCHL P* was included in a set of markers which monitor photosynthesis efficiency to select the healthiest clones in programmes of micro-propagation. Moreover, since *PpCHL P* was observed to decrease with cold stress and during fruit maturation, probing varieties' genomes and RNA with *CHL P* may become a useful tool to screen for those genotypes responding best to cold. Finally, the constitutive or tightly controlled expression of exogenous *CHLP* copies via gene transfer technology may impact on the plant response to mechanical injury (e.g. hail), pathogen attacks, and during fruit shelf life.

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