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ORIGINAL ARTICLE

A fungal transcription factor gene is expressed in plants from its own promoter and improves drought tolerance

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Abstract

Main conclusion **A fungal gene encoding a transcription factor is expressed from its own promoter in *Arabidopsis* phloem and improves drought tolerance by reducing transpiration and increasing osmotic potential.**

Horizontal gene transfer from unrelated organisms has occurred in the course of plant evolution, suggesting that some foreign genes may be useful to plants. The *CtHSR1* gene, previously isolated from the halophytic yeast *Candida tropicalis*, encodes a heat-shock transcription factor-related protein. *CtHSR1*, with expression driven by its own promoter or by the *Arabidopsis UBQ10* promoter, was introduced into the model plant *Arabidopsis thaliana* by *Agrobacterium tumefaciens*-mediated transformation and the resulting transgenic plants were more tolerant to drought than controls. Fusions of the *CtHSR1* promoter with β-glucuronidase reporter gene indicated that this fungal promoter drives expression to phloem tissues. A chimera of CtHSR1 and green fluorescence protein is localized at the cell nucleus. The physiological mechanism of drought tolerance in transgenic plants is based on reduced transpiration (which correlates with decreased opening of stomata and increased levels of jasmonic acid) and increased osmotic potential (which correlates with increased proline accumulation). Transcriptomic analysis indicates that the *CtHSR1* transgenic plants overexpressed a hundred of genes, including many relevant to stress defense such as *LOX4* (involved in jasmonic acid synthesis) and *P5CS1* (involved in proline biosynthesis). The promoters of the induced genes were enriched in upstream activating sequences for water stress induction. These results demonstrate that genes from unrelated organisms can have functional expression in plants from its own promoter and expand the possibilities of useful transgenes for plant biotechnology.

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Abbreviations

ABA	Abscisic acid
DAPI	4′,6-diamino-2-phenylindole
GFP	Green fluorescent protein
GUS	β-Glucuronidase
HSE	Heat-shock element
HSF	Heat-shock transcription factor
IAA	Indolacetic acid
JA	Jasmonic acid

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Introduction

The fact that horizontal gene transfer from unrelated organisms has occurred in the course of plant evolution (Bock 2009; Talianova and Janousek 2011; Schönknecht et al. 2014) indicates that some foreign genes had been useful to plants. This also suggests that complete foreign genes, with their own promoter, may be useful for plant biotechnology, in particular genes from organisms surviving under severe stress conditions because the encoded proteins could be special for stress tolerance (Reed et al. 2013; Schönknecht et al. 2014).

Some time ago we reported that a gene from the halotolerant yeast *Candida tropicalis* improved salt stress tolerance in the model yeast *Saccharomyces cerevisiae*. This gene, named *CtHSR1*, up-regulated in *S. cerevisiae* the *ENA1* gene, encoding a cation-extrusion pump important for sodium stress tolerance (Ali et al. 2001). CtHSR1 has at its N-terminus a domain of about 80 amino acids with homology to the DNA binding domain of transcription factors of the heat-shock response (HSFs) and several low-complexity regions at its C-terminus. The latter are regions characterized by a high level of amino acid (and nucleotide) sequence repetition and tend to occur in disordered regions of transcription factors that could act as activation domains (Alba et al. 2007). CtHSR1, like other HSF-related transcription factors of the Ets family (Landsman and Wolffe 1995), has no homologous regions to the trimerization and activation domains of true HSFs. HSF trimers bind to contiguous arrays (at least 2) of the 5 bp sequence XGAAX arranged in alternating orientation (heat-shock element, HSE) and activate transcription of downstream genes (Sorger 1991). The HSF-related proteins bind as monomers to the GAA trinucleotide of the HSE and activate genes without repeats of this element (Landsman and Wolffe 1995).

Considering that *CtHSR1* improves tolerance to salt stress in *S. cerevisiae* although this yeast has no orthologous transcription factors (Ali et al. 2001), it is plausible that *CtHSR1* could improve tolerance to some stresses in unrelated organisms such as plants. To test this hypothesis we transformed *Arabidopsis thaliana* plants with a genomic fragment containing the *CtHSR1* gene and our results indicate that this fungal gene improves drought tolerance. The promoter of *CtHSR1* drives expression at phloem tissues and the CtHSR1 protein localizes to the nuclei of plant cells. Physiological and transcriptomic studies indicated that CtHSR1 increases jasmonic acid concentration, reduces stomatal aperture and transpiration and increases osmotic potential and proline concentration.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (L.), Heynh. plants (ecotype Columbia-0 or Col-0; Nottingham Arabidopsis Stock Centre, NASC N6673) were grown in the greenhouse and in vitro culture as previously described (Alejandro et al. 2007). The conditions were: 23 °C, 70 % relative humidity and 16-h light (130 µmol photons m^{-2} s^{-1})/8-h dark. Plants of *Nicotiana tabacum* L. (cv. Samsun, kindly donated by Prof. Angelo Spena, Verona, Italy) and *Nicotiana benthamiana* Domin. (kindly donated by Dr. Diego Orzaez, IBMCP, Valencia) were grown in the greenhouse under same conditions as that of *Arabidopsis*.

Plant transformation with a genomic fragment containing the fungal *CtHSR1* gene

A 5829 bp *Bam*HI–*Xba*I genomic fragment from *Candida tropicalis* containing the *CtHSR1* gene (3003 bp of 5′-upstream promoter region, 2187 bp ORF with stop codon and 639 bp 3′-downstream region; see Ali et al. (2001) and GenBank AJ296093.1) was inserted into pBIN19 binary vector (Bevan 1984) and the recombinant plasmid ($P_{CtHSR1}::CtHSR1$) used for transformation of *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) to transform tobacco and strain GV3101::pMP90 (Koncz and Schell 1986) to transform *Arabidopsis*.

For tobacco transformation, seeds of *N. tabacum* cultivar Samsun were surface sterilized and germinated on 1/2 strength MS with 3 % sucrose and 1 % agar. Fully expanded leaves were cut in pieces for transformation. The *A. tumefaciens* LBA4404 containing $P_{CtHSR1}::CtHSR1$ (see above) was co-cultivated with leaf discs and transgenic plants were selected on medium containing 25 mg/l kanamycin. Transgenic plants were confirmed by PCR with primers HSR1Forward (5′-GCACCGCCAGTTCCATCTGG, starting at position 3862 of the genomic fragment) and HSR1Reverse (5′-CACTGGCGTTTCCATTCATAGG, ending at position 4969 of the genomic fragment), amplifying a fragment of 1.1 kb within the coding region. Six transgenic lines segregating 3:1 upon self-pollination (suggesting a single insertion) were raised for three successive generations to get homozygosis and three *CtHSR1* lines were selected for further analyses.

Arabidopsis thaliana Col-0 was transformed with *A. tumefaciens* GV3101::pMP90 containing plasmid $P_{CtHSR1}::CtHSR1$ (see above) by flower infiltration (Bechtold et al. 1993). Transgenic seedlings were selected in medium with 50 mg/l kanamycin and confirmed by PCR as described above. Seven transgenic lines segregating 3:1

upon self-pollination (suggesting a single insertion) were raised for three successive generations to get homozygosis and four *CtHSR1* lines were selected for further analyses and designed H1–H4.

