



Research article

Developmental stimuli and stress factors affect expression of *CIGLP1*, an emerging allergen-related gene in *Citrus limon*

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ABSTRACT

Germins and germin-like proteins (GLPs) constitute an ubiquitous family of plant proteins that seem to be involved in many developmental and stress related processes. A novel GLP cDNA was isolated from *Citrus limon* and structural features and genomic organization were investigated by *in silico* and Southern blots analysis. In lemon, the *CIGLP1* encodes a 24.38 kDa which possesses a conserved motif of plant GLPs proteins. A phylogenetic analysis mapped *CIGLP1* as belonging to the GER3 subfamily into the GLP1 group of large GLP family. *CIGLP1* was differentially expressed in the various organs and was highest in mature fruit. Moreover, expression in the fruit was tissue- and stage-related as well as dependent on agricultural practice (organic vs conventional). *CIGLP1* transcripts increased during the transition from the green (180 days after blooming) to the yellow (240 days after blooming) mature fruit and were strongly enhanced in yellow mature fruit from organic compared with conventional culture. A sudden and systemic increase in *CIGLP1* expression level was observed in leaves injured by wounding, together with an increase of endogenous H₂O₂ amount. Notably, an enhancement of H₂O₂ was observed in fruit peel during transition from green to yellow fruit stage. All together our data showed that *CIGLP1* expression can be modulated in relation to both developmental stimuli and culture practices; evidence is also provided that through an oxidase activity this gene could play a role in fruit maturation as well as in stress responses.

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1. Introduction

The species of the *Citrus* genus are among the most important fruit tree crops in the world and their fruits are highly recommended as components of a balanced diet, particularly for their efficacy in reducing the risk of many serious diseases (Vanamala et al., 2006; Benavente-García and Castillo, 2008). The health-promoting effects of *Citrus* fruits are associated with the presence of many important antioxidants, such as flavonoids and carotenoids, but also vitamins, minerals, dietary fiber and essential oils (Proteggente et al., 2002; Wilmsen et al., 2005). Unfortunately, *Citrus* fruits are also recognized as a source of allergenic reactions for humans, due to the production of compounds such as terpenes,

glycans, and glycoproteins (Saarinen and Kajosaari, 1980; Kajosaari, 1982).

So far, three putative allergens have been isolated and characterized in *Citrus sinensis*: Cit s1 belonging to a germin-like protein (Ahrazem et al., 2006), Cit s2 corresponding to a profilin (López-Torrejón et al., 2005) and Cit s3 belonging to a lipid transfer proteins (Ahrazem et al., 2005). Notably, in independent studies, Cit s1 and Cit s2, have been classified as a new class of major allergens in orange fruits (Crespo et al., 2006). Recently, through a proteomic approach, large amounts of a Cit s1 have also been identified in fruit flavedo of [*Citrus limon* (L.) Burm. f.] cv Femminello (Pignataro et al., 2010).

Germins/germin-like proteins (GLPs), represent a large and highly heterogeneous family of ubiquitous plant proteins belonging to the cupin superfamily (Dunwell et al., 2000; Lane, 2000). Despite the considerable sequence heterogeneity between different germins, they all contain localized amino acidic 'signatures' (Lane et al., 1991). Among these, a key feature is a motif that contains three highly conserved histidines and one glutamate residue involved in the binding with metal ions. Germin sequences also include at least one or two putative N-glycosylation sites at conserved positions.

Abbreviations: SOD, superoxide dismutase; OC, organic cultures; CC, conventional cultures; H₂O₂, hydrogen peroxide; YF, yellow fruit; GF, green fruit; Germin and germin-like proteins, GLPs; hours after damage, (HAD); Quantitative real-time PCR, qRT-PCR.

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Further they are typically hexameric, being trimers of dimers (Carter and Thornburg, 2000).

All germins are cell-wall glycoproteins with robust quaternary structures that show an unusual resistance to detergent treatment, heat denaturation, and degradation by proteases (Carter and Thornburg, 1999; Bernier and Berna, 2001). On the basis of their localization in the extracellular matrix they were assumed to be structural proteins and play a role in response to environmental stress (Bernier and Berna, 2001; Park et al., 2004; Zimmermann et al., 2006; Godfrey et al., 2007; El-Sharkawy et al., 2010; Druka et al., 2002; Davidson et al., 2010; Manosalva et al., 2009; Breen and Bellgard, 2010). However, published data indicate other functions: epidermal specific proteins (Wei et al., 1998), rhicadhesin receptors (Swart et al., 1994) and low-affinity auxin-binding proteins (Ohmiya et al., 1993). Other members of the germin family have catalytic properties: oxalate oxidase (OXO) (Lane et al., 1993; Le Deunff et al., 2004), superoxide dismutase (SOD) (Carter and Thornburg, 2000; Godfrey et al., 2007; Yamahara et al., 1999; Tabuchi et al., 2003; Christensen et al., 2004) and phosphodiesterase (Rodriguez-Lopez et al., 2001). Notably, OXO and SOD activities generate hydrogen peroxide (H_2O_2) a well-known cofactor for both cell wall metabolism and restructuring via either oxidative scission of polysaccharides (Fry, 1998) and/or direct macromolecular cross-linking by peroxidases. Moreover, H_2O_2 , also acts as a signaling molecule for a range of defense reactions (Yamahara et al., 1999; Christensen et al., 2004; Laloi et al., 2004).

Phylogenetic analysis of GLPs from different plants species, associated to functional annotation, proposed a subdivision of this large protein family into different groups which in turn include different subfamilies: from GER1 to GER6 subfamilies [19,23–25 and reference herewith cited]. The GER1 lineage includes proteins, also referred to in the literature as “true germins”, which exhibit OXO activity, a catalytic property unique to this clade. Large diversity of possible functions and enzymatic activities, that do not includes OXO activity is associated to the other GER subfamilies [23 and reference herewith cited].

According to the wide variety of roles proposed for GLPs in planta, GLP genes are expressed in the various organs of plants and are modulated in response to both developmental stimuli and abiotic/biotic stresses (Park et al., 2004; Zimmermann et al., 2006; Godfrey et al., 2007; Davidson et al., 2010; Manosalva et al., 2009; Breen and Bellgard, 2010). However despite the putative allergenic activity of GLP, the modulatory effects of either agricultural or environmental factors on the expression pattern of corresponding genes have not yet been fully investigated. Here, we report the structural features and organ-specific expression of *CiGLP1* (*Citrus limon* *GLP1*) gene, characterized in *Citrus limon* cv Femminello. Gene expression pattern was investigated also in relation to fruit developmental stage and culture practice as well as under stress exposure. We found that *CiGLP1* was modulated in relation to fruit maturation and enhanced by organic agricultural practices and wound stress application. The implication of these results for the implementation of a strategy for lowering putative allergen amount in plant products is discussed.

2. Materials and methods

2.1. Plant material

Lemon [*Citrus limon* (L.) Burm. F.] plants from organic (OC) and conventional (CC) culture (according to the Italian Ministers for Agricultural Food and Forestry Policies ART. 5) were used. In particular, the OC is based on the use of natural compounds for plant nutrition, without using organic fertilizers. Furthermore in OC

practices plant defense is helped by natural pesticides and through a natural balance between beneficial insects and pests.

