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ORIGINAL PAPER

The promoter of a gene encoding an isoflavone reductase-like protein in coffee (*Coffea arabica*) drives a stress-responsive expression in leaves

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Abstract A cDNA clone (designated CaIRL) encoding an isoflavone reductase-like protein from coffee (*Coffea arabica*) was retrieved during a search for genes showing organ/tissue-specific expression among the expressed sequence tags (EST) of the Brazilian coffee EST database. The *CaIRL* cDNA contains a single open reading frame of 946 nucleotides (nt) encoding 314 amino acids (predicted molecular weight of 34 kDa). Several features identified the predicted CaIRL protein as a new member of the PIP family of NADPH-dependent reductases. Expression studies demonstrated that *CaIRL* is expressed exclusively in coffee leaves and its transcript level is markedly increased in response to fungal infection and mechanical injury. Analysis of transgenic tobacco plants harboring a *CaIRL* 5′-flanking region (862 nt) fused to *uidA* reporter gene (GUS) confirmed the responsiveness of the putative promoter to abiotic stress in wounded leaves. In turn, a 5′ deletion to −404 completely abolished promoter activation by abiotic stimulus in transgenic plants. The lack of GUS expression in non-wounded leaf tissues in transgenic tobacco was in contrast to the basal level of *CaIRL* expression observed in non-stressed healthy coffee leaves.

Keywords Coffee · Promoter · Tissue-specificity · Gene expression · Isoflavone-like proteins

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Introduction

Promoters are crucial regulatory sequences that ensure spatial and temporal gene expression. Recovery of highly active promoter sequences, especially those leading to organ/tissue-specific expression, is of great interest to manage the perceived risks and concerns associated with the application of transgenic plant technology. In this context, whereas plant promoters leading to ubiquitous gene expression have been frequently applied in construct design, the employment of those directing tissue-specific expression is still rare. This bias is even more pronounced in perennial species like coffee, due, in part, to the limited availability of such kind of regulatory sequences.

To meet the demand for achieving organ/tissue-specific gene expression in coffee plants, the identification of genes with suitable tissue-specific expression is required. In the current study, as a first step toward promoter isolation in *Coffea arabica*, candidate genes with desired expression patterns were identified from in silico searches in the Brazilian coffee expression sequence tags (EST) database (Vieira et al. 2006). Among the identified genes, a leaf-specific cDNA (named *CaIRL*) encoding a putative isoflavone reductase-like protein (IRL) was validated and selected for further investigation.

Isoflavone reductases (IFR), pinoresinol-lariciresinol reductases (PLRs), and phenylcoumaran benzylic ether reductases (PCBERs) are the founding members of the PIP

family of NADPH-dependent reductases that are involved in the biosynthesis of plant defense metabolites such as lignans and isoflavonoids (Dixon 2001; Kajikawa et al. 2009). Several other homologous reductases that catalyze undetermined enzymatic reactions are also included within this family (Shoji et al. 2002; Kajikawa et al. 2009). The members of this latter group are collectively called IRLs (for isoflavone reductase-like proteins) and knowledge of their function in plants is yet very limited. Insights from the published literature, however, suggest the involvement of these reductase-like proteins in plant response to biotic and abiotic stress (Petrucco et al. 1996; Lers et al. 1998; Salekdeh et al. 2002; Kim et al. 2003a, b).

Herein, the relationship of CaIRL to the other members of the PIP family of NADPH-dependent reductases was determined, and its gene expression in response to biotic and abiotic stresses examined using quantitative RT–PCR. Moreover, the *CaIRL* 5′-flanking region was isolated and its ability to drive leaf-specific expression of a reporter gene evaluated in transgenic tobacco plants. By using these transgenic plants, the effect of abiotic stress in promoter activation was also investigated. We anticipate that the isolated promoter can be used to direct leaf-specific and inducible transgene expression in plants. Once the major pests and pathogens of coffee, such as the common leaf miner and the leaf rust, attack exclusively the leaves, promoters that specifically drive gene expression to this organ represent a promising tool for the development of genetically modified coffee cultivars bearing specific resistance/insecticidal genes.

Materials and methods

Plant material and growth conditions

Freshly harvested coffee tissues were obtained from *Coffea arabica* var. Mundo Novo (IAC 388-17-1) grown under greenhouse conditions (28°C, 60% RH) in Campinas, São Paulo, Brazil. For this, a total of ten 4-month-old coffee plants were used. Flower and fruit samples, at different developmental stages, were collected from 4- to 5-year-old plants of var. Mundo Novo grown under field conditions at Botucatu and Campinas, São Paulo, Brazil. After harvesting, fresh tissue samples were frozen immediately in liquid nitrogen until RNA extraction. Tobacco plants (*Nicotiana tabacum* SR1) were grown in a growth chamber at 22–24°C under a 16 h/8 h light/dark cycle.

RNA extraction and reverse transcription (RT)

Total RNA was extracted from the different organ/tissue samples using Trizol reagent according to manufacturer's instruction (Invitrogen), and contaminating DNA was removed by treatment with RNase-free DNase I (Fermentas). RNA was examined for integrity by denaturing gel electrophoresis and its concentration quantified in a UV spectrophotometer. The samples were stored at −80°C until use.