Arabidopsis transformation with the coding region of *CtHSR1* expressed from the *AtUBQ10* promoter

The ORF with stop codon of *CtHSR1* (2187 bp) was amplified from the 5829 bp *Bam*HI–*Xba*I genomic fragment (see above) by PCR using Phusion High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) and primers HSR1CDSFwBamHI (5′-CG GGATCC AT-GAGTAAGAAAAGCAACAGTACC, *Bam*HI site before start codon underlined) and HSR1CDSRvHistKpnI (5′-GG GGTACC CTAGTGGTGGTGGTGGTGGTGATTT TCTTCTTTTTCAATAGTACTATCC, *Kpn*I site underlined before stop codon and a tail of six CAC histidine codons). PCR-derived constructs were verified by DNA sequencing. After digestion with *Bam*HI and *Kpn*I, the fragment was cloned into plasmid pGPTVII.Hyg. P_{UBQ10}::MCS digested with the same enzymes giving plasmid P_{UBQ10}::*CtHSR1*. PCR-derived constructs were verified by DNA sequencing. pGPTVII.Hyg.P_{UBQ10}::MCS is a derivative of pGPTVII.Hyg (Walter et al. 2004) where the *uidA* (*gusA*) gene was removed and the *AtUBQ10* promoter (Grefen et al. 2010) inserted before the multicloning site. The recombinant plasmid was used for transformation of *A. tumefaciens* strain GV3101::pMP90 to transform *Arabidopsis* as described above. Seven transgenic lines tolerant to 25 mg/l hygromycin with a single insertion were obtained and three were selected for analyses (designed as U1–U3).

Arabidopsis transformation with the coding region of *CtHSR1* expressed from the *35S* promoter with or without GFP fusion

The ORF of the *CtHSR1* gene was PCR amplified with high-fidelity DNA polymerase as described above with primers: HSR1CDSFw + 1 5′-ATG AGTAAGAAAAGCA ACAGTACC (start codon underlined) and either HSR1CD SRv + stop 5′-CTA ATTTTCTTCTTTTTCAATAGTAAC TATCC (stop codon underlined) or HSR1CDSRv-stop 5′-ATTTTCTTCTTTTTCAATAGTAACTATCC (no stop codon) and cloned in the pCR8/GW/TOPO Gateway entry plasmid (Invitrogen-Life Technologies, Carlsbad, CA, USA). The ORF with stop codon was cloned by in vitro recombination into binary plasmids pMDC32 (no GFP) and pMDC43 (GFP at N-terminus of *CtHSR1* ORF) while the ORF without stop codon was cloned into pMDC83 (GFP at C-terminus of *CtHSR1* ORF) (Curtis and Grossniklaus 2003). PCR-derived constructs were verified by DNA

sequencing. The P_{35S}::*CtHSR1*, P_{35S}::*GFP–CtHSR1* and P_{35S}::*CtHSR1–GFP* plasmids were used for transformation of *A. tumefaciens* strain GV3101::pMP90. The *Agrobacterium* containing the P_{35S}::*CtHSR1* plasmid was used to transform *Arabidopsis* as described above and three hygromycin-tolerant homozygous lines with a single insertion (S1–S3) were selected for analysis. The *Agrobacterium* transformants containing plasmids P_{35S}::*CtHSR1–GFP* and P_{35S}::*GFP–CtHSR1* were used for transient expression in leaves of *N. benthamiana* and confocal laser scanning fluorescence microscopy as described below. Transformation of *Arabidopsis* with these plasmids resulted in no detection of GFP.

Transient expression of *CtHSR1–GFP* fusions in leaves of *N. benthamiana* and confocal laser scanning fluorescence microscopy

Agrobacterium tumefaciens GV3101::pMP90 containing GFP-tagged constructs (P_{35S}::*CtHSR1–GFP* or P_{35S}::*GFP-CtHSR1*; see above) were incubated with shaking at 28 °C in Luria–Bertani (LB) broth for 24 h. Cells were harvested by centrifugation ($2500g$ 15 min 4 °C) and resuspended (10 mM MES-KOH, pH 5.6, 10 mM $MgCl_2$, 200 μM acetosyringone) to obtain an absorbance at 600 nm of about 1. These cells were mixed with an equal volume of culture at similar absorbance of strain C58C1 (pBin61 P_{35S}::*p19*), which expresses the silencing suppressor p19 of tomato bushy stunt virus (Voinnet et al. 2003). Bacteria were then incubated for 3–4 h at 28 °C before being injected into young, fully expanded leaves of 20-day-old *N. benthamiana* plants as described (Yang et al. 2000). Epidermal cell layers of agro-infiltrated leaves were examined for fluorescence under a TCS LS confocal laser scanning inverted microscope (Leica Microsystems, Wetzlar, Germany) 3–5 days after infiltration. The samples were treated with DAPI (4′,6-diamino-2-phenylindole; 1 mM) for 5 min prior to obtaining the microphotographs. GFP was excited at 488 nm and emission collected between 500 and 520 nm. DAPI was excited at 358 nm and emission collected at 461 nm.

Arabidopsis transformation with the *GUS* reporter expressed from the promoter of the *CtHSR1* gene (P_{CtHSR1}::*GUS*)

We constructed a promoter–reporter fusion with the promoter of the *CtHSR1* gene driving expression of the *GUS* gene (*gusA*) encoding β-glucuronidase. The genomic region upstream of the *CtHSR1*-coding region (3003 bp) was PCR amplified from the 5829 bp *Bam*HI–*Xba*I genomic fragment (see above) by high-fidelity DNA polymerase as described above with primers pHSR1-Fw-*Bam*HI (5′-CG GGATC CGGATCCATAATAGACAGAGGCAC, including the

genomic *Bam*HI site, underlined) and pHSR1-Rv-*Nco*I (5′-CATG<u>CCATGG</u>TTCTTTTTTGCAATCTTGGGA, with an *Nco*I site, underlined, added after the first nucleotide before the starting codon). After digestion with *Bam*HI and *Nco*I the 3 kb promoter fragment was cloned into binary plasmid pCAMBIA1303 (http://www.cambia.org/daisy/cambia/585.html) digested with the same enzymes, giving recombinant plasmid pCAMBIA1303-P_{CtHSR1}::*GUS*. This plasmid and the original pCAMBIA1303 were used for transformation of *A. tumefaciens* strain GV3101::pMP90 to transform *Arabidopsis* as described above. Five transgenic lines tolerant to 25 mg/l hygromycin with a single insertion were obtained from each construct and three were selected for analyses.

Determination of gene expression by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using NucleoSpin RNA II kit (Macherey–Nagel, Düren, Germany) and 3 μg RNA was reverse transcribed using the Maxima first-strand cDNA synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. PCR amplifications were performed on first cDNA strand corresponding to 150 ng of total RNA and each reaction was performed in triplicate in a total volume of 25 μl. For semi-quantitative PCR analysis of *CtHSR1* expression, the primers HSR1Forward and HSR1Reverse described above were used, the reference gene was *PP2AA3* (see primers given in Supplementary Table S1) and the number of cycles was 25 (S lines with 35S promoter) or 30 (H and U lines with *CtHSR1* and *AtUBQ10* promoters, respectively, and reference gene). Quantitative (real-time) PCR (qRT-PCR) was performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) with the 5× PyroTaq EvaGreen qPCR Mix Plus (ROX) (Cultek S.L.U., Madrid, Spain) according to the manufacturer's protocol. Data are the mean of three biological samples. PCR amplification specificity was confirmed with a heat-dissociation curve (from 60 to 95 °C). Efficiency of the PCR was calculated and different internal standards (*PP2AA3* or At5g55840) were selected depending on the efficiency of the primers (Czechowski et al. 2005). Relative mRNA abundance was calculated using the comparative ΔC_t method according to Pfaffl (2001). Primers for the different genes are described in Supplementary Table S1.