Both OC and CC plants were growing in the area of Rocca Imperiale, Calabria, Italy at: altitude 588 m, latitude 40°06'17.08"N, longitude 16°34'50.83 E and altitude 343 m, latitude 40°06'38.62"N, longitude 16°34'52.27"E, respectively. However, during the sampling period several environmental parameters were recorded in the two culture fields and the mean values did not vary significantly between them (data not shown).

Herbaceous or woody stem portions, fully expanded leaves with petiole, vegetative apical tips (shoot apical meristem and surrounding leaflets) were sampled from OC plants ($n = 10$) during spring (2009–2011). For all the samplings, branches exhibiting similar length and the same number of internodes were selected. Collected samples were rapidly frozen and stored (-80°C).

Green (GF) and yellow (YF) fruits were collected from both OC and CC plants at 180 and 240 days after blooming (DAB), respectively (Cercos et al., 2006). Fruits were collected from all around the external parts of the tree canopy; fruit peel corresponding to the pericarp (i.e. flavedo + albedo) and pulp (endocarp) were separately frozen and stored (-80°C).

2.2. Stress assay conditions

For both wounding and H_2O_2 treatment, OC plants growing in open field were used.

Wounding: Using a sterile tweezer two/three cuts were performed on the half basal part of adult leaves. For gene expression analysis, injured and uninjured leaves ($n = 20$ for each sample) were collected from ten different plants at 0.10, 0.20, 0.30, 1, 2, 4, 6, 8, 10, 12, 24 h after damage (HAD). Basal and apical portions (at least 10 mm from wounded area) of sampled leaves were frozen and stored separately (-80°C). For H_2O_2 quantification, whole injured and healthy leaves ($n = 10$ for each sample) were sampled at the same HADs and immediately processed as described in the following 4.3 section.

H_2O_2 treatment: Detached unwounded leaves ($n = 10$ for each sample) were sprayed four times with a solution of 100 mM H_2O_2 in 0.05% (v/v) Tween 20 at an interval of 2 h. GF and YF fruits ($n = 10$ for each sample) sprayed with Tween 20 solution alone were used as control (Zimmermann et al., 2006). No macroscopically visible tissue damage was induced by H_2O_2 treatment. Samples were collected, rapidly frozen and stored (-80°C) for gene expression analysis.

2.3. H_2O_2 quantification

Collected samples were homogenized in liquid nitrogen with a pellet disrupter. The homogenized samples (0.2–0.3 g each) were mixed with 1 mL of deionized water and the supernatants collected by microcentrifugation of the extract. H_2O_2 concentration was determined using an Amplex Red Hydrogen Peroxide/Peroxidase Assay kit according to the manufacturer's instructions (Invitrogen, Milan, Italy). For each sample, three replicates were sampled.

2.4. RNA isolation and reverse transcription

Total RNA was isolated from 100 mg of plant tissues and processed with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

RNA was treated with DNase I (Roche, Milan Italy) to remove contaminating genomic DNA. DNase I was inactivated at 70°C for 15 min. RNA was precipitated and finally resuspended in 40 μL of RNase-free water. RNA quality was checked by agarose gel electrophoresis and quantified by spectrophotometry (NanoDrop

technologies). Total RNA (3 µg) from each sample was interacted with SuperScript III Reverse Transcriptase and oligo dT(22) for cDNA synthesis, according to the manufacturer's instructions (Invitrogen, Milan, Italy).

2.5. Isolation and sequence analysis of putative GLP1 from *Citrus limon*

cDNA from leaves and fruits were PCR amplified using primers: FW0 (5'-TTATAGGTTTGGCCTCTTG-3') and BW0 (5'-TTATTACATGGCCACAGTCAGC-3'), which were selected from available ESTs for *Citrus sinensis* (<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=orange>).

PCR yielded a 480 bp product, which was homologous to *Citrus sinensis* 1-type GLPs. 5' and 3'RACE was used to obtain the full length gene. cDNA amplification of the full length cDNA was obtained using the primers: FWStart (5'-ATGAAGGTAATTCAATTCT-3') and BWend (5'-CTAATTGCCATTAATGAAC-3') and re-sequencing confirmed the fragment assembly. The 675 bp products were cloned and several of them were sequenced, and nucleotide differences were not encountered, indicating that the same gene was expressed in these tissues.

Introns were located by amplifying leaf genomic DNA (gDNA) with the primers: FWStart/BWend. The sequences of products from cDNA and gDNA, were aligned by ClustalW (<http://www.ebi.ac.uk.clustalw>) and intron size and location was established.

PCR components were: cDNA (100 ng) or gDNA (500 ng), each primer (0.2 µM), dNTPs (0.5 mM), Taq DNA polymerase (2.5 U Go Taq, Promega, Milan, Italy), Taq Buffer (1X), MgCl₂ (2.5 mM), in a final volume of 50 µL. PCR conditions: start cycle at 95 °C for 4 min; 35 cycle at 95 °C for 60 s, 55 °C for 50 s and 72 °C for 60 s, final extension at 72 °C for 7 min. All PCR fragments were cloned into pGEM-T Easy vector system according to the manufacturer's instructions (Promega) and sequenced by the Genechro ENEA service (Rome, Italy). The GenBank accession number of *CIGLP1* cDNA and genomic sequences are JQ941709.

2.6. Alignments and phylogenetics analysis

C. limon GLP1 sequences were aligned with other GLP proteins using ClustalW (<http://www.ebi.ac.uk.clustalw>) and optimized by visual inspection (Bioedit software). A phylogenetic tree was constructed in MegaBlast3 (based on the minimum evolution criterion), using bootstrap values performed on 1000 replicates.

2.7. Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed using a STEP ONE instrument (Applied Biosystems). Primers used for qRT-PCR analysis were designed using Primer3 software according to Yokoyama and Nishitani (2001). Experimentally, optimal primers were identified based upon their ability to meet several standards: (a) robustness: successful amplification over a range of annealing temperatures, (b) specificity: generation of a single significant peak in the melting curve, and (c) consistency: highly reproducible Ct values within the reactions of a triplicate. The primers used for *CIGLP1* were 4R Left Cits1 (5'-CATTCAGTTGGACCAAAG-3') and 4R Right Cits1 (5'-CAGTCAGCTGGAGAGAGGT-3'). The average amplification efficiency of each primer pair was determined, and primers performing poorly were replaced. The average efficiency of all of the primer pairs discussed in this study ranged between 0.95 and 1.0. After checking independent trials of several housekeeping genes, *Elongation Factor 1 alpha* (*EF1*) produced the most reproducible results across various cDNAs, and was used as a

normalization control. The primer sequences of *EF1* (AY498567) are as described by Distefano et al. (2008).

Amplification reactions were prepared in a final volume of 20 µL: Power SYBR Green PCR Master Mix (Applied Biosystems) (2X), dATP-dCTP-dGTP (0.4 mM) and dUTP (0.8 mM), each primers (0.4 µM) and cDNA (25 ng). All reactions were run in triplicate in 48-well reaction plates, and negative controls were included. The cycling parameters were as follows: one cycle at 95 °C for 3 min to activate the *Taq* enzyme, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing-extension at 60 °C for 30 s. After the reaction, in order to confirm the existence of a unique PCR product, the 'melting curve' (Lekanne Deprez et al., 2002) was evaluated by an increase of 0.5 °C every 10 s, from 60 °C to 95 °C. A unique 'melting peak' was obtained in every reaction and the PCR products were verified by 1% agarose gel electrophoresis.

