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The overexpression in *Arabidopsis thaliana* of a *Trichoderma harzianum* gene that modulates glucosidase activity, and enhances tolerance to salt and osmotic stresses

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ABSTRACT

Using the TrichoEST database, generated in a previous functional genomics project from the beneficial filamentous fungus *Trichoderma harzianum*, a gene named *Thkel1*, which codes for a putative kelch-repeat protein, was isolated and characterized. Silencing of this gene in *T. harzianum* leads to a reduction of glucosidase activity and mycelial growth under abiotic stress conditions. Expression of this gene in *Arabidopsis* enhances plant tolerance to salt and osmotic stresses, accompanied by an increase in glucosidase activity and a reduction of abscisic acid levels compared to those observed in wild-type plants. Data presented throughout this article suggest the high value of *T. harzianum* as a source of genes able to facilitate the achievement of producing plants resistant to abiotic stresses without alteration of their phenotype.

Introduction

Trichoderma is a genus that includes cosmopolitan fungi acting as biological control agents, with different mechanisms of action and high biotechnological value (Harman et al., 2004). Due to their ubiquity and rapid substrate colonization, they are commonly used in agriculture and their enzyme systems are widely used in industry. Species of *Trichoderma* are frequently utilized in the biocontrol of soil-borne plant pathogens (Howell, 2003) due to their ability to inhibit or kill plant pathogenic fungi through the production of antifungal antibiotics and/or hydrolytic enzymes (Vinale et al., 2008). In this genus, *Trichoderma harzianum* is the most widely used species employed as a source of active compounds in a variety of commercial biopesticides and biofertilizers (Vinale et al., 2006). Therefore, the isolation of genes from *Trichoderma* spp. and their subsequent transfer to a plant genome may result in a significant improvement in plant defense (Lorito et al., 1998; Calo et al., 2006; Kumar et al., 2009) and resistance to abiotic stresses (Dana et al., 2006; Montero-Barrientos et al., 2010).

In this work, we describe the cloning and characterization of a gene named *Thkel1* from *T. harzianum* CECT2413 (referred to as T34 from now on), which shares sequence similarity to the plant epithiospecifier protein (ESP). As a structural feature, this type of proteins is predicted to contain a series of β-sheets representing kelch domains typically involved in protein–protein interactions (Burow et al., 2007, 2009).

ESPs modify glucosinolate hydrolysis, leading to the formation of nitriles and epithionitriles instead of toxic isothyocianates (Wittstock and Burow, 2007). Six genes encoding kelch proteins with 4–5 domains of this type have been identified in *Arabidopsis* and it has been suggested that they are involved in nitrile formation from glucosinolates (Burow et al., 2009).

Thus, the presence of kelch domains suggests that the *Thkel1* gene may play a role in β-glucosidase–substrate systems. The function of this gene was studied using gene silencing and *Arabidopsis* gene expression strategies in order to analyze the biotechnological implication of *Thkel1* in plant responses to certain types of stresses.

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Materials and methods

Bacterial strains

Escherichia coli DH5α (Invitrogen Life Technologies, Strathclyde, UK) was used as a host for plasmid construction and propagation. This bacterial strain was grown in Luria–Bertani (LB) broth or on LB plates, which were supplemented with ampicillin (0.1 mg ml^{-1}), X-gal (40 μg ml^{-1}) and IPTG (10 μg ml^{-1}) when required.

Fungal strains

Trichoderma harzianum CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) (referred to as T34) was the wild-type strain used in the *Thkel1* silencing experiment.

Plant material

Arabidopsis thaliana wild-type and transgenic plants used throughout this work were the Col-0 ecotype. They were grown in a growth chamber under 40% humidity, a temperature of 22 °C, and with a 16 h light/8 h dark photoperiod at 80–100 μE m^{-2} s^{-1} and in pots containing a 1:3 vermiculite/soil mixture, following the procedure described previously (Montero-Barrientos et al., 2010).

Source of Thkel1 gene: EST database and cDNA library

The L02T34P068R06401 clone (EST 6401) was obtained from the *T. harzianum* T34 cDNA library L02, one of the twenty-six cDNA libraries that were constructed for the TrichoEST project using different mRNA populations expressed under mycoparasitic and nutrient stress conditions. The T34 growth conditions used for preparing the L02 cDNA library are described in Vizcaíno et al. (2006).

