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Early gene expression in the walnut tree occurring during stimulation of leaf hydraulic conductance by irradiance

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Abstract

Leaf hydraulic conductance (K_{leaf}) plays a significant part in plant-water regulation. In walnut leaves, K_{leaf} is stimulated by irradiance and closely relates to the accumulation of *JrPIP2s* aquaporin transcripts, but it is independent of stomatal aperture. To provide an insight into the early molecular events occurred during light-induced K_{leaf}, a large-scale transcriptomic analysis consisting of the cDNA-amplified fragment length polymorphism (AFLP) was carried out on walnut leaves maintained under irradiance or in darkness. Of the total 12 000 transcript-derived fragments (TDFs) obtained using cDNA-AFLP with 128 primer pairs, 187 TDFs were selected after sequencing, and only 93 (49 %) that had been ascribed known functions through *BLAST* searching of the *GenBank* databases. Most of these TDFs correspond to genes whose protein products are involved in cellular regulation (57.9 %) and global metabolism (39.8 %). To validate cDNA-AFLP expression patterns, 30 TDFs were further analyzed using real-time quantitative polymerase chain reaction. Moreover, exposure of leaves to irradiance was accompanied by the modification of the Ca^{2+}-signaling pathway, ubiquitin-proteasome pathway, vesicle trafficking process and expression of multiple transcription factors.

Additional key words: aquaporin, cDNA-AFLP, *Juglans regia*, leaf water transport, plasma membrane intrinsic protein; qRT-PCR.

Introduction

Regulation of water transport is important for the adaptation and survival of terrestrial plants. It provides flexibility so the plant can respond to environmental factors by optimizing the use of water resources. Part of this regulation takes place in leaves, which are the major site of evaporation and photosynthesis. The hydraulic resistance of foliar tissues (R_{leaf}) contributes up to 60 - 80 % of shoot resistance (Yang and Tyree 1993, Nardini and Tyree 1999, Nardini and Salleo 2000) and up to 30 % of the whole plant hydraulic resistance (Sack and Holbrook 2006).

Leaf hydraulic conductance ($K_{leaf} = 1/R_{leaf}$) is defined as the ratio of water flow through the leaf to driving force of the flow, normalized by the leaf area (Sack and Holbrook 2006). K_{leaf} depends upon plant species (Tyree *et al*. 1998, Cosmstock 2000, Brodribb *et al*. 2002) and is modulated by environmental factors, such as water stress (Nardini *et al*. 2003, Nardini and Salleo 2005), temperature (Fredeen and Sage 1999, Cochard *et al*. 2000, 2007, Sack *et al*. 2004) and also by circadian rhythms (Brodribb and Holbrook 2004, Lo Gullo *et al*. 2005). K_{leaf} has long been thought to be mainly associated with the regulation of stomatal aperture, suggesting a minor contribution of aquaporins. This scenario seems to contradict much recent physiological and molecular data, advocating the key role of plasma membrane intrinsic proteins (PIPs) in the determination of K_{leaf}. In the walnut, the stimulation of K_{leaf} by irradiance was insensitive to high concentrations of abscisic acid (an inhibitor of stomatal opening), excluding a major role of stomatal limitation in this process (Tyree *et al*. 2005). On the other hand, in leaves of the *Helianthus annuus*, the presence of $HgCl_2$, an inhibitor of aquaporin activity, prompted a sharp reduction in K_{leaf} (Nardini and Salleo 2005). These findings underpin the idea that a substantial part of leaf water movement occur through the mercury-

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Abbreviations: AFLP - amplified fragment length polymorphism; HPFM - high pressure flow meter; K_{leaf} - leaf hydraulic conductance; K_{root} - root hydraulic conductance; PIP - plasma membrane intrinsic protein; PCR - polymerase chain reaction; qRT-PCR - quantitative real-time PCR; R_{leaf} - leaf hydraulic resistance; TDF - transcript-derived fragment.
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sensitive aquaporin pathway. In addition, aquaporins were reported to be localized in different leaf cells, including bundle sheath (Kaldenhoff *et al*. 1995, Frangne *et al*. 2001) and mesophyll cells (Otto and Kaldenhoff 2000, Hachez *et al*. 2008, Sakurai *et al*. 2008). Stimulation of K_{leaf} in walnut by irradiance was independent of stomatal aperture, but closely related to up-regulation of two isoforms of aquaporins (Cochard *et al*. 2007). Based on physiological investigations, two distinct water pathways have been proposed. The first one, characterized as an aquaporin-independent pathway (water movement occurs *via* the apoplastic and symplasmic routes), takes place both in dark and light conditions and is correlated with basal K_{leaf} values. The second one, identified as an aquaporin-dependent pathway, takes place in response to irradiance and corresponds to high, modulated K_{leaf} values (Voicu *et al.* 2009, Ben Baaziz *et al.* 2012). This high K_{leaf} requires a stimulation of PIP gene expression and de *novo* protein biosynthesis (Cochard *et al*. 2007), assuming that this early regulation of K_{leaf} by irradiance is very complex and may act at multiple levels. Such K_{leaf} regulation can prevent a substantial drop in leaf water potential under high transpiration, thereby providing a physiological advantage for plant growth and development.