The results were analyzed using STEP One Software 2.0 (Applied Biosystems), using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Mean *CIGLP1* expression levels were calculated from three biological replicates, obtained from three independent experiments.

2.8. Southern blot

Total DNA was isolated from lemon leaves frozen in liquid nitrogen, by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Southern blots and hybridizations were performed as described in Bruno et al. (2009). Briefly, 10 µg of gDNA was restricted overnight at 37 °C with *DraI*, *EcoRI* and *EcoRV* endonucleases (Promega), which do not cut in the probe (see Fig. 2A), in a 200 µl final volume.

Digested DNA was precipitated, resuspended and electrophoresed for 20 h at 45 V on 0.8% agarose gel and vacuum blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech). Digoxigenin (DIG)-labeled probe 1 (see Fig. 2A) was obtained after PCR amplification using 10 ng of cDNA, DIG-dNTPs (Roche) and primers FWS (5'-TCTTTGGCCTCTGCCACTGA-3')/BWS (5'-ATTGGAAGTGAATCAAACCT-3').

Digoxigenin (DIG)-labeled probe 2 was obtained after PCR amplification using 30 ng of DNA and primers FWS (5'-TCTTTGGCCTCTGCCACTGA-3')/BWS1 (5'-CCTCTTTTATTACATTAATTA-3') according to the manufacturer's instructions. Prehybridization (3 h at 58 °C) and hybridization (overnight at 58 °C) were carried out in 20X SSC (3 M NaCl, 300 mM sodium citrate), 0.01% sarkosyl (10%), and 0.1% blocking reagent (Roche, Milan) (2.5% prepared in maleic acid buffer, pH 7.5) in a hybridizer (HB-2D, Techne). Detection was performed with anti-DIG-AP (Roche), nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) following the manufacturer's instructions (Roche). Hybridization bands were visualized by keeping the membrane in the dark for 2 h (blocked with Tris-EDTA (50 mM Tris-HCl, pH 8.0, 5 mM EDTA) and then computer scanned.

3. Results

3.1. The lemon GLP1 full-length cDNA and its predicted product

CIGLP1 full-length cDNA (880bp) encompassed an open reading frame (ORF) of 672 bp which encodes 224 amino acid residues. The molecular mass and the isoelectric point (pI) of the predicted encoded protein were 24.38 kDa and 8.82 pI, respectively (ExpASY prediction server (<http://web.expasy.org/translate/>)).

The deduced protein exhibited a high degree of amino acid sequence identity with known GLP homologues from a variety of plants, the highest being 93% homology to *Citrus sinensis* CsGLP1. Multiple alignments of the *CIGLP1* amino acid sequence with other reported GLPs indicated a number of conserved motifs and

The predicted CIGLP1 protein also contains three highly conserved oligopeptides of unknown function which are characteristic of GLPs: boxes A, B, C (Lane et al., 1991; Bernier and Berna, 2001). Box-A contains a conserved cysteine residue (Cys-32), believed to form an internal disulphide bond with a second external cysteine (Cys-49) and to stabilize the protein structure (Woo et al., 2000). Box B (GQRPPHIHPRASEIFVLVLE) and box C (QGLIHVFQFNIG), contain three histidines (His-111, His-113, His-160) and a glutamate (Glu-118) residue involved in binding heavy metal ions (Dunwell and Gane, 1998; Woo et al., 1998). Consistent with GLPs from other plant species (Carter et al., 1998), a single potential N-glycosylation site (Asn-70) was identified in the deduced protein (Fig. 1A). Another important feature of GLPs, present in CIGLP1, is the KGD tripeptide motif sequence, thought to be involved in protein–protein interactions (Fig. 1A) (Laboure' et al., 1999).

3.2. ClGLP1 genomic organization

CIGLP1 harbored one intron (Fig. 2A), which was AT rich (65% on average) and possessed GT/AG or GC/AG splice sites (Kitamura-Abe et al., 2004). The Southern blot analysis on genomic DNA digested with three different enzymes (Fig. 2B) was first carried out with the *CIGLP1* cDNA probe 1 (Fig. 2A) which encompassed the BOX A, B, C conserved domains of Cupins. The hybridization pattern suggested that several members of *GLPs* exist in the lemon cv Femminello genome (Fig. 2B, left panel). Subsequently, genomic probe 2 was

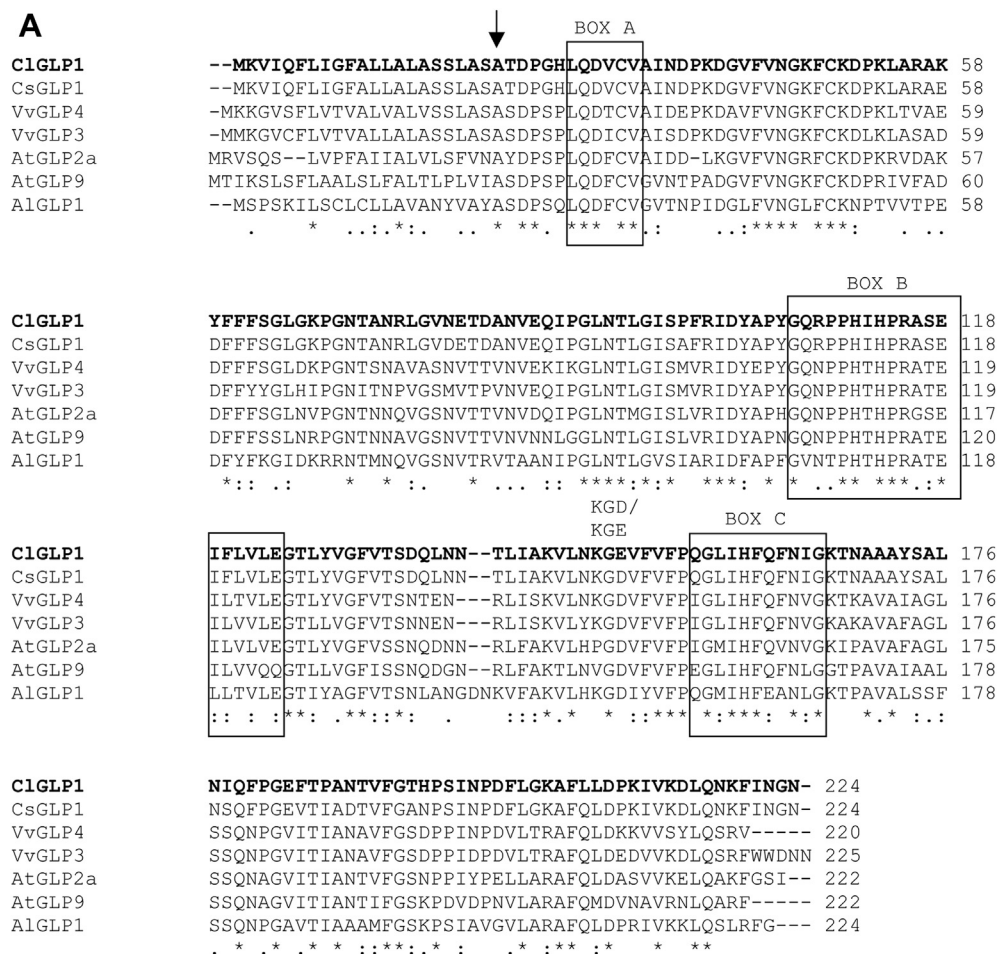


Fig. 1. Features of CIGLP1 protein and its genealogy. (A) Amino acid alignment of predicted protein CIGLP1 (bolded) with homologues from other plant species (Supplemental table). Gaps introduced for better alignment are shown by dashes, asterisks indicate strictly maintained residues. The conserved germin motif, box A, B, and C, are indicated with box. The black arrow indicates the editing site for the transit peptide. Predicted N-glycosylation site for CIGLP1 (N70) is underlined. Putative arginine-glycine-asparagine-like tripeptide are labeled as KGD/KGE. (B) Phylogenetic tree of selected plant germin-like proteins (GLP). A monocupin *Methacoccus jannaschii* protein was used to create an outgroup. Deduced protein sequences were aligned by the multiple alignment program ClustalW. The MEGA3 program was used for the construction of phylogenetic trees. GLP genes from *Citrus limon* are boxed.