Primers and PCR procedures

Primers used in this work are indicated in a table provided as supplementary material. The primer 6401-pA was used to sequence plasmid DNA of the EST 6410 clone to obtain information of the 3′-end of the gene. The *Thkel1* full-length cDNA was amplified by PCR using *Eco*RI-adapted primers kel-1 and kel2-2 and amplified phagemids from L02 cDNA library as template, using the *Taq* polymerase system (Biotools, Madrid, Spain), according to the manufacturer's instructions. PCR was carried out for 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min and 30 s at 72 °C and a 7 min extension at 72 °C. The resulting PCR product was cloned into the vector pGEM-T easy (Promega Corporation, Madison, WI, USA) and sequenced using an automatic sequencer.

Oligos used to make one antisense-pSIL construct: KEL-SpeI, KEL-BamHI, KEL-XhoI and KEL-HindIII. A screen to identify fungal transformants containing the antisense silencing construct was carried out by PCR using the *Thkel1* gene specific primer 6401-2 and primer Pta1, specific to the cloning vector pSIL, using the same conditions as mentioned above.

To generate the construction used in plant transformation, the coding region of the *Thkel1* cDNA was amplified by PCR using the following forward-kel and reverse-kel primers.

Analysis of Thkel1 gene expression

Mycelia from strain *T. harzianum* T34 was obtained following a liquid culture procedure in two steps: (1) the fungus was grown in a minimal medium (Penttilä et al., 1987), containing 2% glucose, at 28 °C for 2 days, (2) biomass was recovered by filtration, washed twice and transferred to MM, without glucose. The following substrates/conditions in separate cultures were included: MM supplemented with 2 or 10% NaCl, or 1 M mannitol at 28 °C for 4, 8 or 24 h; or MM at 4, 28 or 37 °C for 4, 8 or 24 h. In parallel, mycelia from culture conditions above cited but supplemented with 2% glucose were also obtained. Biomass was recovered by filtration, washed twice, frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Gene expression was analyzed by qRT-PCR.

Construction of pSIL-KEL vector and Trichoderma transformation

The method used to silence *Thkel1* gene expression in *T. harzianum* T34 was based on the generation of an intron containing self-complementary "hairpin" RNAs (ihpRNA) (Cardoza et al., 2006). Plasmid pSIL was linearized with *Spe*I–*Bam*HI and then ligated to a 523-bp *Spe*I–*Bam*HI fragment of the *Thkel1* gene. This fragment was in "sense" relative to the orientation of the *tss1* promoter. This construct was later linearized with *Hind*III–*Xho*I and then ligated to the same 523-bp *Hind*III–*Xho*I fragment but in an "antisense" orientation relative to the *tss1* promoter. An intron of 159 bp was introduced between both sense and antisense fragments of the *Thkel1* gene. The resulting plasmid also contained the terminator region of the *cbh1* gene of *Trichoderma reesei* and was designated pSIL-KEL0. In order to transform this cassette in *T. harzianum* T34, pSIL-KEL0 was digested with *Sac*I and then ligated to the same restriction site of plasmid pJL43b1, giving rise to the final construct pSIL-KEL1 (8.29 kb). This was used to transform *T. harzianum* T34 by a protoplast-based method described in Cardoza et al. (2006). A 1.36 kb fragment, containing the region corresponding to the phleomycin resistance gene (*ble*) and the promoter of the *gpdA* gene from *Aspergillus nidulans*, was isolated from the pJL43b1 construct using enzymes *Eco*RV and *Sac*I and used to screen the transformant strains in a DNA blot analysis. In addition, strain T34 was transformed with pJL43b1 and one empty vector transformant, named ThJL43, was included in assays as a control of transformation.

Protein extraction

Total protein extracts were prepared from 0.2 g of fungi or plants. The fungal or plant material was lyophilized and ground in a chilled mortar on ice, transferred to 0.8 ml of cold phosphate buffer (0.1 M, pH 6.8), and vortexed for 1 min at 4 °C. The homogenate was centrifuged at 13,000 × *g* for 20 min at 4 °C, the supernatant was transferred to a clean tube and the protein concentration was determined (Bradford, 1976) with bovine serum albumin used as a standard.