Assay of GUS activity

For qualitative histochemical GUS assays different plant organs from seedlings or adult plants were incubated in buffer with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcU, Duchefa, Haarlem, The Netherlands)

overnight at 37 °C (Naleway 1992). The plant material was cleared in 70 % (v/v) ethanol solution before microscopic observation in a binocular loupe of fluorescence MacroFluo MZZ16F (Leica) or a microscope Eclipse E-600 (Nikon, Chiyoda, Tokyo, Japan). All independent transgenic lines showed identical GUS-staining patterns and only differed in the expression level of GUS.

In the quantitative assay of GUS activity, 25 seedlings of each line were grown in solid MS medium under long-day conditions during 7 days. The samples were ground under liquid nitrogen and resuspended in 250 μl of extraction buffer (100 mM KPO$_4$ pH7.8, 1 mM EDTA, 1 % Triton X-100, 10 % glycerol and 1 mM DTT). Following centrifugation of the crude extract (14,000g, 15 min, 4 °C) proteins were quantified with the Bradford assay (Bradford 1976). For the fluorescent reaction, 5 μl extract was incubated at 37 °C in 100 μl of buffer with 4-methylumbelliferyl β-D-glucuronide (4-MUG) and after 15 min the reaction was stopped with 2 ml of 0.2 M Na$_2$CO$_3$ pH 10.3 (Naleway 1992). Fluorescence of the 4-methylumbelliferone (4-MU) product was quantified with a fluorometer (LS 50B, Perkin Elmer CytoFluor II, Waltham, MA, USA) with an excitation wavelength of 365 nm and an emission wavelength of 445 nm. The activity was given in pmol of 4-MU min^{-1} μg of protein^{-1}.

Transcriptomic analysis with long oligonucleotide microarrays

Two transgenic lines (H1 and H3) with very similar phenotypes (see below under "Results") were utilized together with the control, non-transformed line (Col-0). Three biological replicas were made for each transgenic line versus the control and only genes significantly altered in expression in at least four replicas were considered. For each experiment 30 seedlings of each line were grown in solid MS medium under long-day conditions during 7 days and all samples harvested at the same time of the day (middle of the light cycle). RNA extraction, amplification, labeling, and hybridization were performed as described previously (Bueso et al. 2007), and the analysis of microarray data was as described by Bissoli et al. (2012). Long oligonucleotide microarrays were provided by Dr. David Galbraith (University of Arizona; http://www.ag.arizona.edu/microarray/). Oligonucleotides were from the Operon Arabidopsis Genome Oligo Set version 3.0 (https://www.operon.com/), which contains 29,110 70-mer probes, representing 26,173 protein-coding genes, 28,964 protein-coding gene transcripts, and 87 microRNAs. The design is based on ATH1 release 5.0 of The Institute for Genomic Research Arabidopsis genome annotation database (http://www.tigr.org/tdb/e2k1/ath1/) and release 4.0 of the microRNA registry at the Sanger Institute (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml).

Identification of preferentially located motifs

The methodology has been described in detail (Bernard et al. 2010) and recently utilized in Bueso et al. (2014). We studied a set of 199 known motifs coming from the AGRIS database (Yilmaz et al. 2011), and for each motif, we extracted all occurrences in a promoter set. We defined promoters for 20,687 genes of *Arabidopsis* and for 105 genes out of 108 up-regulated in *CtHSR1* transgenic lines (see above). To define an overrepresented motif we performed a binomial probability to compare percentages between these 105 promoters and the whole promoter set of *Arabidopsis*. Each motif with a probability of less than 5 % was considered significant.

Assay of drought tolerance

Individual *Arabidopsis* plants were grown in square pots of $7 \times 7 \times 6.5$ cm placed in trays, irrigated twice a week to reach water field capacity and drained. After 4 weeks, and with the pots at their water field capacity, irrigation was stopped in half of the samples but continued for the other half. After 2 weeks, pictures were taken and fresh and dry weight of aerial part of 10 plants were determined.

Assay of plant growth in presence of abscisic acid (ABA)

Seedlings were grown on vertically plates for 7 days. Afterwards, plants were transferred to new plates with or without 10 μM of ABA. To score plant growth, fresh weight of each plant was measured after 21 days.

Stomata assays

For stomata assays, leaves of 7-day seedlings were used. Measurements were performed on microphotographs of epidermal cells, which were first incubated for 2 h at 20 °C in agitation and constant light in stomata opening buffer containing 10 mM KCl, and 10 mM MES/Tris, pH 6.2, supplemented or not with 0.5 μM ABA. Then, samples were incubated for 5 min in 0.001 % propidium iodide. Leaves were rinsed with distilled water and placed with the lower surface facing down on a microscope slide and samples were examined for fluorescence under a TCS LS confocal microscope (Leica) with excitation at 535 nm and emission collected at 617 nm. The stomata area and aperture (width/length), and number of stomata were measured with the ImageJ/Fiji software (http://fiji.sc/Fiji). Data were expressed as the average of four experiments with 30–40 stomata measured in each one.

Photosynthesis measurements

For photosynthesis measurements, leaves of 40-day-old plants grown in the greenhouse were used. The conditions were: 23 °C, 70 % relative humidity and 8-h light (130 μmol photons m^{-2} s^{-1})/16-h dark. Instantaneous determinations of net CO_2 assimilation (A_n; μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor (g_s; mol H_2O m^{-2} s^{-1}), transpiration rate (E; mmol H_2O m^{-2} s^{-1}) and quantum yield of photosystem II (Φ SII) were carried out under conditions of saturating light (1200 μmol m^{-2} s^{-1}), a vapor pressure difference of 1–2 kPa, and at 400 ppm CO_2 with an LI-6400 (Li-Cor, Lincoln, NE, USA).

Osmotic potential and cellular solutes

Leaves of 30-day-old plants grown in the greenhouse were used. Samples were ground in liquid nitrogen, centrifuged ($14,000g$ for 15 min) and the supernatant used for different measurements. Osmotic potential was measured by the freeze point depression method using an osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany). K^+ and Na^+ determination was by atomic absorption spectrophotometry (Alejandro et al. 2007), proline content was estimated using ninhydrin acid reagent according to Bates et al. (1973) and soluble sugars by high-performance liquid chromatography (HPLC; see Supplementary Methods). All solutes were expressed as mM in tissue water.

Quantification of indoleacetic acid (IAA), abscisic acid (ABA), and jasmonic acid (JA)

Hormones were quantified in the aerial part of 30-day-old plants grown in the greenhouse. Each sample consisted of material from five plants that was frozen immediately, ground under liquid nitrogen and aliquots of 100 mg of fresh weight were stored at −80 °C until analysis. The detailed procedures of the Plant Hormones Quantification Service of IBMCP (Universidad Politécnica de Valencia-CSIC, Spain) are described under Supplementary Methods.

Reproducibility and statistical analysis of the results

All the experiments were repeated at least three times and samples from *Arabidopsis* lines included 10–30 plants. We calculated the mean and the standard error (SE) and considered differences of the means to be statistically significant when $P < 0.05$ by Student test. In the analysis of 3:1 segregation for homozygosis of transgenic lines, the χ^2 test was employed.