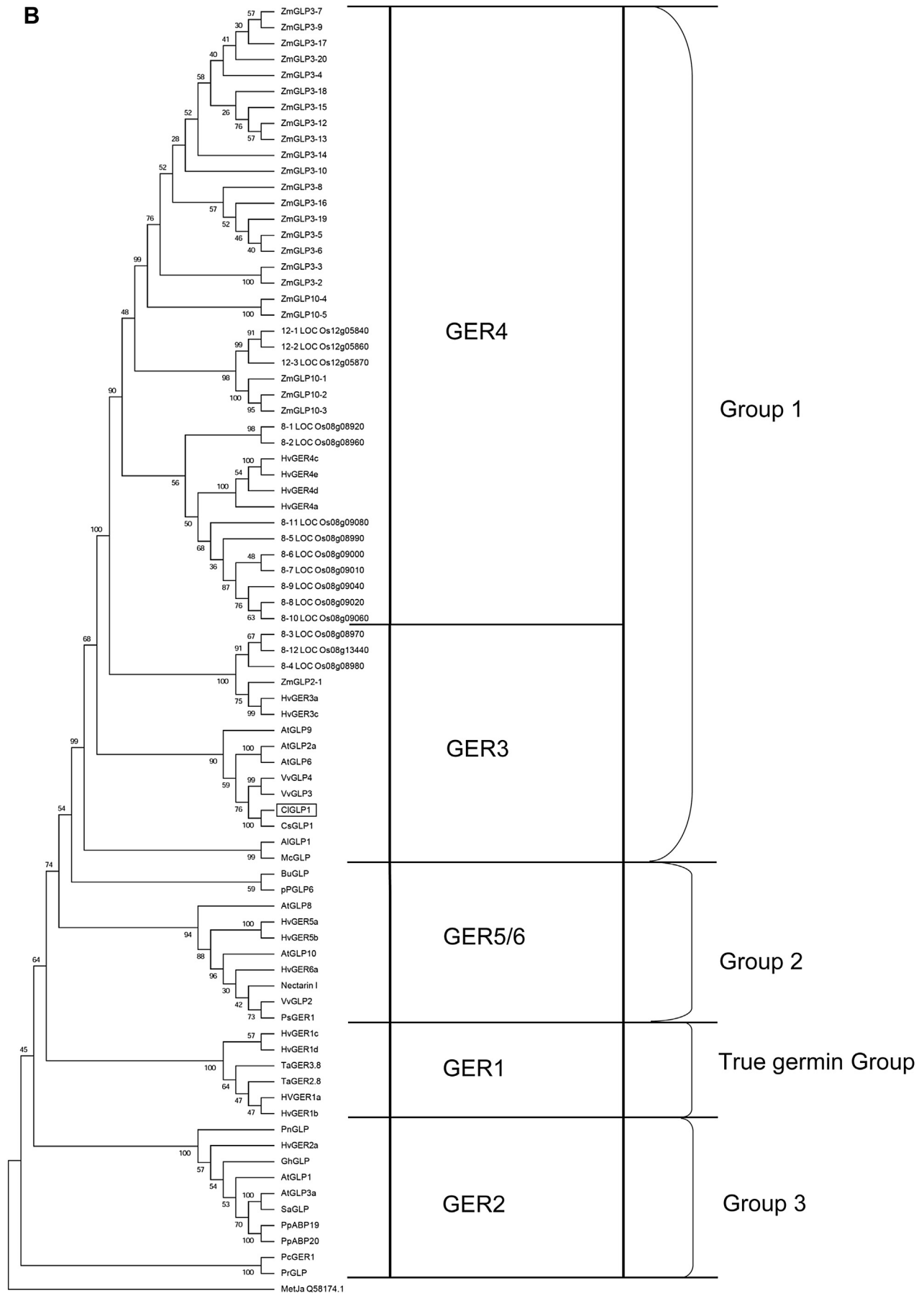


Fig. 1. (continued).

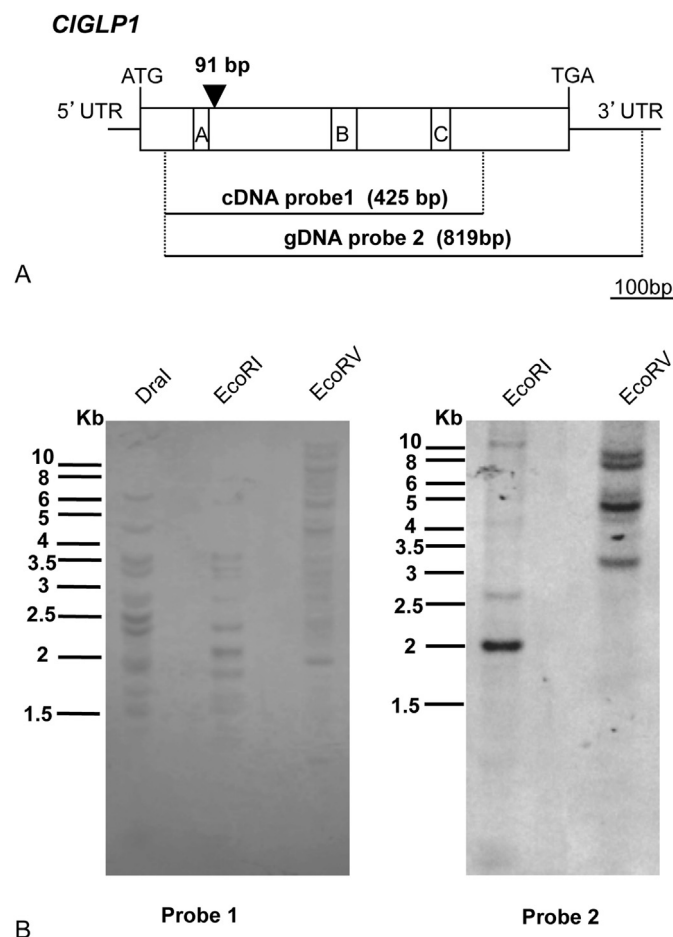


Fig. 2. *CIGLP1* genomic organization. (A) Scheme of lemon *GLP1* gene. The black triangle indicates intron position and size (in base pairs, bp) with respect to the cDNA. Start and stop codons are typed. The cDNA probe 1 spans Box A-C domains, whilst the genomic probe 2 includes intron and a portion of the 3'UTR untranslated regions. (B) Southern blot analysis. gDNA of *Citrus lemon* digested with: *DraI*, *EcoRI*, *EcoRV* and hybridized with cDNA probe 1 (left panel); gDNA of *Citrus lemon* digested with *EcoRI* and *EcoRV* and hybridized with cDNA probe 2 (right panel). The molecular weights of a co-migrating DNA marker are in kilo base pairs (Kb).

used (Fig. 2A) spanning a stretch downstream of BOX A to the 3' UTR. Four signal bands were observed (Fig. 2B, right panel) and their hybridization pattern indicated that there are at least three copies of the *CIGLP1* gene per haploid genome.