Enzyme assay

Protein extracts from untransformed (control) and silenced fungal strains (culture supernatants) or transgenic plants (aereal parts) from different growth conditions were assayed for glucosidase activity according to Dietz et al. (2000) with some modifications. The reaction mixture containing 0.4 ml of 0.1 M citrate buffer (pH 4.8), 0.1 ml of 0.1 M *p*-nitrophenyl-β-D-glucopyranoside (Sigma Chemical, St. Louis, USA) and 0.05 ml of protein extract protein, was incubated at 37 °C for 15 min. The reaction was stopped with 1 ml of 0.2 M Na_2CO_3, pH 3. The amount of liberated *p*-nitrophenol was quantified spectrophotometrically at 405 nm. The activity was expressed as μmol of *p*-nitrophenol released per min and per mg of protein.

Real-time PCR analysis

For the expression analysis of *Thkel1* gene in *T. harzianum* T34, 1 μg of total RNA was used for a RT reaction using the Affinity Script QPCR cDNA Synthesis kit (Stratagene, La Jolla, CA, USA) with a oligo(dT) primer. Then, 1 μL of cDNA was used in the subsequent PCR.

In addition, *Thkel1* expression was comparatively analyzed in *T. harzianum* T34 and transformants Kel4, ThJL43, Kel10 and Kel15 using cDNA synthesized from RNA, which was obtained after fungal growth in MM at 4 °C for 4 h.

Highly purified primers for *Thkel1* (Kel-sil3 and Kel-sil4) and *β-tubulin* (tub-f and tub-r) were used and produced single amplicons of 120 and 71 bp, respectively.

Quantitative real-time PCR was performed using an AB PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Stratagene) as previously described in Montero-Barrientos et al. (2010).

Abiotic stress assays in fungi

Wild-type and three silenced transformant strains were tested for resistance to different types of abiotic stresses by measuring the colony diameters after incubation for 2 or 3 days under different growth conditions. Fungal strains were grown on potato dextrose agar (PDA) medium containing 1 or 5% NaCl, or 0.5 or 1 M mannitol, at 28 °C. Fungal growth was also tested after incubation at 4 °C for 5 weeks.

Vector construction and plant transformation

To generate the construction used in plant transformation, two oligos were designed that allowed subcloning the PCR products into the *Bam*HI–*Sac*I sites of the double-digested pBIN121 vector (see "Primers and PCR procedures" section). The T-DNA region of the pBIN121-*Thkel1* construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere et al., 1985) by electroporation. *Arabidopsis* plants (Col-0 ecotype) were transformed by the floral dip method (Clough and Bent, 1998), and transgenic seedlings were selected on kanamycin medium (50 μg ml^{-1}). From the 75 T1-resistant lines recovered, approximately 65% showed a salt stress-insensitive phenotype in seed germination as compared with untransformed plants. T_2 plants that produced 100% kanamycin-resistant plants in the T_3 generation were considered homozygous for the selection marker and used for further studies.

Abiotic stress assays in plant

Seeds were sowed on solid medium composed of Murashige and Skoog basal salts, 1% (w/v) sucrose and complemented with 350 mM mannitol or 150 mM NaCl to determine sensitivity to inhibition of germination by salt and osmotic stresses. In all cases, seed germination was scored daily to determine the percentage of seeds that had germinated and developed green cotyledons during 5–10 days. At least three replicates of each germination assay (100 seeds per plate) were performed.

To further analyze tolerance of *Thkel1*-overexpressing plants to salt stress, wild-types and transgenic seeds were sown on MS media for one week and after that, similar plants were transferred to soil, grown for 3 weeks and then treated daily with 5 ml of either water or 100 mM NaCl for 10 days.

Extraction and hybridization of nucleic acids

Total RNA was extracted using the TRI reagent (Ambion, Austin, TX, USA) following the manufacturer's protocol. Total DNAs from fungi were extracted following the method of Raeder and Broda (1985), bacterial DNAs were obtained using a routine miniprep procedure, and genomic DNA from *Arabidopsis* plants was extracted using the Plant DNA Isolation kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's recommendations.

DNA blot analysis of the silenced transformant strains was carried out using 10 μg of genomic DNA digested with restriction enzymes *Sac*I and *Sal*I, electrophoresed on 0.7% agarose gels and transferred to a Hybond-N^+ membrane (Amersham, Buckinghamshire, UK). Phleomycin resistance (*ble*) gene was labeled with the DIG High Prime (Roche) kit, respectively, following the supplier's protocols, and used as a probe. Hybridization, washes and detection were performed as previously described (Hermosa et al., 2000).