Equal quantities of total RNA (1 μg) were reverse-transcribed at 42°C using SuperScript III Reverse Transcriptase (Invitrogen) and 2.5 μM of Oligo(dT_{17}VN), according to the manufacturer's recommendations. A similar reaction without reverse transcriptase was also performed as a control to confirm the absence of genomic DNA in subsequent steps. All cDNA samples were analyzed on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) for quantification.

In silico analysis, validation and cloning of the entire *CaIRL* cDNA

To identify genes with organ/tissue-specific expression in *C. arabica*, an in silico analysis was performed by comparing the cDNA libraries prepared from different organs/tissues available in the Brazilian coffee EST database (http://www.lge.ibi.unicamp.br/cafe; Vieira et al. 2006). The leaf-specific expression of *CaIRL* was validated by PCR using a panel of cDNA samples representing different coffee organs/tissues and a primer pair designed based on the EST sequence selected in the mentioned database. A primer pair designed for the amplification of a coffee actin gene (AcF 5′-GACCTCACAGATCACCTCAT-3′ and AcR 5′-GTAGTCTCGTGGATACCAGC-3′) was used as an internal control. PCR amplification was performed with initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 40 s, and extension at 72°C for 1 min.

To obtain the full-length cDNA of *CaIRL*, a 3′-RACE technology was performed using the 3′-RACE System for Rapid Amplification of cDNA Ends according to the manufacturer's protocol (Invitrogen). The gene-specific primers (GSPs) for 3′-RACE were designed based on the validated EST clone, and were as follows: IsofE (5′-GA AGTGATGGATCGTTTGCATGGCGT-3′) and IsofI (5′-G TTGAGCCTGCCTCAAGCTTATACAGAT-3′). Amplified fragments were cloned into pGEM-Teasy and sequenced.

Phylogenetic analyses

The deduced amino acid sequences of CaIRL and the identified NADPH-dependent reductases were aligned using AMAP (Schwartz and Pachter 2007). A Bayesian phylogenetic tree was constructed using MrBayes (Ronquist and Huelsenbeck 2003) with a WGA model of amino-acid substitution. MrBayes was run for 275,000 generations and

trees were sampled every 100 generations, and a consensus tree was estimated by a burn-in of 2,500 trees. Protein maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel 2003). PhyML was performed using an input tree generated by BioNJ assuming the WAG substitution model and a gamma distribution of rates among sites (four categories, γ shape parameter 1.256). The statistical support was made by SH-like support according to the aLTR algorithm. Trees were displayed using Dendroscope (Huson et al. 2007).

Promoter cloning, construction of the expression cassettes, and tobacco transformation

The *CaIRL* promoter was isolated using the GenomeWalker Universal Kit (Clontech) according to the manufacturer's instructions. For that, two gene-specific primers were designed based on the validated EST clone (IsoGPS1 5′-CTCCCAAACTCTTGAAGCTCTCTAGGTTG-3′ and IsoGSP2 5′-GGATGGTAAACTACTGTTGCAAATTG G-3′). This PCR-based genome walking procedure resulted in the amplification of a 0.86 kb DNA fragment (which included the upstream region of *CaIRL* extended from its start codon) that was cloned into the pGEM-Teasy vector (Promega) and sequenced. Putative *cis*-elements within the amplified fragment were analyzed using PLACE (Higo et al. 1999) and PlantCARE (Lescot et al. 2002) databases.

To generate pCaIRL:GUS, which contains the *uidA* reporter gene (GUS) under the control of the 5′-flanking region of *CaIRL*, the cloned walking product was amplified by PCR using forward and reverse primers containing overhanging *Hin*dIII and *Nco*I recognition sites, respectively, and *Pfu*. The PCR product was digested and subcloned into the *Hin*dIII and *Nco*I sites of pCAMBIA-1381z (CAMBIA). A 5′ deletion version ($\sim$0.4 kb) of the *CaIRL* promoter was created by PCR amplification using specific primers harboring *Hin*dIII and *Nco*I recognition sites and inserted, as described, into pCAMBIA-1381z to generate pΔCaIRL:GUS.

The resulting constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 and used to transform tobacco leaf discs as described (Horsch et al. 1985). Putative transformants (a total of 20 per construct) were selected in MS medium supplemented with hygromycin. The integration of the expression cassettes in the plant genome was confirmed by PCR using primers directed against the *CaIRL* promoter. Only selfed T1 progenies were used in subsequent assays.

Biotic and abiotic stress assays

For the biotic stress assay, equally aged sets of *C. arabica* var. Mundo Novo plants were kept in a growth chamber (16 h/8 h light/dark; 23°C; 70% RH) for at least 1 week, before being inoculated with the coffee leaf-rust fungus *Hemileia vastatrix* Berk and Br. race II, that elicits a compatible reaction in coffee. The urediniospores (100 mg) were harvested in a *C. arabica* field in Campinas, São Paulo, Brazil, and diluted in 10 ml of sterile water under dark conditions.