Mechanisms responsible for perception and transduction of the signal of changing irradiance have been widely documented in relation to germination, flowering, phototropism and etiolation. However, little is known about the mechanism through which irradiance can regulate K_{leaf} and aquaporin expression. As a first step for elucidation of this task, amplified fragment length polymorphism-based mRNA fingerprinting (cDNA-AFLP) has been carried out on leaves exposed to irradiance for short time periods. 93 transcript-derived fragments (TDFs) were identified and grouped into ten functional categories. The efficiency of cDNA-AFLP was evaluated by monitoring the expression profile of 30 TDFs. This work provides the possibility to investigate the link(s) between these identified TDFs, aquaporins and stimulation of K_{leaf} by irradiance.

Materials and methods

The experiments were performed during the summer of 2006 and 2008, on leafy branches sampled from 15-year-old walnut trees (*Juglans regia* L. cv. Franquette) growing in the Institut National de la Recherche Agronomique (INRA) orchard near Clermont-Ferrand (South-central France). Leafy branches were randomly sampled from the sun-exposed part of the tree, and immediately recut under water. They were then enclosed in a black plastic bag and kept in full darkness at high value of humidity for 24 h before their use in this study. Only the mature developed leaves from the branches were used for experiments.

Measurement of leaf hydraulic conductance was carried out at room temperature (25 °C) as previously described Cochard *et al*. (2007). Briefly, the experiment consists of pushing degassed and pressurized water into the petiole of an excised leaf under a pressure P of 0.15 MPa. Irradiance of approximately 600 µmol m^{-2} s^{-1} at leaf level was provided by two 400 W iodine lamp. Water flow values (F, mmol s^{-1}) were recorded every 30 s using a high pressure flow meter (HPFM; *Bronkhorst*, Montigny Les Cormeilles, France) connected to computer. Leaf hydraulic conductance (K_{leaf}, mmol m^{-2} s^{-1} MPa^{-1}) was calculated as: $K_{leaf} = F/(P \times LA)$, where LA was the total leaf area [m^2]. F was measured on leaves exposed to irradiance for 120 min, and then during darkness for 120 min. Then leaves were immediately immersed in liquid nitrogen and stored at -80 °C.

Total RNA was extracted according to the method of Chang *et al*. (1993) and quantified in Clermont University using a spectrophoto-meter *ND-1000* (*Nanodrop*, France). First-strand cDNA was synthesized from 2 µg total RNA using *oligo(dT)$_{30}$* AFLP primer and *AMV* reverse transcriptase (*Promega*, Madisson, WI, USA). Second-strand cDNA was made using 25 U of DNA polymerase I (*Promega*) and blunted with 6 U of T_4 DNA polymerase (*Promega*). cDNA double strand ($cDNA_{ds}$) were successively purified with 1 volume of isoamyl: phenol:chloroform (1:25:24) and 1 volume of isoamyl: chloroform (1:24), and precipitated using 3 volumes of 100 % ethanol added to 0.1 volume of ammonium acetate (3 M). Extracted $cDNA_{ds}$ were quantified using a *Nanodrop* spectrophoto-meter and checked by electrophoresis on a 2 % agarose gel. The AFLP reactions were performed according to Vos *et al*. (1995) and Bachem *et al*. (1996) with minor modifications. 500 ng of $cDNA_{ds}$ were digested by restriction enzyme pairs *Eco*RI/*Msp*I or *Eco*RI/*Mse*I (10 U each) (*BioLabs*, Ozyme, France). The restriction fragments were ligated to specific double strand adaptors with 400 U of T_4 DNA ligase (*Promega*) at 16 °C for 4 h. The PCR pre-amplification step was carried out on 0.004 cm^3 of ligated products diluted 10-fold in sterile water using the combinations *Eco*RI/*Msp*I or *Eco*RI/*Mse*I primers without selective nucleotides and 1 U of *Red GoldStar*™ Taq polymerase (*Eurogentec*, Seraing, Belgium). The PCR cycling conditions consisted in an initial step of 2 min at 94 °C followed by 25 cycles (94 °C for 30 s ; 55 °C for 1 min ; 72 °C for 1.30 min). The PCR products were diluted 20-fold in water and 0.004 cm^3 served as a matrix for selective amplification with 64 possible combinations of *Eco*RI adaptator (+N) and *Msp*I or *Mse*I adaptator (+NN) primers. PCR selective amplification consisted in 35 cycles including 11 touchdown cycles (65 - 56 °C in 0.7 °C step) subsequently maintained at 56 °C for the last 24 cycles. A final step of 72 °C for 10 min was adopted. About 0.003 cm^3 of heat denatured PCR product was resolved on a 5 % denaturing polyacrylamide (*Bio-Rad*, Marne la Coquette, France) sequencing gel containing

7 M urea. The amplicon bands were visualized by silver staining (0.1 %) according to Chalhoub *et al*. (1997). The reproducibility of the method was tested by comparing the pattern of two independent cDNA-AFLP procedures on three samples with 5 primer sets (data not shown).