For RNA blot analyses, 20 μg of total RNA was separated on a 1.2% formaldehyde–agarose gel and transferred to a Hybond-N^+ membrane. Hybridization, washes and detection were performed as previously described (Montero-Barrientos et al., 2008).

Stomatal conductance measurements

Stomatal conductance was measured essentially as described in Arbona et al. (2009) with a LCpro+ portable infrared gas analyzer (ADC Bioscientific Ltd., Hoddesdon, UK) fitted with an *Arabidopsis* leaf chamber (16.5 mm window diameter) under ambient CO_2, humidity and light. Measurements on fully expanded leaves ($N = 6$) were taken after instrument stabilization.

Hormone analysis

Hormone extraction and analysis of aereal parts of *Arabidopsis* plants were carried out as described in Durgbanshi et al. (2005).

Results

Isolation of Thkel1 gene

A BlastX alignment of the EST 6401 showed high homologies with hypothetical proteins of *Aspergillus flavus* (e^{-105}) (accession: XP_002384414), *Aspergillus oryzae* (e^{-105}) (accession: XP_001827175) or *Gibberella zeae* (e^{-98}) (accession: XP_386004) containing kelch domains and, among others, with a nitrile specific protein (NSP)-5 from *A. thaliana* (e^{-35}). The ORF of 1014 bp was registered in the GenBank under the accession number EU399786 and encodes a polypeptide of 338 amino acids with a calculated molecular mass of 36.45 kDa, and a hypothetical pI of 5.27 (accession: ACA03183). The protein contained four putative kelch domains, predicted after an analysis for protein domain identification. A neighbor-joining (NJ) tree (Fig. 1) was generated after the alignment of ThKEL1 with 20 other GenBank-retrieved amino acid sequences, representing kelch-repeat proteins from fungi, humans, bacteria and plants. Two independent clades containing kelch-repeat proteins were identified, where fungal kelch-repeat proteins formed a major subclade with a bootstrap value of 100%. The ThKEL1 tertiary structure was predicted using the Swiss-PDB viewer software (http://www.expasy.org/spdbv7) resembling the circularized structure of kelch-repeat proteins (data not shown), according to Prag and Adams (2003).

A single hybridization band was detected in *T. harzianum* T34 (data not shown), indicating that the *Thkel1* gene exists as a single copy in T34. Additionally, one homologous gene was also identified in the three online *Trichoderma* available genomes (*T. reesei*, *T. virens* and *T. atroviride*), showing an amino acid identity about 55% with ThKEL1.

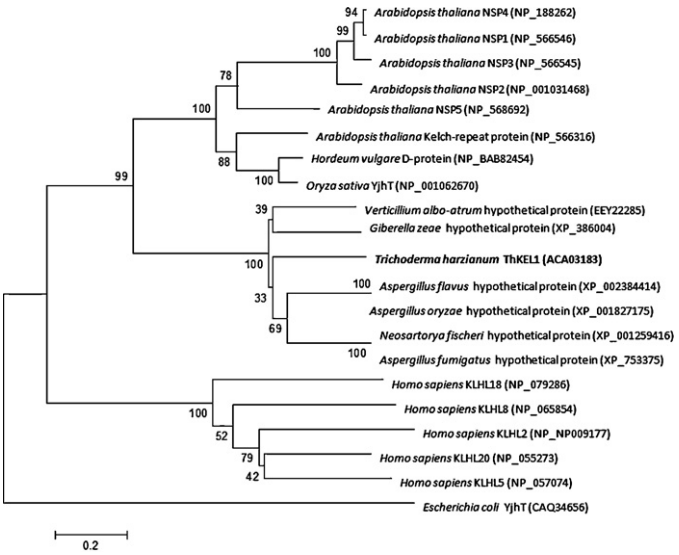

Fig. 1. Neighbor-joining (NJ) tree of protein ThKEL1 from *T. harzianum* T34 and the other twenty kelch-repeat proteins from plants, fungi, humans and bacteria. Amino acid sequences were aligned using the CLUSTAL X algorithm (Thompson et al., 1997) and the NJ tree was constructed with the MEGA software (Kumar et al., 2001). Sequences are indicated using their GenBank accession numbers. The bar represents five substitutions per 200 amino acids.