3.3. *CIGLP1* expression is differentially expressed in plant organs

qRT-PCR analysis was used to determine *CIGLP1* expression in the different organs of plant.

Transcript accumulation was found to be low in woody stem (WS) and fully expanded leaf (EL), while it was progressively higher in petiole (P), vegetative apical tip (AT), herbaceous stem (HS) and peaked in YF fruits (Fig. 3).

3.4. In fruit tissues *CIGLP1* expression is modulated in relation to maturation stage and culture practice

To further characterize the expression profile of *CIGLP1* in the fruit, we used qRT-PCR analysis to evaluate gene expression level separately in peel and pulp of OC fruits at two different developmental stages: immature (GF, 180 DAB) and mature (YF, 240 DAB). In GFs, relatively low *CIGLP1* mRNA levels were detected in the peel while gene expression was undetectable in the pulp (Fig. 4A). In

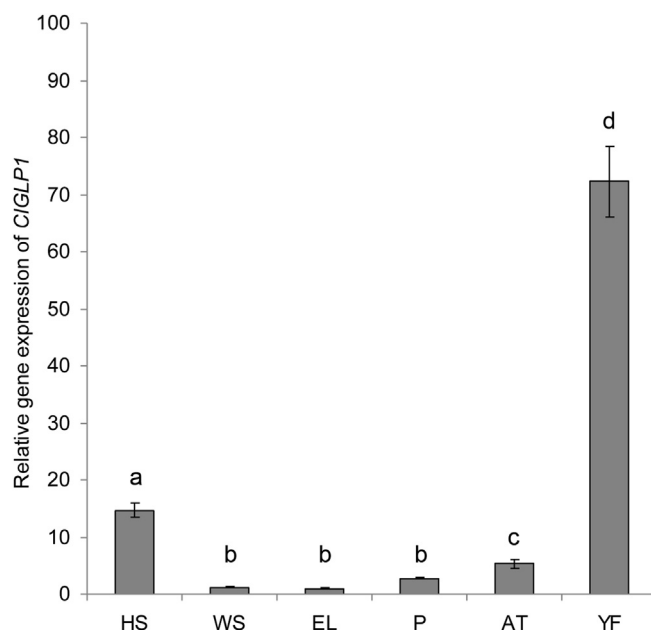


Fig. 3. Levels of *CIGLP1* expression in various organs of lemon evaluated by qRT-PCR analysis. The results are presented as a proportion of the highest value after normalization with respect to *elongation factor 1-alpha (EF1)* expression level. The results represent the average value of three independent biological replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Turkey's Honestly Significant Difference (HSD) test. HS, herbaceous stem; WS, woody stem; EL, fully expanded leaf; P, petiole; AT, vegetative apical bud and YF, mature fruit.

YFs, a prominent 12- and 175-fold increase of *CIGLP1* transcript level was detected in peel and pulp respectively (Fig. 4A).

To determine whether expression level of *CIGLP1* could be influenced by different culture practices, *CIGLP1* expression was examined in peel and pulp of mature YFs produced from conventional culture compared with organic culture (Fig. 4B). A lower level of *CIGLP1* transcript was detected in both peel and pulp tissues of fruits grown under conventional culture compared with those from organic culture (3.6- and 3.9- fold decrease) (Fig. 4B).

3.5. Do oxidative conditions modulate *CIGLP1* expression?

Based on the putative superoxide dismutase activity of predicted *GIGLP1* protein, as indicated by *in silico* and phylogenetic analysis (Fig. 1A, B), *CIGLP1* expression was investigated in response to wounding and related to either endogenous level or exogenous supply of H_2O_2 .

Gene expression was monitored in wounded leaves at different time intervals, from 0.10 to 24 HAD, compared with un-wounded leaves, used as control (Fig. 5). To verify whether the wounding signal was transduced systemically, basal and apical portions of the same leaf were separately analyzed. It was evident that in the basal and apical portions of control leaves *GLP1* transcripts accumulated at quite comparable level (Fig. 5). A sudden and strong increase of *CLGLP1* expression (8 fold change, at 0.20 HAD) was detected in the basal portion of wounded leaves compared to the un-wounded control leaves (Fig. 5). Starting from 1HAD, transcript level progressively decreased and became almost comparable to control value at 8 HAD (Fig. 5). While, in the apical portion (10 mm from the wounded area), there was a less pronounced and progressive increase of transcripts from 1 until 6 HAD (3.5 fold change), followed by a decrease to the control value at 10 HAD (Fig. 5).

To relate gene expression with oxidative conditions, endogenous H_2O_2 was measured in both control and wounded leaves at