Selection of differentially expressed transcript-derived fragments (TDFs) was visually made on AFLP-derived gels. These identified TDFs were excised from dried gels, eluted into 0.02 cm^3 of *Taq* polymerase buffer (1×) (*Qiagen*), heated at 94 °C for 4 min and then left to diffuse overnight at 4 °C. PCR re-amplification was performed with 0.01 cm^3 cDNA extracted using the same conditions and the non-selective *Eco*RI and *Msp*I or *Mse*I primers used for the non-selective amplification. PCR products were checked on a 2 % agarose gel and cloned into *pGEM®T-easy* vector (*Promega*) and then transfected into competent *Escherichia coli DH5α* (*Promega*) in accordance with the manufacturer's instructions. One clone pre-amplified fragment was sequenced (*Cogenics*, *Genome Express*, Rhône Alpes, France). Analysis of the nucleotide sequence as well as potential relative translated sequences was carried out using respectively *BLASTN* and *BLASTX* algorithmic tools (Altschul *et al*. 1997) from publicly available *GenBank* non-redundant genes/transcripts databases (http:// www.ncbi.nlm.gov/BLAST). In parallel, sequences were compared with the expressed sequence tag (EST) databases using the *BLASTN* algorithm by targeting *Juglans* organism (*NCBI* web server; *taxid 16718*) to gain potential further information. Sequences were manually assigned to putative functional categories based on the scientific literature analysis and the information reported for each sequence by *The Gene Ontology Consortium* (http://www.geneontology.org). Sequence data from this article can be found in the *GenBank*/*EMBL* data libraries under *GenBank* accession numbers GR410235 to GR410424.

Real-time quantitative RT-PCR (qPCR) was used to assess the expression pattern of 30 TDFs (third of 93 identified TDFs by cDNA-AFLP analysis), and of two *JrPIP2s* (*JrPIP2;1*, AY189973 and *JrPIP2;2*, AY189974) in leaves. Experiments were carried out after 0, 5, 10, 15, 30, 60 and 120 min under irradiance and 120 min after return to darkness). *Juglans regia* 18S rRNA gene (AF399876) was chosen as an internal reference for normalization of transcript abundance of the target genes. Three independent leaves were used for each time point.

All sets of primers and the related annealing temperature are described on http://www.sakretal2011.blogspot.com/). The amplification efficiency of all primer sets was checked routinely (data not shown).

Total RNA was extracted according to the method of Chang *et al*. (1993), and 2 µg was used for the single-stranded cDNA synthesis, using *SuperScript III* (*Invitrogen*, Carlsbad, CA, USA) following the manufacturer's instructions. qPCR amplification was performed using an *iCycler iQ* (*Bio-Rad*, Hercules, CA, USA) in 0.03cm^3 reaction mix using platinum *Taq* DNA polymerase (*Invitrogen*) according to the supplier's instructions, 10 µM specific primers (supplementary data 2, http://www.sakretal2011.blogspot.com/) and 0.5 mm^3 SYBR green I (1/1000, *Sigma,* Lyon, France). The PCR conditions were 94 °C for 3 min for denaturation, followed by 35 cycles of 94 °C for 20 s, 50/61 °C for 20 s and 72 °C for 20 s for amplification. Relative quantification (Qr) of the target genes was determined by comparison with the reference gene and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) where $\Delta\Delta C_T$ is calculated as follows:

$\Delta\Delta C_T = (C_{Tt0} - C_{Ttx})_{Target}/(C_{Tt0} - C_{Ttx})_{18s}$, where C_T represents threshold cycle number of real-time PCR, tx represents time x for any point of the kinetic and t0 corresponds to the darkness control. Values were represented as $\log_2$ of Qr. For each investigated gene, three independent technical replicates were performed in addition to a second biological replicate. Values described represent means ± standard deviation.

For water flow parameters, each value is mean of 5 independent measurements. The effect of the different treatments on leaf hydraulic conductance was assessed by one-way *ANOVA* followed by a Tukey HSD post-hoc test. We considered the results statistically different at $P < 0.01$.

Results

Response of K_{leaf} and aquaporin transcripts to irradiance: In order to re-confirm the correlation between the pattern of K_{leaf} and the transcript level of two aquaporin isoforms (*JrPIP2;1* and *JrPIP2;2*) real-time quantitative RT-PCR was carried during a 120 min irradiance and 120 min after return to darkness (Fig. 1). Our data showed that the kinetics of K_{leaf} paralleled those of *JrPIP2s*. The abundance of both *JrPIP2s* transcripts increased significantly within the first 15 min of irradiance. Maximum values of K_{leaf} and transcript levels of *JrPIP2s* were achieved 1 h after the onset of irradiance. A concomitant drop in K_{leaf} and PIP2s transcripts occurred after return to darkness.