Leaves (six per plant) from the second pair of plagiotropic shoots from the apex of 4-month-old coffee plants (a total of three plants) were inoculated with an aqueous suspension of fresh urediniospores (10 mg/ml). Inoculated leaves were not detached from the plants. Leaves were randomly sampled from each inoculated plant at different time-points after inoculation (0, 8, 12, and 24 h), pooled, and immediately deep-frozen. To confirm the infection by the leaf-rust fungus, some inoculated leaves were maintained in plants. Leaves from non-inoculated plants served as control.

For mechanical wounding treatment, coffee plants at the same developmental stage and physiological conditions as described above were used. Mechanical wounding was performed using a blade affixed to a scalpel essentially as described by Ganesh et al. (2006). In brief, leaf blades were mechanically injured by making a series of three longitudinal cuts through the leaf parallel to the midrib on each side. One leaf of each plant was collected before treatment and used as control. Samples were collected at 4, 8, and 12 h after injury.

In tobacco, wounding was performed by crushing the leaf lamina with forceps, and samples were collected at 4, 8, 12, and 24 h after injury. Non transgenic plants stressed in the same manner were used as control.

Quantitative real-time PCR (qPCR)

CaIRL transcript levels were assessed by real-time PCR using an ABI Prism 7300 Sequence Detection System (PE Applied Biosystems, USA). Reactions were carried out in 96-well optical reaction plates (Applied Biosystems) using the kit Platinum SYBR Green qPCR SuperMixUDG with ROX (Invitrogen). PCR cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles each of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Each reaction consisted of 10 µl containing 2 µl of cDNA (10 ng/µl) and 0.2 µM of each amplification primer, and was run in triplicate. Confirmation of amplicon specificity was based on the dissociation curve at the end of each run and by product visualization after electrophoresis on an 8% polyacrylamide gel.

In all reactions leading with coffee samples, *CaUbiquitin* (Ganesh et al. 2006) was used as housekeeping gene to normalize levels of *CaIRL* transcripts. Gene-specific primers were designed using the Primer Express 2.0

software (Applied Biosystems). Primer sequences for *CaIRL* and *CaUbiquitin* were as follows: CaIRL forward (5′-CGGCACCGGATACATTGG-3′); CaIRL reverse (5′-TTGGGTGCCCTGCTTTTG-3′); CaUb forward (5′-AACATTGAGGGTGGTTCTGTTC-3′) and CaUb reverse (5′-GCAGAAAACCAACTAAGACCTAACAA-3′).

The level of GUS expression in leaves of transgenic tobacco plants harboring the *CaIRL* promoter (or its shorter version) was also assessed by qPCR in a time–course study using the following primer pair: GUSF (5′-TTGCCAACGAACCGGATAC-3′) and GUSR (5′-GCCAGTGGCGCGAAATATT-3′). In this case, GUS expression was normalized using the tobacco α-tubulin gene (5′-GCATATCGATCCACATTGGTCAG-3′ and 5′-GAGCTGCCTGTATGTTCCAGTCC-3′).

Data analysis

Cycle threshold (C_t) values were determined for both *CaIRL* and *CaUbiquitin* for each sample, and relative quantification of *CaIRL* transcript was determined using the comparative CT method ($\Delta\Delta C_t$) as described (Livak and Schmittgen 2001). Amplification efficiencies were derived from the amplification plots using the program LinRegPCR (Ramakers et al. 2003). A value of two was used in calculations. The same procedure was applied for relative quantification of GUS expression in transgenic tobacco leaves.

Histochemical analysis

Histochemical localization of GUS activity was performed 4 h after stress by incubating whole transgenic plants (or controls) with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) as described (Jefferson et al. 1987).

Results

The electronic screening in the Brazilian coffee EST database allowed the identification of clusters with restricted pattern of expression, thus predicted to be organ/tissue-specific, as well as others that displayed expression in more than one organ/tissue. In order to independently verify the validity of these predicted EST-based tissue profiles, different candidate genes were randomly selected and their organ/tissue-specific expression evaluated in a set of five coffee tissues using qualitative RT–PCR. Among the validated candidate genes, one encoding an isoflavone reductase-like protein was shown to be specifically expressed in non-stressed healthy leaves (Fig. 1a) as compared to the expression of a control housekeeping gene for which almost equal amounts of a single amplification product were observed in all organ/tissues examined (Fig. 1b). Due to the plausible importance of reaching leaf-specific expression in coffee, this gene (named *CaIRL*) was selected for further investigation and promoter cloning.