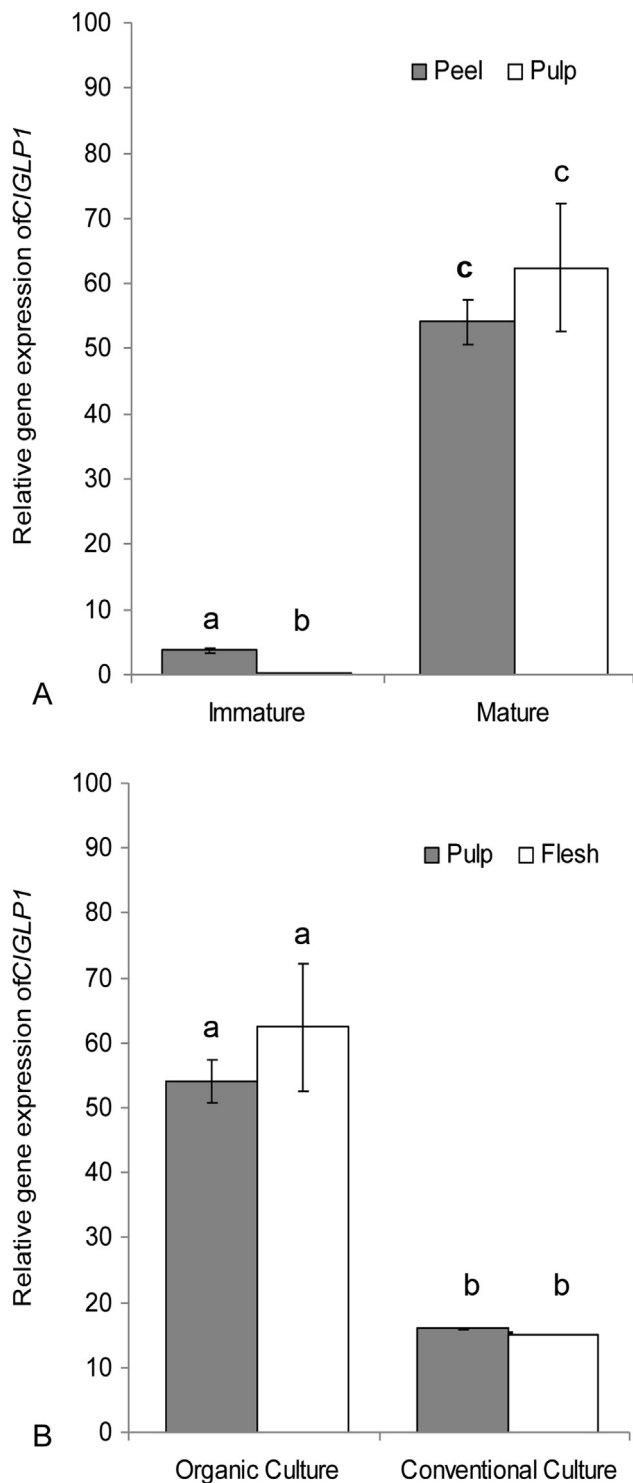


Fig. 4. Levels of *CIGLP1* expression in fruit of lemon evaluated by qRT-PCR analysis (A) Relative expression of *GIGLP1* gene in different tissues of immature and mature lemon fruits from organic culture. (B) Relative expression of *GIGLP1* gene in different tissues of mature fruits from different culture practice: organic vs conventional culture. The results are presented as a proportion of the highest value after normalisation with respect to *elongation factor 1-alpha* (*EF1*) expression level. The results represent the average value of three independent biological replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Turkey's Honestly Significant Difference (HSD) test.

the same HADs above described as well as in the peel of GF and YF (Fig. 6). A transient and bifasic increase of endogenous H_2O_2 amount was detected in the leaf after wounding. In particular H_2O_2 started accumulating at 0.20 HAD, reaching the maximal accumulation at 1HAD; thereafter H_2O_2 amount progressively decreased until 4HAD and picked again at 6HAD, lowering to initial value within 12 HAD (Fig. 6A). Likewise, a higher amount of endogenous H_2O_2 was detected in the peel of YF compared with GF fruits (Fig. 6B). *CIGLP1* expression was evaluated also in unwounded leaves sprayed with H_2O_2 . The results showed that in healthy tissues gene expression level did not change following this treatment (Fig. 1S).

4. Discussion

Comparison of the full length cDNA sequence isolated from *Citrus limon* cv Femminello with the germin family indicated that the *CIGLP1* gene belongs to this family of recently identified *Citrus* allergens present in a taxonomically wider range of species including both monocot and dicot species (Godfrey et al., 2007; Davidson et al., 2010; Manosalva et al., 2009; Breen and Bellgard, 2010).

The Southern blot results with a probe encompassing Cupin genomic DNA supported the presence of a multigene family also in the 'Femminello' genome, while the hybridization pattern with a probe corresponding to *CIGLP1* 3'UTR indicated that the least three copies of identified gene is likely to be present x haploid genome. However, the absence of any nucleotide polymorphism in the cloned PCR products of the full length cDNA, strongly suggests that the three copies of *CIGLP1* are due to a recent duplication event.

The predicted *CIGLP1* protein exhibited the typical and highly conserved signature elements of this protein family. Phylogenetic analysis showed that *CIGLP1* belongs to GER3 subfamily into the GLP1 group, for which superoxide dismutases (SOD) activities have been verified in other species (Carter and Thornburg, 2000; Godfrey et al., 2007; Yamahara et al., 1999; Tabuchi et al., 2003; Christensen et al., 2004). Moreover, within GER3 subfamily *CIGLP1* exhibited the highest homology with Os 8-12, Os 8-4 and Os 8-3 of rice, that were found to be involved in stress responses (Davidson et al., 2010).

In line with the expression pattern of other homologous *GLP* genes (Park et al., 2004; Godfrey et al., 2007; El-Sharkawy et al., 2010; Himmelbach et al., 2010), *CIGLP1* was differentially expressed in plant organs. A very high level of *CIGLP1* transcripts was detected in the mature YFs. Note that the development of citrus fruit occurs in three clear-cut stages (Bain, 1958). In the earliest phase (phase I) fruit growth is slow through cell division. Thereafter, in the rapid growth period (phase II) fruit increases in size by cell enlargement and water accumulation. Finally, growth is mostly arrested and fruit undergoes a non-climateric ripening process (phase III) marking the conversion of chloroplasts to chromoplasts, a change influenced by both nutrient availability and hormones, in particular ethylene (Iglesias et al., 2001). In the present work a strong increase of *CIGLP1* expression was detected in the fruit of *Citrus limon* cv Femminello at the ripened YF stage compared with GF stage. This result is in line with the up-regulation of *Ps-GLP1* and 2 genes detected during fruit ripening in *Prunus salicina*, (El-Sharkawy et al., 2010). Moreover, the up regulation of *CIGLP1* in lemon YFs was associated with high H_2O_2 production. Notably our phylogenetic analysis indicated putative SOD activity for the deduced protein. Therefore, *CIGLP1* up-regulation which occurs during fruit ripening may well be related to cell wall restructuring and loosening via oxidative scission of polysaccharides (Fry, 1998). Consistently, an involvement of GLP in cell wall remodeling has been reported during somatic embryogenesis in hybrid larch (*Larix kaempferi* x *Larix decidua*) (Mathieu et al., 2006).

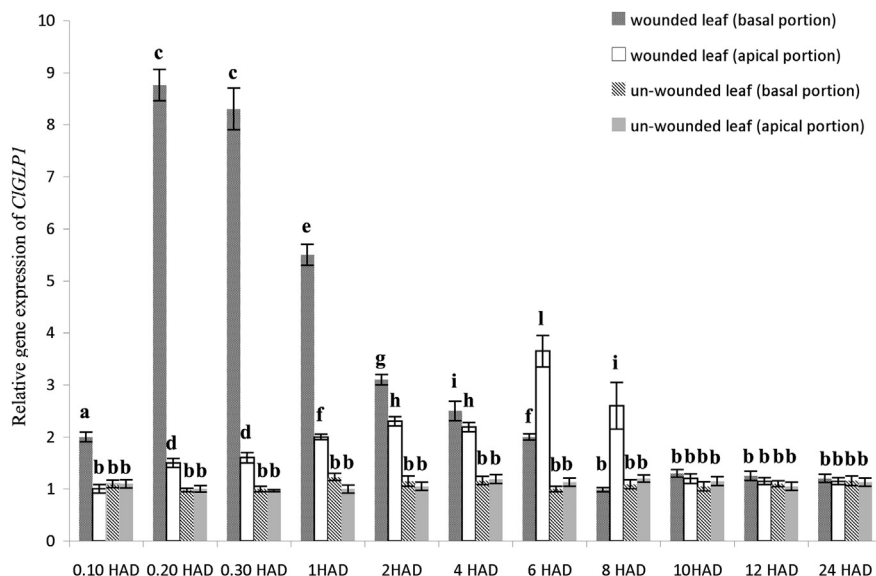


Fig. 5. Time-course of *CIGLP1* expression levels analyzed by qRT-PCR in the apical and basal portion of *Citrus limon* mature leaves at different hours after wounding (HAD). Un-wounded mature leaves were also analyzed at comparable times and used as control. The results are presented as a proportion of the highest value after normalisation with respect to *elongation factor 1-alpha (EF1)* expression level. The results represent the average value of three independent biological replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Turkey's Honestly Significant Difference (HSD) test.