Isolation of radiation-responsive TDFs: Overall, the analysis of the 128 primers with 3 × 2 neighboring selective nucleotide combinations (*Eco*RI-N3 and *Mse*- or *Msp*I-N2) yielded a profiling of about 12 000 TDFs. Their sizes ranged between 50 to 1 300 bp, and all bands > 50 bp were analyzed. A comparison of cDNA-AFLP patterns revealed 592 TDFs with differentially up- and down-modulated expression between assayed samples. These regulations were defined herein as having clear differential band intensities under irradiance and in darkness for one or more of the early time points. From this pool, a subset of 225 quantitatively reliable TDFs was chosen due to their earlier responsiveness to

irradiance (15 and/or 30 min). Among them, 126 were up-regulated and 99 down-regulated genes. 38 TDFs were redundant sequences. Finally, 187 TDFs were used as queries for a bioinformatic characterization to search significant similarities and potential structural homologies with public nucleotide and amino acid sequence databases, using *BLASTN* and *BLASTX*, respectively. A minimal expectation value corresponding to the probability that a homology could be random was fixed at 10^{-4}, except for a short sequence TDF046 (78 bp) which had an *E*-value of 1.1 for 87 % of identities with the ethylene responsive element binding factor type 5 (*ATERF5*) of *Arabidopsis thaliana* (BAA97157). Nucleotide sequences of all TDFs identified through this study have been recorded and are available in the *GenBank/EMBL* sequence database.

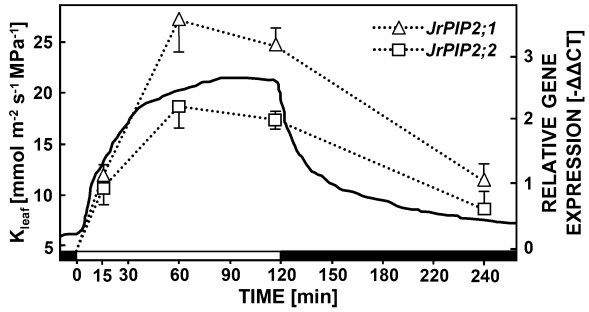

Fig. 1. Real-time quantitative RT-PCR analyses of the relative expression rate of genes encoding aquaporines *JrPIP2;1* and *JrPIP2;2* in walnut leaves at 15, 60 and 120 min under irradiance and 120 min after return to dark. Relative gene expression was normalized by comparing ΔΔCt of the *JrPIP2s* genes and *J. regia* 18S rRNA gene, where the starting state quantity was the dark control point 0 min. K_{leaf} values are mean of five independent leaves and PCR values are mean of three independent measurements.

Functional classification of radiation-responsive TDFs: A functional classification of the TDFs identified in this research was carried out according to *Gene Ontology* (http://www.geneontology.org). A total of 93 TDFs were identified, and divided into 10 categories containing their putative functions (suppl. data 4, http://www.sakretal2011.blogspot.com/). The largest number of TDFs was related to signal transduction (19 TDFs; 20.5 %). This category was followed by maintenance protein (14 TDFs; 15 %), DNA/RNA maintenance (12 TDFs; 12.9 %), binding regulatory proteins (9 TDFs; 9.7 %), fatty acid metabolism (9 TDFs; 9.7 %), cellular metabolism (9 TDFs; 9.7 %), and sugar metabolism (7 TDFs; 7.5 %). These first four categories, involved in regulator modules in management and transmission of cellular signal, comprise 54 TDFs (57.9 %). The lowest number of TDFs was found for three functional categories: stress and defense-related proteins (6 TDFs; 6.4 %), transport facilitation (5 TDFs; 5.4 %), and metabolism (3 TDFs; 3.2 %). 21 TDFs showed similarities with protein sequences found in others plant species but were devoid of any known function. 68 TDFs returned no similarity match with known sequences, including 31 up-regulated and 37 down-regulated genes after exposure to light (suppl. data 3; http://www.sakretal2011.blogspot.com/). These "anonymous ESTs" can not be identified because *1*) their sequences are too short to reveal significant homology, *2*) their amplified sequences were located in the highly variable gene sequence, or *3*) they have never been cloned before, because of the scarcity of molecular research that covers *Juglans* taxon. Finally, 5 TDFs showed no similarity with sequences from other green organisms, but significant similarities with prokaryote sequences revealing a natural contamination artifact. These fragments were not further investigated.

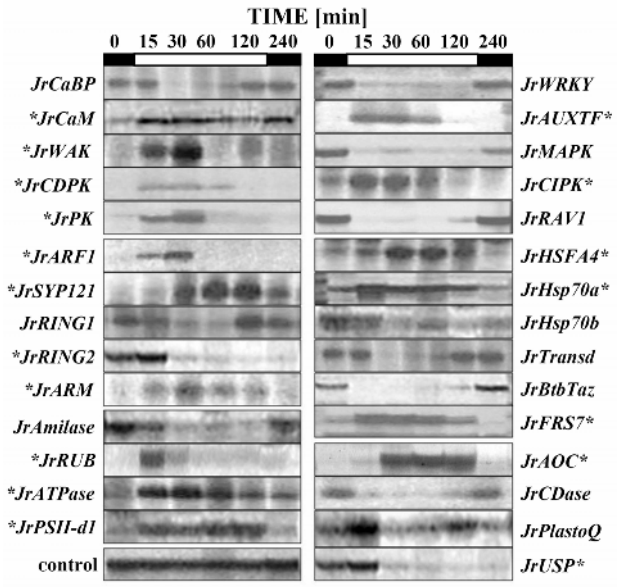

Fig. 2. Putative functional classification categories of the early differentially-expressed light-regulated transcript-derivated fragments (TDFs) identified by cDNA-AFLP in walnut leaves. Predictive functions were established on the basis of the best matches obtained from *BlastX* and *BlastN* searches to sequences in the *NCBI* database. Expected values greater than e^{-04} are considered not statistically significant (* corresponds to the up-regulated TDFs under light).