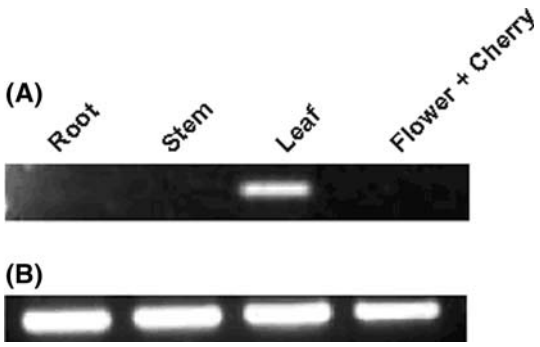

Fig. 1 Qualitative RT–PCR analysis of the distribution of *CaIRL* transcripts in different organ/tissues of coffee. **a** Products generated using *CaIRL* specific primers. **b** Products generated using primers directed against the coffee actin gene, used as control. Products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized by UV transillumination

Sequence analysis of the *CaIRL* cDNA and predicted polypeptide

As already mentioned, the starting EST clone used for cloning the full-length *CaIRL* cDNA was retrieved during a coffee EST database screening for genes showing organ/tissue-specific expression. Interestingly, the corresponding contig was composed of sequences derived from cDNA libraries constructed from non-stressed leaves and from leaves infected with the rust fungus *H. vastatrix*, respectively. The starting EST clone was found to contain a ~0.87 kb insert that covered the 5′-untranslated region (UTR; 280 nt) and part of an open reading frame encoding an IRL protein. To retrieve the entire *CaIRL* cDNA, a 3′-RACE PCR was performed on leaf cDNA using gene-specific primers designed based on the sequence of the starting EST clone. In this case, three individual RACE clones were sequenced and found identical. The nucleotide sequence of the resulting 3′-RACE product overlapped that of the original EST and both were merged into a complete cDNA. The resulting full-length sequence contained a single open reading frame of 946 nucleotides (nt) encoding a polypeptide of 314 amino acids (aa) with an estimated molecular mass of 34 kDa. A database search using BLAST revealed that the deduced amino acid sequence of *CaIRL* shared significant overall similarity to different members of the PIP family of NADPH-dependent reductases. In this context, the closest homologue was a *Pyrus communis* IRL (63% aa identity; AAC24001) followed by a *Populus trichocarpa* PCBER 3 (62% aa identity; EEE87743).

In order to more accurately place *CaIRL* in this family, a Bayesian phylogenetic tree was created using a selection of IFR, PCBER, PLR, and IRL protein sequences from different plant species. Sequences of two other PIP-enzymes, a eugenol synthase (EGS; Louie et al. 2007) and a pterocarpan reductase (PTR; Akashi et al. 2006), were also included in this phylogeny. As it can be seen from Fig. 2, the Bayesian tree divided the reductases into three main supergroups: the PCBER-derived supergroup, the EGS supergroup, and the PLR supergroup. Interestingly, no change in overall tree topology was observed using PhyML, thus confirming the reliability of the obtained tree. In this tree, CaIRL was located in the PCBER-derived supergroup, which includes a number of reductases with determined enzymatic activity (PTR, PCBER and the leguminous IFRs), falling within the PCBER L2 group. In this group, CaIRL was placed in close proximity to a *Lotus japonicum* PTR (PTR2; Akashi et al. 2006), placement that was strongly supported by both the posterior probability (93) and aLTR (79). The PCBER L2 group also encompassed the tobacco A622 IRL (Shoji et al. 2002) and two IRLs from *Arabidopsis thaliana* and *Zea mays*, respectively. The close evolutionary relationship between PTR and PCBER was previously observed (Akashi et al. 2006).

Effects of biotic and abiotic stresses on *CaIRL* expression in coffee

To test whether the expression of *CaIRL* is affected by biotic and abiotic stresses, transcript accumulation in coffee plants exposed to fungal infection and mechanical injury was monitored by quantitative real-time PCR.

To characterize the kinetics of induction of *CaIRL* transcripts in response to fungal infection, leaves were analyzed at various times after challenging with *H. vastatrix*. As it was observed for other IRL genes, *CaIRL* gene expression is responsive to biotic stress (Fig. 3a). When leaves were challenged with the fungus, a progressive increase in *CaIRL* transcript levels as compared to 0 h (non)-stressed control was observed, peaking at 12 h post inoculation (Fig. 3a). A rapid and marked increase in *CaIRL* expression was also observed following mechanical injury of the leaves (75-fold after 4 h treatment) (Fig. 3b).