Results

Arabidopsis plants transformed with the *CtHSR1* gene with its own promoter region or with the *UBQ10* promoter are more drought tolerant than controls

The initial experiment of plant transformation with a genomic fragment including the *CtHSR1* gene was done in tobacco. As indicated in Supplementary Fig. S1, transgenic plants have bigger leaves than control plants under irrigation conditions (Supplementary Fig. S1a), have a better aspect than control plants after drought treatment (Supplementary Fig. S1b) and could recover from drought stress upon re-watering while control plants could not survive (Supplementary Fig. S1c). As similar results were obtained with three transgenic tobacco lines (data not shown) we decided to further investigate the phenomenon in the model plant, *A. thaliana*.

As indicated in the left part of Fig. 1, four lines of *Arabidopsis* transformed with a genomic fragment including the *CtHSR1* gene exhibited better drought tolerance than the control line (untransformed Col-0 or wild type). The aspect of transgenic plants grown under irrigation conditions was indistinguishable form that of control plants (Fig. 1a, left panel). After drought treatment, however, the leaves of control plants were brownish while in the transgenic lines most leaves remained green (Fig. 1b, left panel). Under irrigation conditions both the fresh and dry weights of the aerial parts of the transgenic plants were slightly smaller (20–35 %) than those of control plants (Fig. 1d, left part). After drought treatment the fresh weight of transgenic aerial parts was 50–80 % greater than that of control plants while the dry weight of transgenic aerial parts was 5–15 % smaller than that of control plants (Fig. 1e, left part). These results indicate that transgenic plants have slightly less growth than control plants under irrigation conditions but retain much more water during drought treatment. The water content under irrigation conditions was similar in control and transgenic plants (9.4–9.8 g water per g dry weight) but after drought treatment transgenic plants retained about twice as much water (4.0 versus 2.3 g water per g dry weight, respectively). Actually, control plants could not recover from the drought treatment (irreversible wilting) while transgenic plants continued growth upon irrigation (data not shown). As indicated in Fig. 1c, the phenotypes observed in the transgenic lines are similar despite some differences in expression levels of *CtHSR1*. This can be explained if a threshold level of expression already results in maximal drought tolerance.

To demonstrate that the phenotype of drought tolerance conferred by a genomic fragment including the *CtHSR1*

gene is due to expression of the coding region of this gene, we have expressed this 2184 bp ORF from two types of promoters driving expression in most plant tissues: the strong 35S promoter (Benfey and Chua 1990) and the moderate *Arabidopsis AtUBQ10* promoter (Norris et al. 1993; Grefen et al. 2010). As indicated at the right part of Fig. 1, transgenic plants from three different lines expressing the *CtHSR1* ORF from the *AtUBQ10* promoter were more drought tolerant than control plants, reproducing the results obtained when *CtHSR1* ORF was expressed from its own fungal genomic promoter. On the other hand, three transgenic lines expressing the *CtHSR1*-coding region from the 35S promoter exhibited no phenotype (Supplementary Fig. S2). This suggests that moderate expression of *CtHSR1*, but not strong expression, confers the drought tolerance phenotype.

The fungal gene *CtHSR1* is expressed in phloem tissue of Arabidopsis from its own promoter

Having confirmed a phenotypic effect in transgenic plants expressing the *CtHSR1* gene from its own genomic promoter region and from the *AtUBQ10* promoter, we investigated if the gene is actually expressed in the transgenic plants. First we performed semi-quantitative RT-PCR and compared expression from the genomic promoter region (P_{CtHSR1}::*CtHSR1*), from the UBQ10 promoter ($P_{AtUBQ10}$::*CtHSR1*) and from the 35S promoter (P_{35S}::*CtHSR1*). As indicated in Fig. 1c and in Supplementary Fig. S2c, using semi-quantitative RT-PCR we could detect expression in the aerial part of transgenic lines with the three promoters but not in control plants (Col-0 or wild type). However, in the case of the P_{CtHSR1}::*CtHSR1* and $P_{AtUBQ10}$::*CtHSR1* plants 5 more amplification cycles (30 versus 25) were needed to obtain similar levels of PCR product than in P_{35S}::*CtHSR1* plants. This indicates that expression from the first two promoters was about 32-fold lower than from the 35S promoter.

To identify tissues where the *CtHSR1* promoter is active, we constructed transgenic plants with the 3 kb genomic region upstream of the *CtHSR1*-coding region (containing the fungal gene promoter) driving the expression of the GUS reporter gene. The reporter was expressed at vascular bundles of different organs (Fig. 2a) and specifically at phloem tissue (Fig. 2b).

We have quantified the level of GUS expression driven by the *CtHSR1* promoter by determining in extracts of transgenic plants the specific activity of β-glucuronidase and compared with the activity of plants transformed with the GUS gene under the 35S promoter (plasmid pCAMBIA1303). In the P_{CtHSR1}::*GUS* plants the activities of three lines were in the range of 0.5–5 pmol
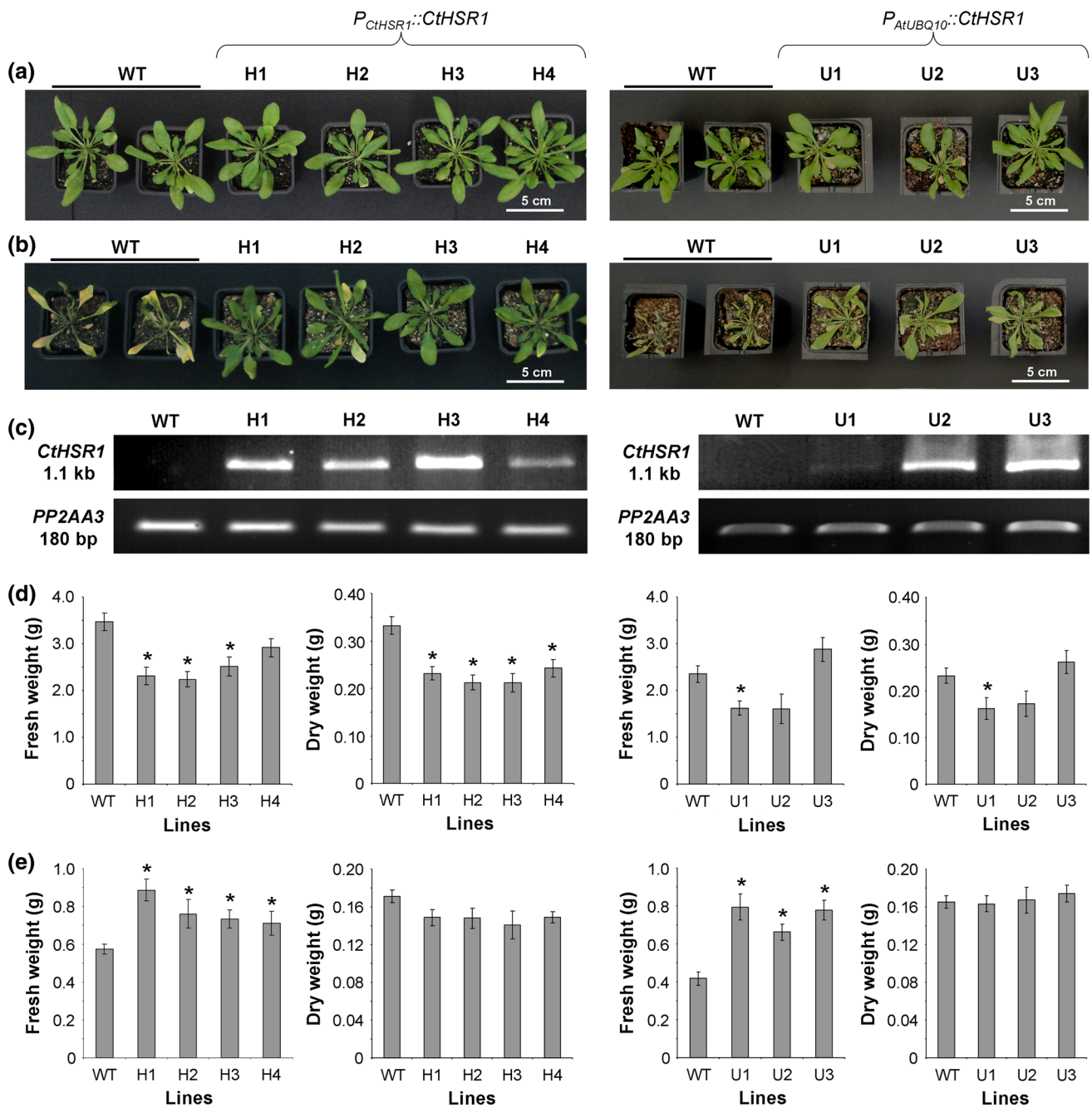
Fig. 1 Drought tolerance of *Arabidopsis* lines transformed with a genomic fragment containing the *CtHSR1* gene (*left part*, P_{CtHSR1}::HSR1, transgenic lines H1 to H4) and with the *AtUBQ10* promoter driving expression of the coding region of *CtHSR1* (*right part*, $P_{AtUBQ10}$::*CtHSR1*, transgenic lines U1 to U3). Results with the control line (Col-0 or WT) and 3–4 *transgenic lines* are shown. **a** Aspect of representative plants of the indicated *lines* after 6 weeks of irrigation. **b** Aspect of representative plants of the indicated *lines* after 4 weeks of irrigation and 2 weeks without irrigation. **c** Expression of *CtHSR1* gene in control (Col-0 or WT) and transgenic plants as determined by semi-quantitative RT-PCR with 30 cycles of amplification. **d** Average fresh and dry weight (mg) of the aerial part of plants of the indicated lines after 6 weeks of irrigation. **e** Average fresh and dry weight (mg) of the aerial part of plants of the indicated lines after 4 weeks of irrigation and 2 weeks without irrigation. *Bars* indicate standard error and *asterisks* significant differences ($P \leq 0.05$) with control line according to the Student's *t* test. It must be indicated that in the experiment of the right part of the figure the growth of control and transgenic plants was smaller than in the experiment of the left part due to different periods of growth