Pattern expression of 30 TDFs: The efficiency of cDNA-AFLP was evaluated by real-time quantitative RT-PCR (qPCR) analysis, conducted on 30 TDFs (Fig. 3). No significant difference was found between data of cDNA-AFLP and those of qPCR experiments, highlighting the robustness and reproducibility of this approach. Of these 30 selected radiation-responsive TDFs, 11 genes were related to signal transduction (Fig. 3*A*,*B*), 5 to binding regulatory proteins (Fig. 3*C*), 2 to fatty acid metabolism, 1 to cell wall strengthening, 2 to stress defense (Fig. 3*D*), 1 to sugar metabolism, 3 to photosynthesis (Fig. 3*E*), 2 to synthesis *de novo* and vesicles trafficking of protein and 3 to ubiquitination (Fig. 3*F*).

Among the TDFs of the signal transduction category,

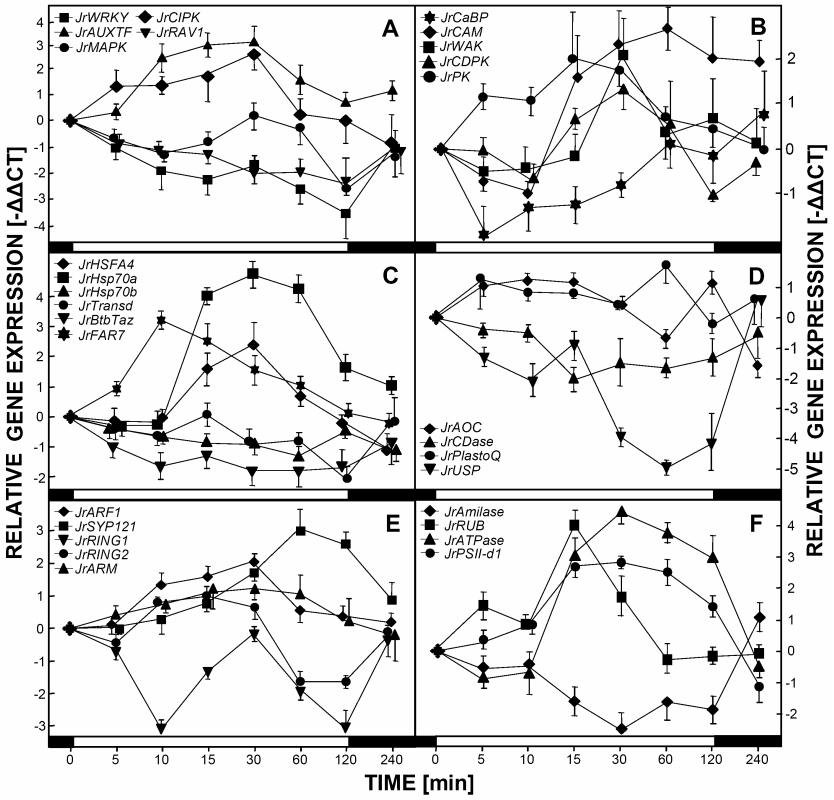

Fig. 3. Real-time quantitative RT-PCR analyses of the expression of selected TDFs in walnut leaves after 5, 10, 15, 30, 60 and 120 min under irradiance and 120 min after return to dark. TDFs were selected from the 7 following functional categories: transduction of signal (*A*, *B*), protein regulation (*C*), cell-wall integrity and stress defense (*D*), photosynthesis (*E*), ubiquitination and vesicle trafficking (*F*). Relative gene expression was normalized by comparing ΔΔCt of genes of interest and *J. regia* 18S rRNA gene, where the starting level was the dark control point 0 min. Relative transcript abundance were represented as log of Qr. Values are means of three independent measurements.

JrCIPK (calcineurin interacting protein kinase), *JrCAM* (calmodulin), *JrCDPK* (Ca^{2+}-dependent protein kinase) and *JrCaBP* (calmodulin-like) were identified. Transcript levels of *JrCIPK*, *JrCDPK and JrCAM* were transiently up-regulated by irradiance, and peaked after 15 min (6-fold), 30 min (3-fold) and 60 min (5-fold), respectively. Except for *JrCAM*, *JrCIPK* and *JrCDPK* returned to its basal expression after 120 min, and remained constant when leaves were back in darkness. Conversely, *JrCaBP* expression significantly and transiently decreased, to reach its lowest level after 60 min, before returning to basal levels when leaves were back in darkness (Fig. 3*B*).

In the same functional categories, transcript levels of *JrAUXTF* (auxin response transcription factor-like protein) and *JrPK* (receptor serine/threonine kinase) were found to be transiently up-regulated by irradiance and peaked after 15 min (Fig. 3*A*,*B*), whereas those of *JrWRKY* (WRKY-type transcription factor), *JrRAV* (AP2 domain transcription factor RAV1-type) and *JrMAPK* (mitogen activated protein kinase) were down-regulated in response to irradiance. Up-regulation of *JrWAK* (wall-associated kinase) peaked at 15 min, but was preceded by a diminution within the first 10 min of irradiance.