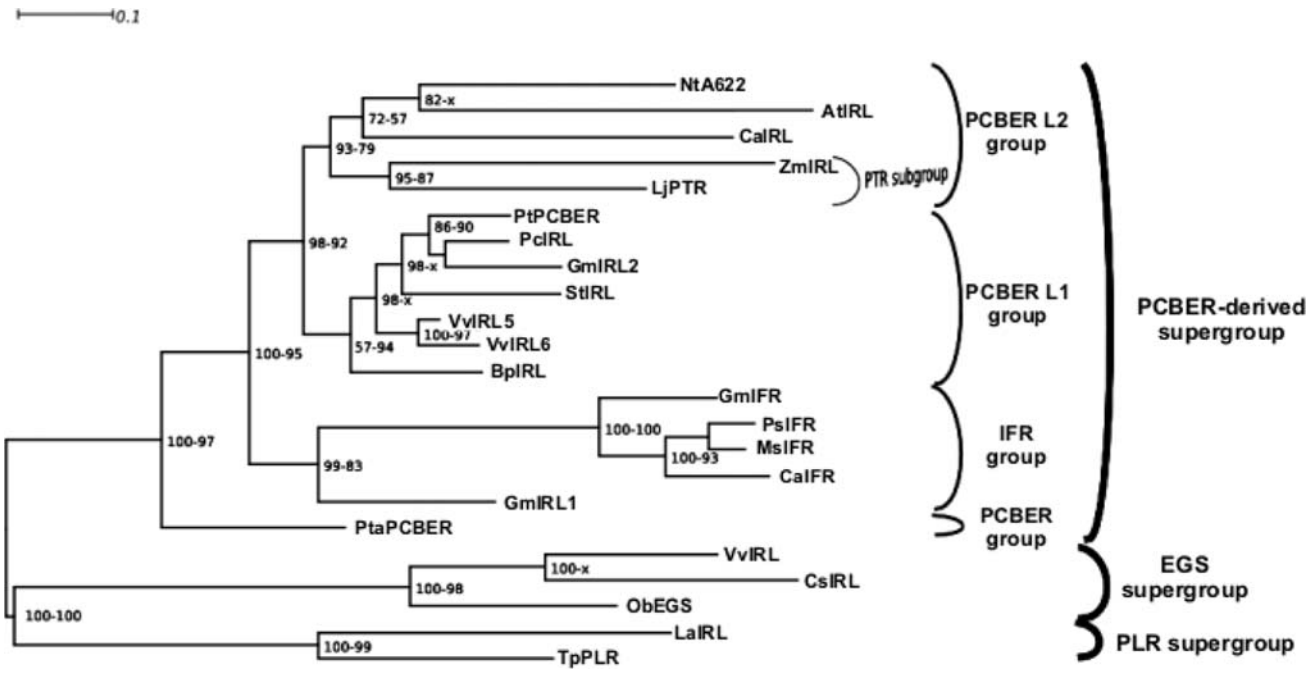

Fig. 2 Bayesian phylogenetic tree of CaIRL and other NADPH-dependent reductases. At the nodes, *numbers* to the left indicate Bayesian posterior probability calculated with MrBayes program, and to the right the SH-like support according to the aLTR algorithm from PhyML. The *scale bar* represents 0.1 substitutions per site. Accession numbers are: *Arabidopsis thaliana* IRL (AtIRL; NP_173385.1), *Betula pendula* IRL (BpIRL; AAG22740.1), *Citrus sp* IRL (CsIRL; CAA73220.1), *Glycine max* IRL1 (GmIRL1; AAF17577.1), *Glycine max* IRL2 (GmIRL2; AAF17578.1), *Lupinus albus* IRL (LaIRL; AAB67729.1), *Nicotiana tabacum* A622 (NtA622; BAA05866.1), *Pyrus communis* IRL (PyIRL; AAC24001.1), *Solanum tuberosum* IRL (StIRL; CAA63056.1), *Vitis vinifera* IRL (VvIRL; CAI56330.1), *Vitis vinifera* IRL5 (VvIRL5; CAI56334.1), *Vitis vinifera* IRL6 (VvIRL6; CAI56335.1), *Zea mays* IRL (ZmIRL; NP_001105699.1), *Lotus japonicus* PTR (LjPTR; BAF34842.1), *Cicer arietinum* IFR (CaIFR; CAA43167.1), *Glycine max* IFR (GmIFR; CAA06027.1), *Medicago sativa* IFR (MsIFR; CAA41106.1), *Pisum sativum* IFR (PsIFR; AAB31368.1), *Pinus taeda* PCBER (PtaPCBER; AAC32591.1), *Populus trichocarpa* PCBER (PtPCBER; XP_002313788.1), *Ocimum basilicum* EGS (ObEGS; ABD17321.1), *Thuja plicata* PLR (TpPLR; AAF63507.1)

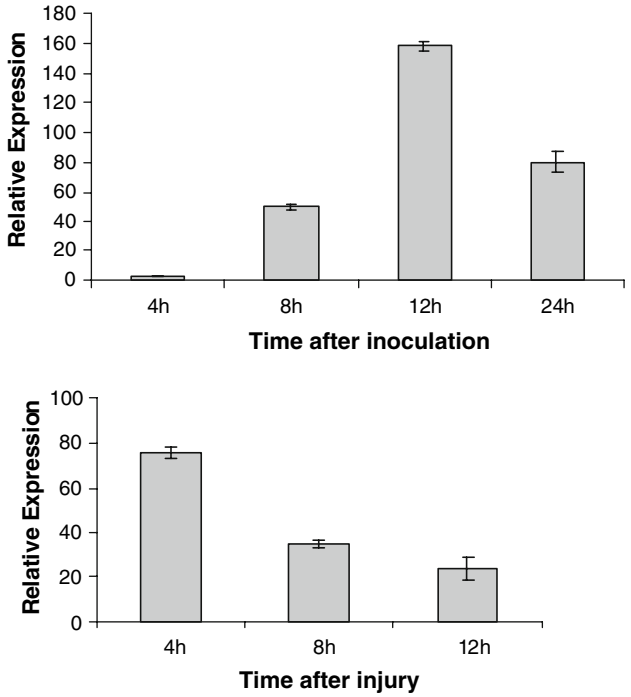

Fig. 3 Time–course expression analysis of *CaIRL* in *Coffea arabica* leaves following inoculation with *H. vastatrix* (**a**), and mechanical injury (**b**). Transcript levels in inoculated/injured leaves were normalized to the expression of an ubiquitin gene (*CaUbiquitin*). For each time point, leaves from three independent treated plants were pooled for RNA extraction. Each time-point represents average data with standard errors from three replicates

However, this induction was followed by a progressive decrease in transcript accumulation at 8 and 12 h post injury. It is noteworthy that the activation of *CaIRL* expression by mechanical injury was faster than that observed in response to fungal inoculation.