4-methylumbelliferone min^{-1} µg protein^{-1} (Supplementary Fig. S3) while in the P_{35S}::*GUS* plants the activity range of four lines in the same units was 30–100 (data not shown). Therefore, the fungal promoter works in *Arabidopsis* at levels 1–5 % than the 35S promoter, in agreement with the RT-PCR results discussed above.
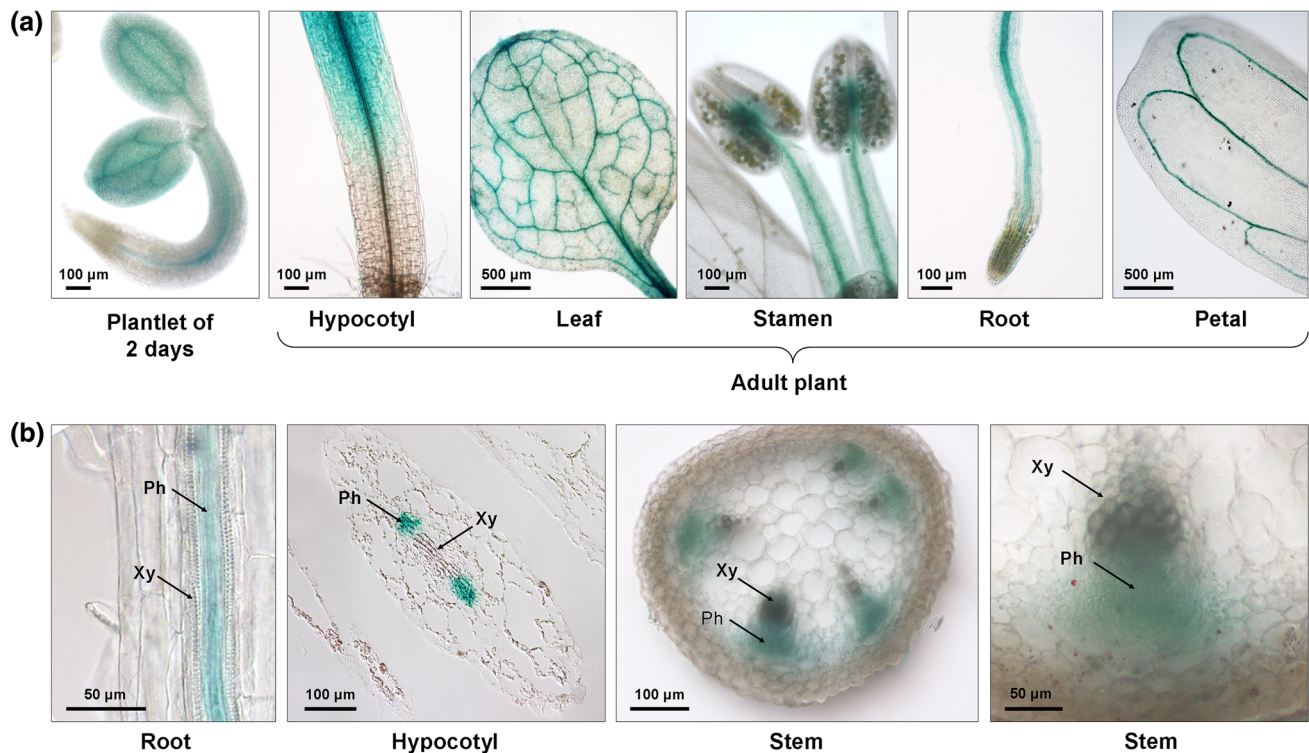
Fig. 2 The promoter of the fungal gene *CtHSR1* drives expression of GUS at phloem tissue. Transgenic *Arabidopsis* plants containing the GUS reporter gene expressed from the promoter of the *CtHSR1* gene were analyzed for β-glucuronidase activity by the histochemical method described in "Materials and methods". **a** Expression at vasculature in a plantlet of 2 days and in hypocotyl, leaf, stamen, root and petal of adult plants. **b** Expression at phloem tissue in root, hypocotyl and stem of adult plants

The effect of stresses on the activity of the *CtHSR1* promoter was investigated in $P_{CtHSR1}::GUS$ transgenic plants. The activity of β-glucuronidase was not significantly affected by 2-h treatment of 7-day plantlets with 125 mM NaCl or by increasing temperature to 42 °C but 250 mM mannitol resulted in a modest induction of 1.2–1.5-fold in 2 of the three lines investigated (Supplementary Fig. S3).