As concern binding regulatory proteins, two sets of TDFs could be distinguished (Fig. 3*C*) according to their response to irradiance, *JrHsp70a* (heat shock protein 70), *JrHSFA4* (heat shock transcription factor) and *JrFAR7* (FAR1-related sequence 7) underwent early up-regulation. Transcript levels of *JrHsp70a* peaked after 10 min, and those of *JrHSFA4* and *JrFAR7* did so after 30 min. Conversely, transcript accumulation of *JrTransd* (transducine-like), *JrHsp70b* (heat shock protein 70) and *JrBtbTaz* [protein with an N terminal BTB/POZ (broad-complex, tramtrack, and bric-a-brac/poxvirus and zinc finger) domain, a central TAZ (transcriptional adaptor zinc finger) domain and a C terminal calmodulin-binding domain (CaMBD)] were down-regulated.

For the category cell wall and stress defense-related (Fig. 3*D*), three TDFs [*JrXTR* (xyloglucan endotransglycosylase), *JrUSP* (universal stress protein) and *JrCDase* (ceramidase)] were down-regulated during irradiance. The highest inhibition was found for *JrXTR* and *JrUSP* after a 2-h exposure. In contrast, *JrPlastQ* and *JrAOC* were slightly up-regulated. Most of these TDFs returned to their basal level when leaves were back in darkness.

Most of TDFs concerning photosynthesis (Fig. 3*E*),

were early and transitory up-regulated by irradiance. These TDFs [*JrRUB* (ribulose biphosphate carboxylase), *JrATPase* (ATP synthase) and *JrPSII-d1* (protein D1 of photosystem 2)] peaked after 15 min. Under the same experimental condition, *JrAmylase* (amylase β-type) was down-regulated and the highest reduction was observed at 30 min after irradiance.

The majority of genes concerning ubiquitination and trafficking were up-regulated by irradiance [*JrARF1* (ADP-ribosylation factor 1), *JrARM* (armadillo/catenin repeat family protein) and *JrSYP121* (syntaxin 121)] (Fig. 3*F*). The highest stimulation was found for *JrSYP121* after 60 min. Expression pattern of *JrRING2* was marked by a slight stimulation during the first 30 min, followed by a down-regulation until 120 min of irradiance. *JrRING1* (RING-finger copine like protein) was early and strongly down-regulated by light. Expression of all these TDFs returned to its basal level when leaves were back in darkness.

Discussion

The cDNA-AFLP approach offered, for the first time, a large review of dynamic modulations in the transcriptome profiling of *Juglans* leaves under exposure to irradiance. 93 TDFs were selected due to the sensitivity and reliability of their expression pattern, and to their significant similarity with known proteins. In addition, the data of qPCR experiments carried out on 30 pre-selected TDFs were consistent with those of cDNA-AFLP (Fig. 3).

Ten functional categories were identified (suppl. data 4, http://www.sakretal2011.blogspot.com/). Cellular regulation including 3 major categories (signal transduction, protein and nucleotide matrices maintenance, and binding regulatory protein) was the largest with 54 TDFs. Here, the hypothetical link between one, or many, functional category and K_{leaf} is discussed.

Four TDFs encoding ribulose bisphophate carboxylase (*JrRUB*), protein J of photosystem II, protein D1 of photosystem II (*JrPSII-d1*) and a chloroplastic ATP synthase β-subunit (*JrATPase*) were found to undergo early and significant up-regulation by irradiance (Fig. 3*E*). These results might suggest a link between mechanisms regulating photosynthesis and K_{leaf} including *JrPIP2s*. This hypothesis may fit in with recent data showing direct evidence of a role for the PIP homologue of *Nicotiana tobacum* NtAQP1 in CO_2 transport in leaves and its localization in both the plasmalemma membrane and chloroplast envelope (Flexas *et al*. 2007, Uehlein *et al*. 2008). Within the latter, NtAQP1 could account for ~90 % of CO_2 permeability. An important future task will be to determine whether stimulation of K_{leaf} by irradiance could be related to stimulation of photosynthesis and mesophyll conductance (g_m) to CO_2 (Evans *et al*. 2009).

One of the TDFs exhibited a high homology with gene encoding the cell wall enzyme xyloglucan endotransglycosylase (*JrXTR*) (Fig. 3*D*). The XTR is able to cut and graft xyloglucan molecules to each other (Thompson *et al*. 1997, Ito and Nishitani 1999), and thereby contributes to the loosening of cell walls (Hayashi 1989, Fry 1989, McCann *et al*. 1990). The inhibition of the transcript level of *JrXTR* modified the extensibility of the cell wall, required for the regulation of cell pressure potential. Such an event coupled with a stimulation of *JrPIP2s* (Fig. 1) could promote a high leaf water content and pressure potential in our experimental conditions.