Promoter cloning and functional characterization in transgenic tobacco

The 5′-flanking region of the *CaIRL* gene was cloned by using a PCR-based genome-walking (GW) technique. For that, two gene-specific oligonucleotides were designed based on the 5′-end of the validated EST sequence available in the Brazilian coffee EST database. A fragment of ~0.86 kb obtained after *Dra*I library amplification was cloned into the pGEMT-easy vector (Promega) and its complete nucleotide sequence determined. BLAST analysis of the obtained sequence against the Brazilian coffee EST and GenBank databases did not reveal any homology with any sequence, thus confirming that the amplified genomic region corresponded to an unidentified portion of the coffee genome. Moreover, the relationship between the starting coffee EST and the fragment amplified by GW was confirmed by PCR using genomic DNA and primers targeting both sequences (not shown).

By using computer analysis, several putative regulatory *cis*-elements were identified within the amplified fragment, including a number of elements associated with stress-related responses. As shown in Fig. 4, a typical W-box element containing the consensus sequence TTGACC was identified within the *CaIRL* 5′-flanking region. W-boxes are binding sites for WRKY plant-specific transcription factors involved in stress-induced gene expression (Eulgem et al. 1999; Dong et al. 2003). In this context, the presence of five copies of a 15-base conserved sequence (TAATTTCTGACCTTA; gray boxes in Fig. 4) carrying the W-box core motif TGAC (underlined) was particularly remarkable. Two other putative *cis*-regulatory elements (GT-1 box and TGTCA) previously described as being involved in the activation of defense-related genes were also identified. The sequence TGTCA is a motif recognized by a plant homeodomain transcription factor associated with disease resistance responses in rice (Luo et al. 2005). The GT-1 element (GAAAAA) is involved in the regulation of a gene activated by pathogen infection and salt stress (Park et al. 2004). The analyzed region also bears an ACCTACC motif that has been associated with wound-, elicitor-, and light-responsive gene expression (Palm et al. 1990). The presence of such regulatory sequences within the amplified fragment is compatible with the observed induction of *CaIRL* expression following fungal inoculation and mechanical injury (Fig. 3). Additionally, consistent with the regular feature of eukaryotic promoters, a putative TATA-box (ATTATA) was found 44 nt upstream of the putative transcription start site (first nt of the 5′UTR of the *CaIRL* cDNA).

To determine whether the isolated 5′-flanking region contained promoter activity, the amplified 0.86 kb fragment was subcloned into the promoter-less pCAMBIA-1381z vector (CAMBIA) upstream of the GUS reporter gene to generate pCaIRL:GUS. The resulting construct was firstly analyzed in transient expression in coffee leaves and shown to possess promoter activity (data not shown).

To further investigate the spatial and temporal regulation of the *CaIRL* promoter region, the construct CaIRL:-GUS was stably transformed into tobacco plants. To allow a preliminary evaluation of the importance of the identified *cis*-regulatory elements in promoter activity, a shorter promoter version (a 5′ deletion resulting in a ~0.4 kb fragment; Fig. 4) was also generated by PCR and transformed into tobacco plants. Several independent transgenic lines (12 for CaIRL:GUS and 10 for ΔCaIRL:GUS) were obtained in which the integration of the expression cassette in the plant genome was confirmed by PCR using primers directed against the *CaIRL* promoter (not shown). Promoter activity of the GUS fusions was evaluated in selfed T1 progeny by histochemical staining and quantified in RT-qPCR assays.