The fungal HSR1 protein fused to GFP localizes to the cell nuclei in leaves of *N. benthamiana*

We have used transient expression in leaves of *N. benthamiana* of an AtHSR1–GFP protein fusion driven by the 35S promoter to determine the subcellular localization of the fungal transcription factor in transgenic plants. As indicated in Fig. 3, the CtHSR1–GFP fusion protein concentrates in spots coincident with those stained with the nuclear dye DAPI. This indicates a nuclear location as expected from a transcription factor recognized by the nuclear localization machinery. Similar results were obtained with GFP–CtHSR1 fusions (data not shown). It must be indicated that GFP alone, without a targeting sequence, is known to accumulate diffusely in the cytoplasm and nucleus and only concentrates in nuclei when

fused to proteins containing nuclear localization signals (Chiu et al. 1996), as seems to be case with GFP–CtHSR1 fusions.

Physiological basis of drought tolerance in transgenic plants expressing *CtHSR1*

To understand the physiological mechanisms of drought tolerance conferred by expression of *CtHSR1*, we have determined in transgenic and control plants several physiological parameters. *Arabidopsis* plants transformed with a genomic fragment including the *CtHSR1* gene were used for these experiments.

The inhibition of seedling growth by abscisic acid (ABA) was similar in control and transgenic plants (Supplementary Fig. S4), suggesting that ABA sensitivity was not affected by expression of *CtHSR1*. On the other hand, both in the absence and presence of 0.5 µM ABA, the opening of stomata in the leaves of *CtHSR1* transgenic plants is 13–14 % smaller than in the leaves of control (non-transgenic) plants (Fig. 4a). Stomata density in transgenic leaves is slightly less than in control leaves but the differences are not significant (Fig. 4b) and the stomata surface is very similar in control and transgenic leaves (Fig. 4c).
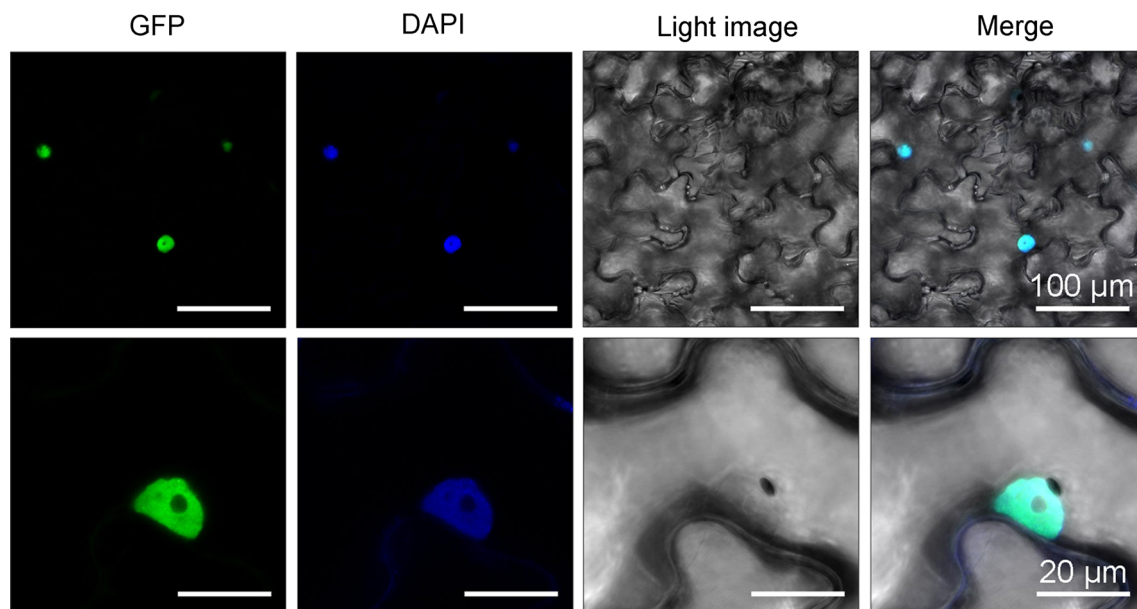
Fig. 3 A CtHSR1–GFP protein fusion localizes to the cell nucleus of epidermal cells of *Nicotiana benthamiana*. *Panels* with the fluorescence of GFP, DAPI staining of nuclei, light microscopy image and combination of pictures (*merge*) are shown

These results were corroborated by measurements of gas exchange with an LI-6400 Portable Photosynthesis System. As indicated in Table 1, both the stomatal conductance and the transpiration rate were significantly less in transgenic *CtHSR1* lines than in control plants (on average 20–25 % less both parameters) and this was expected from the smaller stomatal opening of the transgenic lines indicated above. The photosynthetic rate and the quantum yield of photosystem II were not significantly different between control and transgenic lines, although we observed a tendency for less photosynthetic rate in transgenic lines (Table 1). This could explain the slightly reduced growth rate of transgenic plants as compared with controls.

The osmotic potential of transgenic lines was significantly greater than that of controls (Table 1). The observed 5 % increase in osmotic potential corresponds to a solute concentration of about 18 mM. Looking for candidate solutes we found that the concentrations of neither soluble sugars (sucrose, glucose and fructose; Supplementary Table S2) nor major cations (sodium and potassium; Supplementary Fig. S5) were significantly different between control and transgenic leaves. A different situation, however, was encountered by measuring proline concentrations, which were twofold greater in transgenic lines than in controls, both under irrigation and under drought conditions (Fig. 5).

We have also determined the concentration of some hormones important for drought tolerance. As indicated in Table 2, the concentration of ABA was similar in control and transgenic lines but in the case of JA and auxin transgenic lines contained concentrations 2.1-fold and 1.3-fold greater, respectively, than control plants.

Expression of *CtHSR1* alters the transcriptome of *Arabidopsis*

To identify molecular mechanisms for the drought tolerance conferred by expression of *CtHSR1*, we have performed a transcriptomic analysis of two *CtHSR1* transgenic lines with similar phenotypes (H1 and H3) versus control (non-transformed) *Arabidopsis*. No significantly repressed genes were found but 108 genes were up-regulated at least 1.5-fold as compared with the control, with a maximum increase of fourfold (Supplementary Table S3). The validation by qRT-PCR of the up-regulation of some of these genes is shown in Supplementary Fig. S6.

This set of genes is enriched in some functional categories related to biotic and abiotic stress such as "Response to wounding", "Response to jasmonic acid", "Response to ethylene", "Response to abscisic acid", "Response to water deprivation", "Response to oxidative stress" and "Regulation of transcription" (Supplementary Table S4). It includes relevant genes to the physiology of the *CtHSR1* transgenic plants such as *P5CS1* and *LOX4*. *P5CS1* encodes isoform 1 of Δ-1-pyrroline-5-carboxylate synthetase, the rate-limiting step of proline biosynthesis in vegetative tissues that is required for proline accumulation under osmotic stress (Székely et al. 2008). *LOX4* encodes an isoform of 13-lipoxygenase, the rate-limiting step for JA biosynthesis during plant defense against herbivores (Ozalvo et al. 2014).