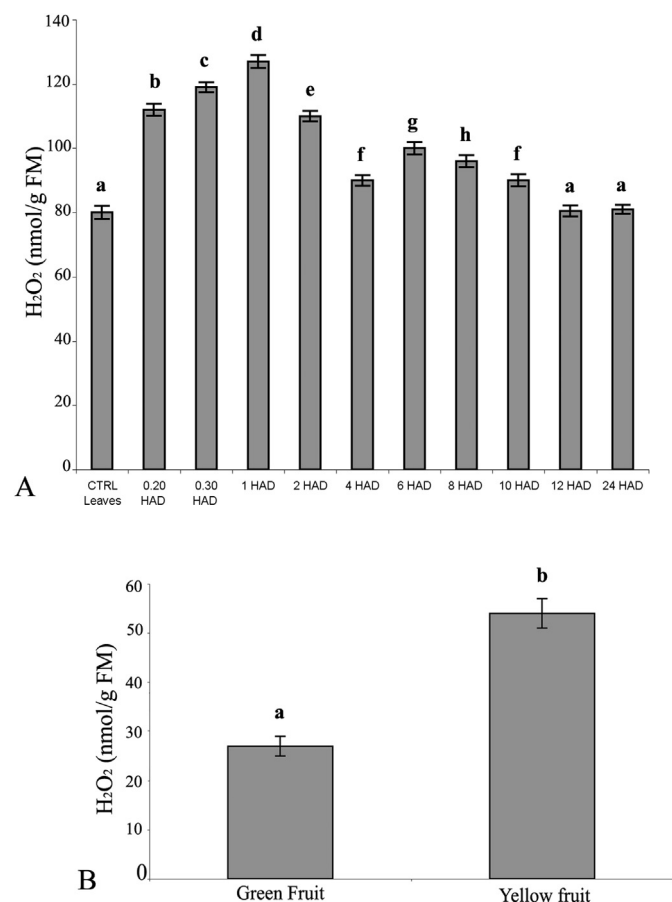


Fig. 6. H₂O₂ amount: (A) in control and wounded leaves, at different hours after wounding (HAD); (B) in the peel of green and yellow fruits. The results represent the average value of three independent biological replicates. Means with common letters are not significantly different at $P \leq 0.05$, according to Turkey's Honestly Significant Difference (HSD) test.

A relationship between *CIGLP1* transcriptional activity and endogenous H₂O₂ amount was observed also in wounding assay. Indeed, the rapid wound-induced expression of *CIGLP1* (0.10–0.20 HAD) in injured leaves was followed by increased H₂O₂ production, further supporting the putative SOD activity of *CIGLP1*. In this context it is worthy noting that also in rice, SOD activity of OsGER3 and OsGER4 was induced by wounding beside pathogen attack (Manosalva et al., 2009). Moreover, the enhancement of *CIGLP1* expression after wounding is consistent with the strong and rapid wound-induced activity of OsGLP8–10 promoter observed in transgenic tobacco plants (Davidson et al., 2010; Dunwell et al., 2008) as well as with the presence in such promoter of several responsive elements for wounding and defense (Davidson et al., 2010). On the other hand, in plants reactive oxygen species may accumulate following both abiotic and biotic stresses, and a large number of isoenzymes, related to cellular detoxification, are expressed by plants as a component of the complex defense mechanism to combat biological invaders (Dietz et al., 2006). Therefore, in line with detected SOD activity for members of GLPs family from different species, it has been demonstrated an involvement of these proteins in response to biotic stresses (i.e salt, drought, heavy metal) as well as in the defense against viruses, bacteria and, fungi (Zimmermann et al., 2006; Godfrey et al., 2007; Davidson et al., 2010; Breen and Bellgard, 2010; Wei et al., 1998; Christensen et al., 2004). For example, TaGLP4 and HvGLP4 contribute to disease resistance in wheat and barley respectively (Christensen et al., 2004). Members of the OsGLP family contribute collectively to disease resistance because silencing of several OsGLP genes confers susceptibility to distinct fungal pathogens, (Manosalva et al., 2009; Banerjee and Maiti, 2010). Recently, GLP gene clusters have been identified in specific QTL regions related to disease resistance in both rice and barley (Druka et al., 2002; Davidson et al., 2010; Manosalva et al., 2009; Breen and Bellgard, 2010). Further related information is available from studies dealing with the infection of grape berries by powdery mildew pathogen (Dunwell et al., 2008) as well as with the infection of wheat by fungal pathogen (Troshina et al., 2007). Notably, in this last case the spread of fungal hyphae into leaf mesophyll is associated with H₂O₂ production in the infected tissues (Troshina et al., 2007).

Based on all these data, CIGLP1 may also play a role against biotic stress through the putative SOD activity. Interestingly such role well fits with the upregulation of CIGLP1 transcription detected in OC fruits compared to CC ones as well as with data in the literature reporting a high level of Cit s1, a GLP protein, in the peel of *Citrus limon* cv Femminello fruits from OC (Pignataro et al., 2010). OC practice is based on the promotion of natural cycles and on the “non-use” of chemical compounds for both plant nutrition and crop protection against insects and pests. Consequently, plant response mechanisms to cope with biotic stresses, including the synthesis of protein with a protective role like GLPs, could be more highly expressed in absence of effective crop protection strategies such in OC practice compared with CC conditions.

In this context we may recall that in *Citrus sinensis*, Cits1, the homologous protein to CIGLP1, was classified as a major orange allergen, (Crespo et al., 2006). Our term aim is to identify organic growth conditions to obtain lemon fruits with desirable traits that include: aroma, flavor and a low content of allergenic protein. So far, our data indicate that the expression of CIGLP1 gene in lemon fruit can be modulated by culture practice. Further work should be required to verify whether and how interrelated seasonal (i.e. temperature, raining), culture (i.e. altitude, irrigation) and post-harvest storage (i.e. temperature, humidity) conditions could influence CIGLP1 expression. Such additional insights could be indeed essential for defining the thresholds of gene expression modulation and planning culture and post-harvest storage condition by which allergen production can be reduced without compromising plant agronomic traits and disease resistance.

Contributions

L.B., N.D.S and M.B.B. designed research; L.B., N.D.S., D.I. performed research; L.B., N.D.S., D.I., A.C. and M.B.B analyzed data and discussed results; L.B., N.D.S and M.B.B. wrote the paper.

L.B. and N.D.S. contributed equally to this work.

All authors contributed to improving the paper and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2014.03.003>.