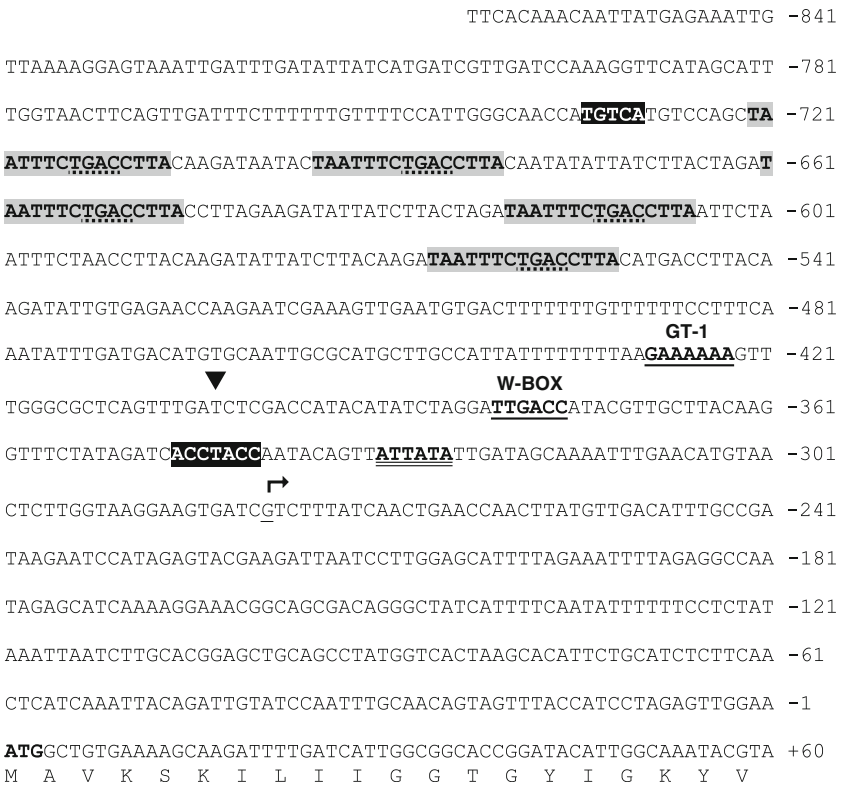

Fig. 4 Nucleotide (nt) sequence of the *CaIRL* 5′-flanking region. *Numbers* indicate the positions relative to the translational start codon ATG (marked in *bold*) with the adenine assigned as position +1. The first 20 amino acids of CaIRL are shown below the nt sequences. The *bent arrow* indicates the first nt (underlined G) of the 5′UTR region of the *CaIRL* cDNA as found in the coffee EST database. The locations of the putative *cis*-elements of interest identified using PLACE and PLANTCARE are indicated. The potential TATA-box is *double-underlined*. The GT-1 and W-box consensus sequences are underlined and labeled. The different copies of a conserved sequence carrying a W-box related core motif (TGAC) are indicated on a *gray* background. The sequences of two additional elements associated with stress responses in plants are indicated by *black boxes*. The 5′ end of the fragment corresponding to the shortest promoter construct is marked by an *inverted triangle*

In tobacco seedlings harboring the full-length *CaIRL* promoter, GUS activity was histochemically detected only in leaves that were wounded by crushing the leaf lamina with forceps (Fig. 5a). The responsiveness of CaIRL:GUS to mechanical injury was further confirmed in a time–course expression analysis using RT-qPCR. In this case, a rapid increase in *uidA* transcript accumulation was observed 4 h after the injury, decreasing thereafter (Fig. 5b). Although peaking at the same time, the observed induction was much lower than the one detected in treated coffee leaves. Interestingly, a sharp increase was observed at 24 h after injury. In contrast, no induction of GUS expression was evident in similar induction kinetic assays using wounded leaves from plants carrying the truncated version of the *CaIRL* promoter. In these plants, GUS expression was very weak and barely detected by histochemical examination.

Overall, these results demonstrate that the full-length version of the *CaIRL* promoter directs a reporter gene expression that is stimulated by mechanical injury as the expression from the native *CaIRL* gene. In transgenic plants, however, mechanical wounding induced the accumulation of GUS only in injured leaf tissues. Our experimental data also suggest that the whole set of elements present in the longest version of the *CaIRL* promoter are essential for its activation by mechanical stress.

Discussion

In this study, we have isolated and sequenced a full-length cDNA from coffee leaves that encodes an isoflavone reductase-like protein, namely *CaIRL*, a new member of the PIP family of NADPH-dependent reductases. Members of this family are found in different plant species, and a number are involved in the biosynthesis of important defense compounds that can also act as signaling molecules mediating bacterial or fungal symbioses (Dixon 2001).

In contrast, very little is known about the biochemical and molecular functions of the so-called IRL members of this family. Functional insights into their possible roles have originated from gene expression analysis, especially