A significant outcome of this study was the identification of TDFs involved in the first signal transduction step, including kinases [wall associated kinase (*JrWAK*); serine/threonine kinase (*JrPK)*, CBL-interacting protein kinase (*JrCIPK*), calcium-dependant protein kinase (*JrCDPK*), mitogen activated protein kinase (*JrMAPK*)], receptor kinase (Ferronia receptor-like) and two calcium-dependent proteins [camoduline (*JrCAM*), calcium binding protein (*JrCaBP*)]. These comprise one of the higher modulated categories. Many of these TDFs share the common property of being associated with calcium, one of the major chemical mediators for living organisms. Here, 4 TDFs related to Ca^{2+}-signaling and encoded for the four major groups of calcium-binding proteins in plants [CaM, CaBP, CIPKs and CDPKs (Snedden and Fromm 2001, Luan *et al*. 2002, Sanders *et al*. 2002)] were found to be regulated by irradiance (Figs. 3*A*,*B*). The response of aquaporins to calcium variation has been shown in plants. Indeed, the fluctuation of Ca^{2+} concentration can affect either expression of respective genes (Maathuis *et al*. 2003), or activities of aquaporins (Johnson and Chrispeels 1992, Johansson *et al*. 1996, Alleva *et al*. 2006, Van Wilder *et al*. 2008).

There were 9 early modulated putative transcription factors: an AP2domain RAV1-subfamily, an ethylene responsive binding factor 5-type, an auxin responsive factor, a homeotic transcription factor, 2 heat shock transcription factors (see below), and 3 WRKY transcription factors. Among these, WRKY TF are a large family of regulatory proteins (Eulgem *et al*. 1999, Wei *et al*. 2008), defined as master regulators of plant response to biotic and abiotic stimuli (Du and Chen. 2000, Pandey and Somossich 2009, Ren *et al*. 2010). However, few studies have described the involvement of these transcription factors in light signaling. The only available data indicated that an over-expression of WRKY89 enhances UV-B tolerance and disease resistance in rice plants (Wang *et al*. 2007). Here, we showed, for the first time, that 3*WRKY* TDFs were early down-regulated in response to irradiance. These new findings might emphasize the importance of WRKY factors in transcriptional reprogramming of plant in responses to irradiance, probably acting as negative regulators. The expression pattern of *JrWRKY* revealed a negative

correlation with K_{leaf} dynamics and the expression pattern of *JrPIP2s* (Fig. 3*A*,*C*). As K_{leaf} was closely related with transcript abundance of two walnut aquaporins, *JrPIP2,1* and *JrPIP2,2* (Fig. 1), the identification of a *JrWRKY*-responsive *cis*-element on the promoter of these aquaporins might support a possible role of WRKY TF in induction of K_{leaf} by irradiance.

Of particular interest is the identification of two genes that encode putative regulatory proteins. Two of them are discussed: FRS7 (for FAR1-related Sequence 7), and BTB and TAZ domain protein. They were found to be up- and down-regulated, respectively, in leaves under irradiance (Fig. 3*C*). FRS7 protein is a member of the nuclear FRS protein family that appears to be linked to the phytochrome signaling pathway (Lin and Wang 2004). These interact with diverse partners to control a broad range of signaling processes (Wang and Deng 2002), as being essential positive signal transducers for phyA-mediated FR-high irradiance responses (HIRs) in response to white light. With respect to *JrBtbTaz* TDF, this is one member of a BTB protein subfamily (Ren *et al*. 2007, Robert *et al*. 2008, Mandadi *et al*. 2009). The role of BTB proteins in mediating signaling pathways of diverse stimuli were recently documented (Mandadi *et al*. 2009). In this respect, the *Arabidopsis thaliana* gene BT2 is maximally expressed in the dark and it is under circadian control (Mandadi *et al*. 2009). Consistently with these data, *JrBtbTaz* was found to be down-regulated under irradiance, and almost returned to its high level when leaves were back in darkness. It will be useful to determine whether these two transduction modulators could bind directly to transcription factors targeting aquaporin genes.

The effect of irradiance can also be extended to components involved in regulation of proteins, including heat stress proteins (HSPs). These proteins play an essential role in maintenance and/or restoration of protein homeostasis (Baniwal *et al*. 2004) under normal and stress conditions (Lee and Vierling 2000). The modulation of their related genes is under the control of heat shock transcription factors (HSFs). Under our experimental conditions, a transient transcript accumulation encoding five HSP (18, 40, 70, 101 kD) and two HSFs (HsfA2 and HsfA4-types) was observed (Fig. 3*C*). This is rather unexpected because HSPs have been commonly studied in heat stress contexts. Here, we paid particular attention to three of them, the chaperone *JrHsp70* and the two heat stress transcription factors, *JrHsfA2* and *JrHsfA4*-types, which showed simultaneous early differential transcript abundance (Figs. 3*C* and 4). The protein product of *Hsp70* is implicated in trafficking and stabilization of newly synthesized proteins (Baniwal *et al.* 2004), including membrane channel proteins such as K^+-channel (Negulyaev *et al*. 1996). We have identified two *HSP70* isoforms which each harbored contrasted modulation: *JrHsp70a* was up-regulated whereas *JrHSP70b* was down-regulated. Such a regulation supports the idea of an auto-repression in this class of chaperones (Kim and Schöffl 2002). Under high irradiance, increasing the K_{leaf} may be essential not only to supply CO_2 and water for challenged cells, but also for leaf cooling. Thus, HSP70 may contribute to the aquaporin protein structure strengthening and so safeguard their transport function when the temperature of leaves increases with differential water movements. The similar role of *JrHSFA* in K_{leaf} induction may be possible, due to the significance of the *de novo* protein biosynthesis in this process (Cochard *et al*. 2007). Therefore, it is tempting to speculate that HSFA4 and HSFA2 differentially expressed in our light irradiance condition may be the potential transcription factors which control the modulation of the *JrHSP70* and *JrPIP2s* genes. A detailed analysis of *cis*-regulating motifs within their promoters would provide answers to such assumptions.