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Gene Name	Accession no.	Plant species
12-1_LOC_Os12g05840	NP_001066188.1	<i>Oryza sativa</i>
12-2_LOC_Os12g05860	NP_001066189.1	<i>Oryza sativa</i>
12-3_LOC_Os12g05870	Q2QXJ1.1	<i>Oryza sativa</i>
8-1_LOC_Os08g08920	NP_001061155.1	<i>Oryza sativa</i>
8-10_LOC_Os08g09060	NP_001061163.1	<i>Oryza sativa</i>
8-11_LOC_Os08g09080	NP_001061164.1	<i>Oryza sativa</i>
8-12_LOC_Os08g13440	NP_001061303.1	<i>Oryza sativa</i>
8-2_LOC_Os08g08960	NP_001061156.1	<i>Oryza sativa</i>
8-3_LOC_Os08g08970	NP_001061157.1	<i>Oryza sativa</i>
8-4_LOC_Os08g08980	NP_001061158.1	<i>Oryza sativa</i>
8-5_LOC_Os08g08990	Q6YZA6.1	<i>Oryza sativa</i>
8-6_LOC_Os08g09000	NP_001061160.1	<i>Oryza sativa</i>
8-7_LOC_Os08g09010	NP_001061161.1	<i>Oryza sativa</i>
8-8_LOC_Os08g09020	NP_001061162.2	<i>Oryza sativa</i>
8-9_LOC_Os08g09040	NP_001175415.1	<i>Oryza sativa</i>
AtGLP1	BAA78563.1	<i>Atriplex lentiformis</i>
AtGLP1	U75206	<i>Arabidopsis thaliana</i>
AtGLP10	NP_191761.1	<i>Arabidopsis thaliana</i>
AtGLP2a	X91957	<i>Arabidopsis thaliana</i>
AtGLP3a	ATU75195	<i>Arabidopsis thaliana</i>
AtGLP6	AAB51572.1	<i>Arabidopsis thaliana</i>
AtGLP8	NP_187244.1	<i>Arabidopsis thaliana</i>
AtGLP9	NP_193199.1	<i>Arabidopsis thaliana</i>
BuGLP	AB036797	<i>Barbula unguiculata</i>
CIGLP1	AFN02126.1	<i>Citrus Limon</i>
CsGLP1	XP_006479011.1	<i>Citrus sinensis</i>
GhGLP	AF116537	<i>Gossypium hirsutum</i>
HVGER1a	DQ647619	<i>Hordeum vulgare</i>
HvGER1b	BI949639	<i>Hordeum vulgare</i>
HvGER1c	BY847725	<i>Hordeum vulgare</i>
HvGER1d	BG366014	<i>Hordeum vulgare</i>
HvGER2a	DQ647620	<i>Hordeum vulgare</i>
HvGER2b	BY837622	<i>Hordeum vulgare</i>
HvGER3a	DQ647621	<i>Hordeum vulgare</i>
HvGER3c	DN178806	<i>Hordeum vulgare</i>
HvGER4a	CD056200	<i>Hordeum vulgare</i>
HvGER4c	DQ647622	<i>Hordeum vulgare</i>
HvGER4d	DQ647623	<i>Hordeum vulgare</i>
HvGER4e	BQ762852	<i>Hordeum vulgare</i>
HvGER5a	DQ647624	<i>Hordeum vulgare</i>
HvGER5b	AV923347	<i>Hordeum vulgare</i>
HvGER6a	DQ647625	<i>Hordeum vulgare</i>
McGLP	P45852.1	<i>Mesembryanthemum crystallinum</i>
MetJa	Q58174.1	<i>Methanococcus jannaschii</i>
Nectarin I	Q9SPV5.1	<i>Nicotiana plumbaginifolia</i>
PcGER1	AAC99473.1	<i>Pinus caribaea</i>
PnGLP	D45425	<i>Ipomoea nil</i>
PpABP19	Q9ZRA4.1	<i>Prunus persica</i>
PpABP20	O04011.1	<i>Prunus persica</i>

<i>PpGLP6</i>	XP_001782709.	<i>Physcomitrella patens</i>
<i>PrGLP</i>	AAC05146.1	<i>Pinus radiata</i>
<i>PsGER1</i>	AJ250832	<i>Pisum sativum</i>
<i>SaGLP</i>	P45854.1	<i>Sinapis alba</i>
<i>TaGER2.8</i>	M63223	<i>Triticum aestivum</i>
<i>TaGER3.8</i>	M63224	<i>Triticum aestivum</i>
<i>VvGLP2</i>	DQ673106	<i>Vitis vinifera</i>
<i>VvGLP3</i>	AY298727	<i>Vitis vinifera</i>
<i>VvGLP4</i>	EF064172	<i>Vitis vinifera</i>
<i>ZmGLP10-1</i>	AFW56909.1	<i>Zea mays</i>
<i>ZmGLP10-2</i>	AFW56910.1	<i>Zea mays</i>
<i>ZmGLP10-3</i>	AFW56912.1	<i>Zea mays</i>
<i>ZmGLP10-4</i>	AFW56915.1	<i>Zea mays</i>
<i>ZmGLP10-5</i>	NP_001152473.1	<i>Zea mays</i>
<i>ZmGLP2-1</i>	NP_001141258.1	<i>Zea mays</i>
<i>ZmGLP3-1</i>	n/a	<i>Zea mays</i>
<i>ZmGLP3-10</i>	AFW61075.1	<i>Zea mays</i>
<i>ZmGLP3-11</i>	AFW61070.1	<i>Zea mays</i>
<i>ZmGLP3-12</i>	AFW61071.1	<i>Zea mays</i>
<i>ZmGLP3-13</i>	AFW61072.1	<i>Zea mays</i>
<i>ZmGLP3-14</i>	AFW61073.1	<i>Zea mays</i>
<i>ZmGLP3-15</i>	AFW61074.1	<i>Zea mays</i>
<i>ZmGLP3-16</i>	AFW61075.1	<i>Zea mays</i>
<i>ZmGLP3-17</i>	AFW61076.1	<i>Zea mays</i>
<i>ZmGLP3-18</i>	AFW61077.1	<i>Zea mays</i>
<i>ZmGLP3-19</i>	AFW61078.1	<i>Zea mays</i>
<i>ZmGLP3-2</i>	AFW61061.1	<i>Zea mays</i>
<i>ZmGLP3-20</i>	AFW61079.1	<i>Zea mays</i>
<i>ZmGLP3-3</i>	AFW61062.1	<i>Zea mays</i>
<i>ZmGLP3-4</i>	AFW61063.1	<i>Zea mays</i>
<i>ZmGLP3-5</i>	AFW61064.1	<i>Zea mays</i>
<i>ZmGLP3-6</i>	AFW61065.1	<i>Zea mays</i>
<i>ZmGLP3-7</i>	AFW61066.1	<i>Zea mays</i>
<i>ZmGLP3-8</i>	AFW61067.1	<i>Zea mays</i>
<i>ZmGLP3-9</i>	AFW61068.1	<i>Zea mays</i>

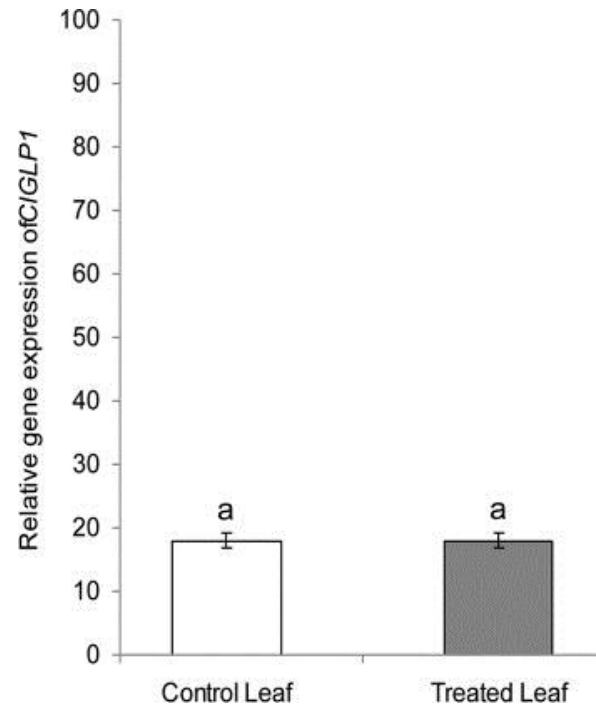


Fig. 1S. Effect of exogenous treatment with H2O2 on the expression level of CIGLP1 analyzed by qRT-PCR in mature leaf. The results are presented as a proportion of the highest value after normalisation with respect to elongation factor 1-alpha (EF1) expression...

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