Fig. 4 Stomatal aperture, but not density or size, is decreased by expression of *CtHSR1*. Results with the control line (Col-0 or WT) and 3 transgenic lines (*H1* to *H3*) expressing *CtHSR1* from its own promoter are shown. **a** Stomata aperture in the absence (*filled bars*) and presence (*empty bars*) of 0.5 μM ABA as measured by the ratio wide/length of the opening cavity determined by microscopy. **b** Stomata density. **c** Stomata size without ABA. **d** Micrographs of the stomata of the different lines without ABA utilized to determine the above data. Values are the mean of 4 determinations from micrographs as in **d** with 20–30 stomata. *Bars* indicate standard error and *asterisks* significant differences ($P \leq 0.05$) with control line according to the Student's *t* test
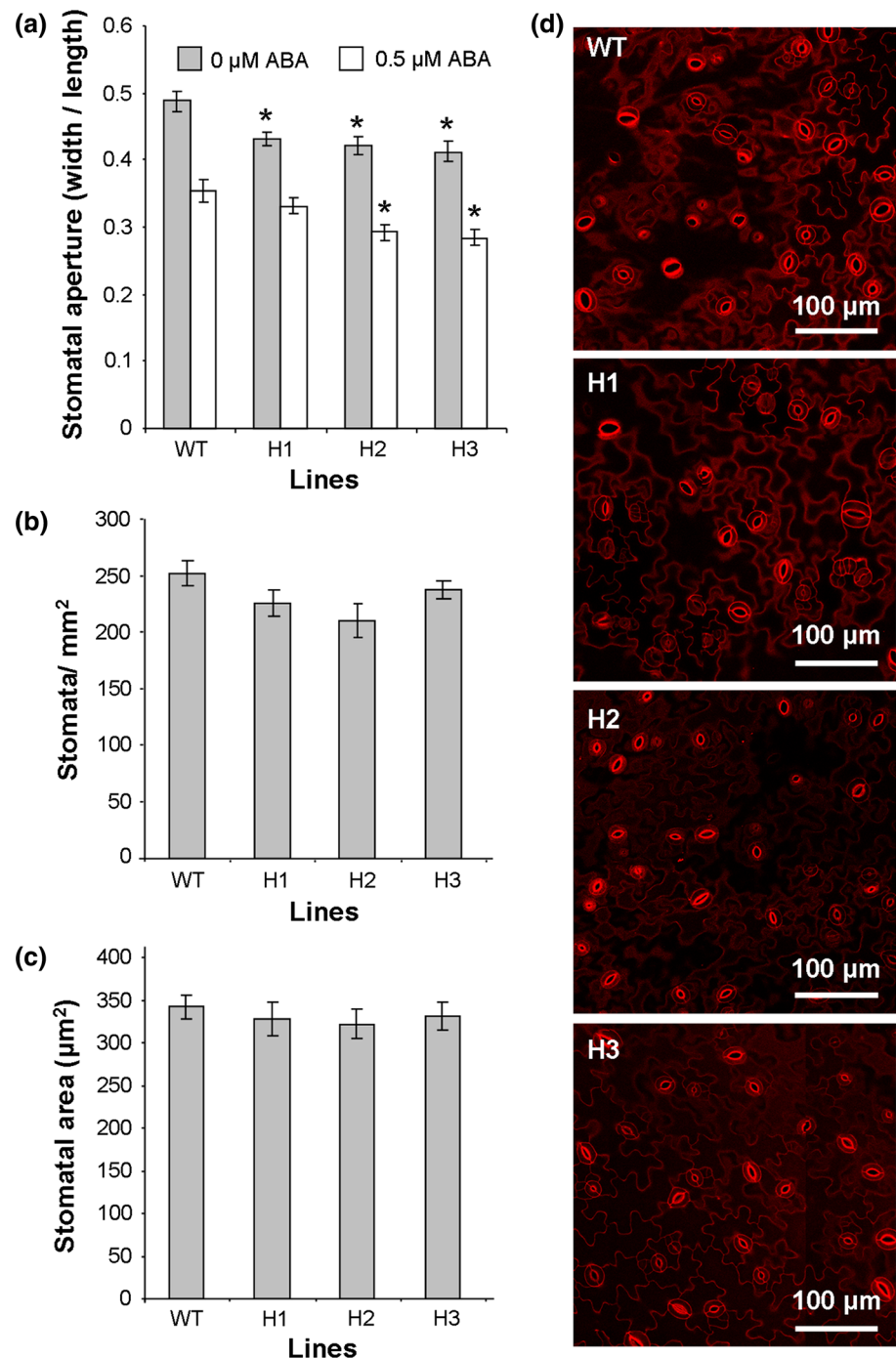
A comparison was made between the set of genes up-regulated by expression of *CtHSR1* in *Arabidopsis* and microarray experiments in the NASCArrays data base (http://affymetris.arabidopsis.info/narrays/experimentbrowse.pl). The most significant overlaps of the 108 *CtHSR1*-induced genes were found with genes induced by osmotic stress (43), JA (23) and heat stress (12) (Supplementary Table S5a–d).

Motifs enriched in the promoters of *CtHSR1*-induced *Arabidopsis* genes

We have studied preferentially located motifs in the promoter of *CtHSR1* up-regulated genes as compared with all *Arabidopsis* genes. The two most enriched motifs are the CGCG box of genes involved in ethylene and ABA signaling (Yang and Poovaiah 2002) and the ABRE element

Table 1 Effect of *CtHSR1* gene expression in *Arabidopsis* on the photosynthetic rate (A_n, μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), quantum yield of photosystem II (Φ SII), and osmotic potential (Ψ, Osm kg of fresh weigth^{-1})

Line	A_n	G_s	E	Φ SII	Ψ
WT	7.4 ± 1.0	0.12 ± 0.01	2.1 ± 0.1	0.16 ± 0.02	0.389 ± 0.003
H1	6.4 ± 0.4	0.10 ± 0.00*	1.8 ± 0.1*	0.16 ± 0.00	0.402 ± 0.002*
H2	7.3 ± 0.7	0.08 ± 0.01*	1.5 ± 0.1*	0.16 ± 0.01	0.420 ± 0.005*
H3	6.5 ± 0.3	0.10 ± 0.00*	1.8 ± 0.1*	0.14 ± 0.02	0.401 ± 0.005*
H4	7.2 ± 1.0	0.08 ± 0.01*	1.5 ± 0.2*	0.17 ± 0.03	0.405 ± 0.007*

Reported values represent the mean ± SE of 5 independent determinations. Asterisks indicate significant differences with controls (Col-0 or WT) at $P < 0.05$ (Student's *t* test)
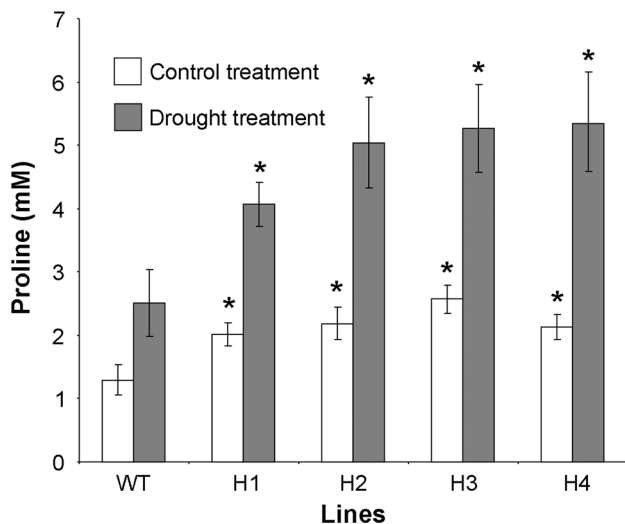
Fig. 5 The concentration of proline is higher in leaves of *CtHSR1* transgenic plants than in control plants (Col-0 or WT). Values are the mean of three experiments where proline was determined in leaf samples from 10 plants of every line. *Open bars* correspond to plants grown under irrigation conditions while *filled bars* after a drought treatment as described in Fig. 1. *Bars* indicate standard error and *asterisks* significant differences ($P \le 0.05$) with control line under the same conditions, according to the Student's *t* test

Table 2 Hormone concentrations (ng g^{-1} fresh weight) in transgenic *Arabidopsis* lines (H1 and H3) versus control (Col-0 or WT)

Lines	ABA	JA	IAA
WT	7.6 ± 0.7	69.9 ± 2.8	1.72 ± 0.04
H1	10.2 ± 0.8	162.7 ± 26.5*	2.25 ± 0.17*
H3	8.1 ± 0.1	142.6 ± 13.7*	2.42 ± 0.04*

Reported values represent the mean ± SE of 3 independent determinations. Asterisks indicate significant differences with WT at $P < 0.05$ (Student's *t* test)

of genes responding to ABA (Yamaguchi-Shinozaki and Shinozaki 2005). Although less significant, some enrichment of the WRKY transcription factor-binding box was observed (Table 3).