Modification of vesicle trafficking upon irradiance is consistent with the up-regulation of the vesicle-targeted genes *ADP-ribosylation-factor 1(ARF1*) and *SYP121* (syntaxin 121) (Fig. 3*F*). ARF1 proteins are considered central to orchestrating asymmetrical cell vesicle trafficking, and are primarily localized in Golgi apparatus and a subpopulation of post-Golgi vesicles (Matheson *et al*. 2008). Concomitant induction of *SYP121*, a *SNAP receptor/protein anchor* [SNAP for soluble NSF (N-ethylmaleimide-sensitive fusion protein) - attachment protein] located in *cis*-Golgi cisternae, reinforces the indication of enhanced protein trafficking between endoplasmic reticulum (ER) and Golgi. The modification of *trans*-Golgi vesicle trafficking as well as endocytosome (spherical bodies in Fig. 4) seems to be under the control of clathrin coat adaptators, themselves regulated by ARFs (Ooi *et al*. 1998). Interestingly, the steady state level of certain PIP subfamily aquaporins in *Arabidopsis* undergo a constitutive cycling mediated by clathrin-coated vesicles, and a possible in-cell internalization through the sorting *Nexin 1* (SNX1) endosomes (Paciorek *et al*. 2005, Dhonukshe *et al*. 2007, Jaillais *et al*. 2008). The use of *Brefeldin A* and/or *Tyrphostin A23*, an inhibitor of cargo protein trafficking from endosomes to the plasmalemma membrane and a structural analog of tyrosine which interferes with clathrin function, may highlight the involvement of such a trafficking pathway in the light stimulation of *JrPIP2s*.

In the same way, a TDF exhibited high homology with a gene encoding ceramidase (*JrCDase*) (Fig. 3*D*) that is a central enzyme in sphingolipid metabolism and signaling (Worrall *et al*. 2008). By cleaving fatty acids from ceramides (knows as sphingosine), this produces a sphingosine that in turn is phosphorylated by a sphingosine kinase to form sphingosine-1-phosphate (S1P). Sphingosine and S1P are two motile second messengers, found in the plant kingdom. In addition, S1P is an active Ca^{2+}-mobilizing messenger in guard cells during drought and abscissic signaling (Ng *et al*. 2001). Down-regulation of ceramidase by irradiance may mean that perception of a signal elicits a fluctuation of cytosolic Ca^{2+} content *via* production of S1P in leaves. Because these compounds can cross the entire lipid bilayer, they

seem to be particularly important in membrane domain formation and vesicle trafficking, vesicle endocytosis, and diverse cellular signaling pathways (Rohrbough *et al*. 2003, Helms and Zurzolo 2004). However, the means by which ceramide acts as a signaling molecule are not clear.

In this research, twelve genes putatively related to the proteolysis underwent early and significant up-regulation by irradiance (suppl. data 4, http://www.sakretal2011.blogspot.com/). Among the most significant, we found a gene encoding E2 ubiquitin protein ligase, and various isoforms of E3 ubiquitin ligases and RING-finger copine-like protein and two F-box proteins. Among the F-box protein family, a transducin was reported to be up-regulated under irradiance and plays an important role in the protein degradation by acting as components of SKP cullin F-BOX protein ligases that selectively act on a subset of proteins including β-catenin. A gene encoding an armadillo/catenin repeat family protein (*JrARM)* was among the radiation-responsive TDFs identified. These findings fit in with recent research highlighting the role of ubiquitin-/26S proteasome-mediated protein degradation in the light-signaling pathway (Hoecker 2005). In addition, the role of ubiquitination in the aquaporin function was directly shown by Lee *et al*. (2009). These authors established novel links in plants between ubiquitination and aquaporin trafficking and degradation, with an implication of physical interaction of a RING membrane anchor E3 ubiquitin ligase to aquaporin (*AtPIP2;1*). Such a regulation of aquaporin by ubiquitination is reminiscent of the hypertonicity-dependent degradation of mammalian AQP1 (Leitch *et al*. 2001). To uphold this hypothesis in our experimental conditions, pharmacological investigations based on the utilization of 26 proteasome inhibitor (MG132) will be carried out in the near future.

In conclusion, K_{leaf} increases within minutes after exposure to irradiance in parallel with the transcription of two members of *JrPIP2s*. However, such a short time underpins the idea that these physiological events cannot be only explained through a single transcription of aquaporin genes. By obtaining early temporal expression profiles, the diversity of up- and down-regulated TDFs suggest that regulation of K_{leaf} might be paralleled with very complex processes, and that further analyses are certainly required to identify which ones could directly be involved in light-induced K_{leaf}. These considerations may lay the foundations of the mechanistic aspects of light-mediated regulation of both hydraulic conductance and aquaporin expression, and their integration could be central for a deeper understanding of the leaf-water relations.

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