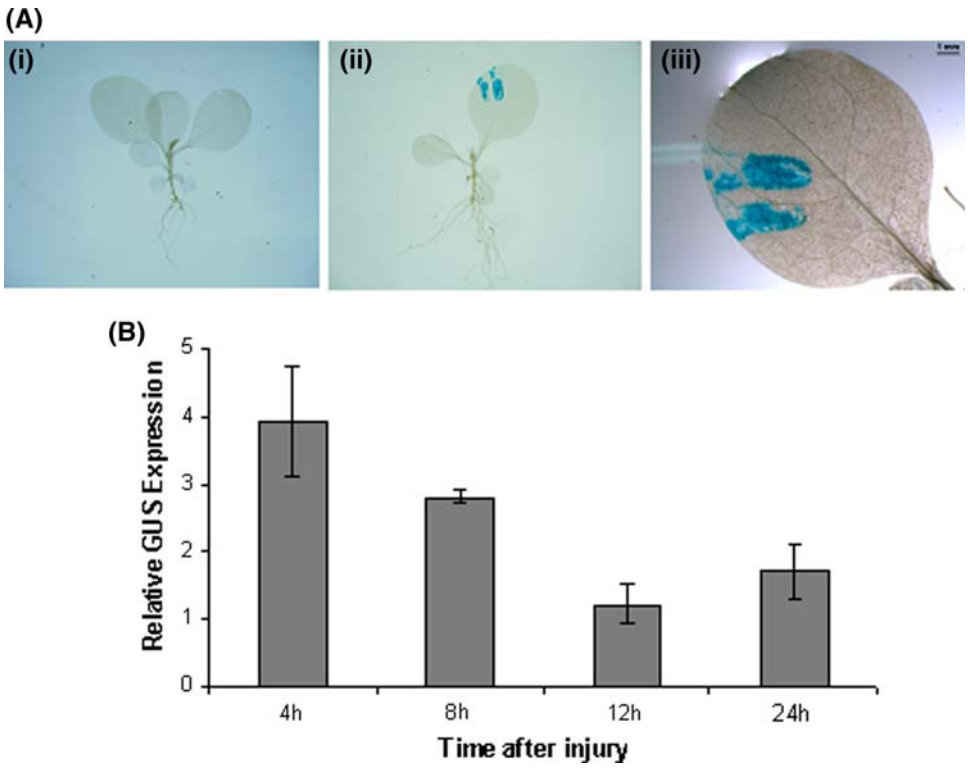

Fig. 5 Analysis of GUS expression in tobacco plants transformed with the full-length version of the *CaIRL* promoter fused to GUS reporter gene. **a** Histochemical localization of GUS activity in a non transgenic control seedling (*i*) and in a seedling of a representative transgenic line submitted to leaf injury (*ii*); a higher magnification of the injured leaf is also shown (*iii*). **b** Relative quantification by RT-qPCR. Transcript levels in injured leaves were normalized to the expression of a α-tubulin gene. Relative expression is computed based on the corresponding gene expression at time zero (untreated control). Each time-point represents average data with standard errors from three replicates

those leading with stress response. As result, *IRL* genes have been shown to be induced by both biotic and abiotic stress in different plant species, including rice, grapefruit, and tobacco. In rice, a gene encoding an IRL protein (*OsIRL*) was shown to be up-regulated by inoculation with the rice blast fungus, *Magnaporthe grisea*, as well as by treatment by fungal elicitor and jasmonic acid (Kim et al. 2003a). In a complementary study using suspension-cultured rice cells, an increase in OsIRL protein abundance in response to the fungus (or its elicitor) was also observed (Kim et al. 2003b). In grapefruit, Lers et al. (1998) identified an *IRL* gene that was concomitantly induced by UV irradiation and fungal (*Penicillium digitatum*) infection. In addition, this gene was also shown to be wound-inducible.

Consistent with the aforementioned published data, the expression of *CaIRL* accurately reproduced the inducible expression pattern observed for other IRL genes, being rapidly up-regulated by incoming abiotic and biotic stimuli. Accordingly, the 0.86 kb of its 5′-flanking region directed GUS expression in transgenic tobacco leaves in a wound-inducible manner. In contrast, the shorter version of the promoter (~0.4 kb) establishes a very weak GUS expression in leaves that was not inducible. This indicates that the *cis*-elements present within the deleted region are essential for the activation of *CaIRL* promoter by wounding. In contrast, the elements present in the 0.4 kb promoter version (including a W-box and an ACCTACC motif) were not sufficient to support promoter activation. Interestingly, the deleted region bears five copies of a conserved sequence (TAATTTCTGACCTTA) carrying a W-box core motif TGAC that may contribute to promoter induction. Consistent with that is the fact that the promoters of genes regulated by WRKY transcription factors normally show clustered occurrences of W-boxes within a kilobase (an average of four copies according to Maleck et al. 2000). However, further functional analyses are necessary to confirm this possibility. Taken together, these data suggest that *CaIRL* undertake a role in stress adaptation being activated during the early stages of the stress response.

The highly *CaIRL* promoter-driven GUS expression in wounded leaves contrasts the expression patterns observed for other IRL members of the NADPH-dependent reductases family identified so far. In this context, only preferential gene expression in certain organs/tissues has been described. Examples include the potato *IRL* gene that shows a pollination-enhanced expression in pistils (van Eldik et al. 1997), and the tobacco *A622* gene, which is predominantly expressed in roots but is also perceived in stems (Hibi et al. 1994; Shoji et al. 2002). An interesting structural feature of the *CaIRL* promoter is the presence of a putative GT element that was previously determined to be involved leaf-specific transcriptional activation (Villain et al. 1996). On the other hand, the absence of GUS expression in undamaged tobacco tissues is in contrast to the basal expression of *CaIRL* observed in non-stressed healthy coffee leaves. This discrepancy may be attributed

to the existence of different regulatory mechanisms between coffee and tobacco, including the presence of host-specific *trans*-acting factors, or to the lack of essential regulatory elements within the cloned promoter region. Such kind of differential expression has already been reported for the alfalfa isoflavone reductase promoter that displays different developmental expression patterns in alfalfa and tobacco transgenic plants (Oommen et al. 1994).

A specific relationship between CaIRL and a known PTR from *Lotus japonicum* (PTR2) was found to be strongly supported in our phylogenetic analyses. PTR2 catalyzes the production of an isoflavan (−)-vestitol from pterocarpan medicarpin in this model legume (Akashi et al. 2006).

Finally, considering the current initiatives to limit gene expression to certain organs/tissues of genetically engineered plants, the *CaIRL* promoter may be of great utility to drive transgene expression in an inducible manner in leaves.

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