Discussion

Our results indicate that a fungal gene with its own promoter is expressed in plants and confers a useful phenotype such as drought tolerance. As this may be relevant for horizontal gene transfer from fungi to plants in the course of evolution (Bock 2009; Talianova and Janousek 2011; Schönknecht et al. 2014) and for biotechnological applications, we investigated the functionality of the fungal promoter in plants, the expression of the encoded protein and the mechanisms of the conferred drought tolerance.

We have shown that the promoter region of the *CtHSR1* gene drives expression of the GUS reporter at the *Arabidopsis* phloem. This fungal promoter functions in plants with moderate strength, similar to the *AtUBQ10* promoter but much less active than the 35S promoter. The fact that similar phenotypes are observed in transgenic plants with either the *CtHSR1* promoter (phloem specific) or with the *AtUBQ10* promoter (general expression) indicates that phloem expression is sufficient for the phenotypes and that expression in other tissues is not deleterious. Although *cis*-elements determining phloem expression are not completely characterized (Yin and Beachy 1997; Saha et al. 2007), some potential sequences such as the ASL (GCAX$_{17-18}$GCA), GATA (AX$_3$GATA) and CCCC boxes are present in the *CtHSR1* promoter (Supplementary Fig. S7). It is interesting that expression of *CtHSR1* from moderate promoters such as its own one or the *AtUBQ10* promoter results in drought tolerance but expression from the strong 35S promoter results in no phenotype. No silencing at the level of mRNA was observed with the strong promoter (Supplemental Fig. S2) and, therefore, a plausible mechanism is aggregation and degradation of the over-produced protein (Tyedmers et al. 2010). A similar situation has recently been found by overexpressing the transcription factor ATHB25 in *Arabidopsis* with *AtUBQ10* and 35S promoters, when only in the first case was a phenotype observed (Bueso et al. 2014). This may also explain that transgenic *Arabidopsis* plants with

Table 3 Motifs overrepresented in 105 promoters of *CtHSR1* up-regulated genes versus the promoters of 20,687 *Arabidopsis* genes

Motif	Sequence	Window	% HSR	% AT
CGCG box	CGCG	(−150, −50)	24.5	6.5
ABRE (GCCACGT)	GCCAC	(−180, −50)	20.0	11.0
WRKY box (TTGACY)	TGAC	(−170, −50)	68.5	54.0
TATA box	TATAWA	(−50, 0)	50.2	30.2

"Window" refers to positions from the transcription start point where the motif is significantly enriched and "% HSR" and "% AT" to the per cent of promoters from genes up-regulated in *CtHSR1* plants (% HSR) and from all *Arabidopsis* genes (% AT) that have the motif within the indicated window. Y = C or T; W = A or T

plasmids $P_{35S}::CtHSR1–GFP$ and $P_{35S}::GFP-CtHSR1$ exhibited no fluorescence signal (see "Materials and methods").

Transient expression in leaves of *N. benthamiana* of a fusion protein CtHSR1–GFP indicates that CtHSR1 localizes in plant cells at the nucleus, as expected from a transcription factor. Therefore, we investigated the transcriptome of the transgenic *Arabidopsis* plants expressing *CtHSR1*. Only about hundred genes were altered in expression by CtHSR1, all of them up-regulated. Some of these genes could be direct targets of the fungal transcription factor but as there are 16 transcription factors induced by CtHSR1 (Supplemental Table S3), some of the up-regulated genes may be secondary targets. For example, expression of CtHSR1 up-regulates several ERF transcription factors and it has been described that ERF1 induces the expression of defense genes such as *P5CS1* (Cheng et al. 2013). Also, two transcription factors up-regulated by CtHSR1, BZIP1 and ZFP1, induce the expression of several genes involved in drought tolerance (Sun et al. 2012; Han et al. 2014). The most enriched motifs in the promoters of up-regulated genes are the CGCG box (Yang and Poovaiah 2002) and a partial version of the ABRE box (GCCAC; Yamaguchi-Shinozaki and Shinozaki 2005). This motif of only 5 nucleotides is probably not a real ABRE because it lacks two crucial nucleotides (the last GT; compare the complete ABRE motif in Table 3 with the partial one enriched in the promoter of genes up-regulated by CtHSR1). The primary targets of CtHSR1 can only be speculated. It probably binds a single repeat (GAA) of the heat-shock element (Landsman and Wolffe 1995), a sequence too short for specificity. A plausible mechanism is that CtHSR1 interacts with some other transcription factor and the proximity of the two recognized sequences determines the specific binding to a few promoters.

We have characterized the physiological parameters of the *CtHSR1* transgenic plants and the observed drought tolerance may be explained by two observations. In the first place, transgenic plants have decreased stomatal opening and conductance, decreased transpiration and better water retention (Figs. 1, 4; Table 1). This also explains the slightly reduced growth of the transgenic plants under irrigation conditions because of slightly reduced photosynthetic rate. This stomata behavior could be the consequence of the doubling of JA concentration in transgenic plants (Table 2) because this hormone induces closing of stomata (Acharya and Assmann 2009; Montillet and Hirt 2013). The increase in JA correlates with increased expression of *LOX4*, a gene encoding a lipoxygenase of leaf mesophyll cells involved in JA synthesis (see http://bbc.botany.utoronto.ca/efp/). The small increase in auxin concentration may be a secondary effect of the increase in JA (Hentrich et al. 2013).

The second clue to the drought tolerance conferred by *CtHSR1* was the increase in osmotic potential of the transgenic plants (Table 1). One of the solutes contributing to this increase is proline, whose concentration is doubled in transgenic with respect to control plants (Fig. 5). Although this can only explain a small part of the change in osmotic potential, proline is a special protecting solute (osmolyte) whose levels correlate with stress tolerance (Ramanjulu and Bartels 2002; Kavi Kishor and Sreenivasulu 2014). The increase in proline in the *CtHSR1* transgenic plants correlates with the increased expression of the *P5CS1* gene, encoding Δ1-pyrroline-5-carboxylate synthase, the rate-limiting enzyme in the biosynthesis of proline (Székely et al. 2008). Additional up-regulated genes that could directly contribute to drought tolerance include those encoding the chaperones DNAJ11 and DJC22 and one LEA2 protein (At2g27080) (Ramanjulu and Bartels 2002).

In conclusion, a fungal transcription factor gene expressed in plants from its own promoter confers drought tolerance by altering the transcriptome and some physiological parameters related to water stress. Clearly, this is a case of serendipity but it cannot be discarded that expression of other foreign transcription factors may also result in beneficial effects for plants under certain conditions, paving the way for biotechnological applications and, in the evolutionary timescale, horizontal gene transfer.

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