Expression analysis of Thkel1 in T. harzianum T34

We investigated, in 4-, 8- and 24-h-old cultures, the *Thkel1* expression levels in the presence or not of 2% glucose, and under abiotic stress treatments (NaCl, mannitol) (Fig. 2A and B). We also analyzed the gene expression after fungal growing at different temperatures (4, 28 or 37 °C) for 4 h. The *Thkel1* transcript levels from *T. harzianum* T34 grown in cultures supplemented with different amounts of the above compounds were compared with those detected in control cultures (MM with or without glucose). The expression values of the target gene were normalized to the expression value of the *β-tubulin* gene. The highest gene expression was detected in 4-h cultures without glucose, and particularly when the fungus was grown at 4 °C. Fig. 2B shows that *Thkel1* expression in the presence of abiotic agents was lower when glucose was present in the medium.

Silencing of Thkel1 in T. harzianum T34 and characterization of transformants

In order to functionally characterize the *Thkel1* gene, plasmid pSIL-KEL1 was constructed and transformed in *T. harzianum* T34. Seventeen transformants showing phleomycin resistance were obtained and the integration of the pSIL-KEL1 plasmid was analyzed in their DNAs by PCR with the oligonucleotides 6401-2 and Pta 1 as the primers. A 410 bp fragment was amplified in 5 of the 17 transformed strains. The copy number of the pSIL-KEL1 cassette in the *T. harzianum* transformants was analyzed by DNA blot, using the 1.36 kb fragment containing the region corresponding to the *ble* gene and part of the *gpdA* promoter as a probe, and *Sal*I and *Sac*I digested DNAs (Fig. 3). No signal was observed in the lane containing T34 DNA (lane 6) and 2–4 highlighted bands were detected in the lanes corresponding to the five transformants (lanes 1–5). As the probe did not contain *Sal*I or *Sac*I restriction sites, between two and four copies of the transformation cassette had been integrated into the silenced-transformants, of which Kel4, Kel10 and Kel15 were selected for further characterization.

We compared the expression level of *Thkel1* in three *Thkel1*-silenced transformants (Kel4, Kel10 and Kel15) and the empty

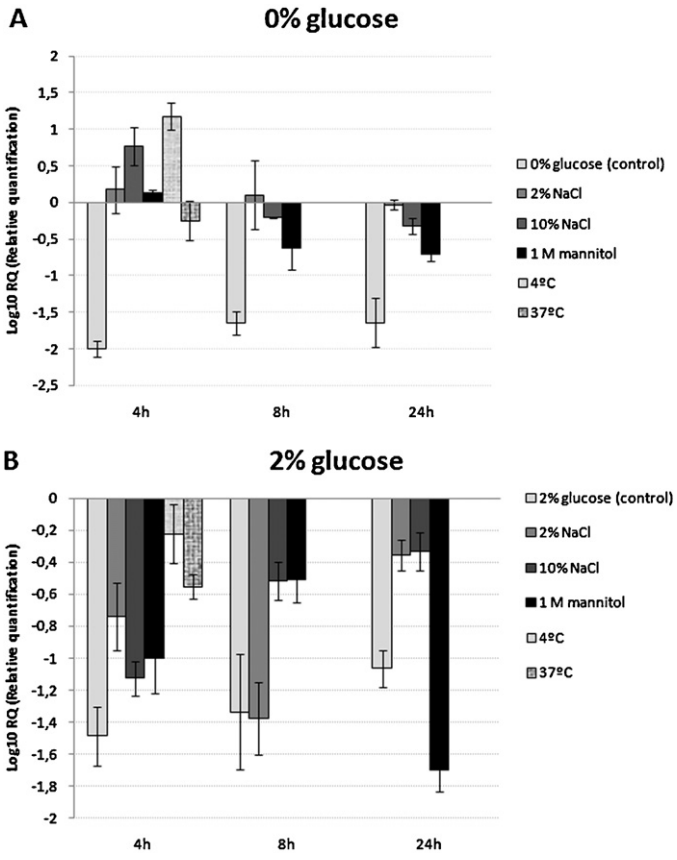

Fig. 2. qRT-PCR quantification of *Thkel1* transcript in *T. harzianum* T34, using *β-tubulin* as reference gene. The experiment was carried out with total RNA (20 μg) extracted from mycelia of *Trichoderma harzianum* T34 grown in MM (Penttilä et al., 1987), without glucose or with 2% glucose, and under the following conditions: 2 or 10% NaCl, or 1 M mannitol at 28 °C for 4, 8 or 24 h; and at 4, 28 or 37 °C for 4 h.

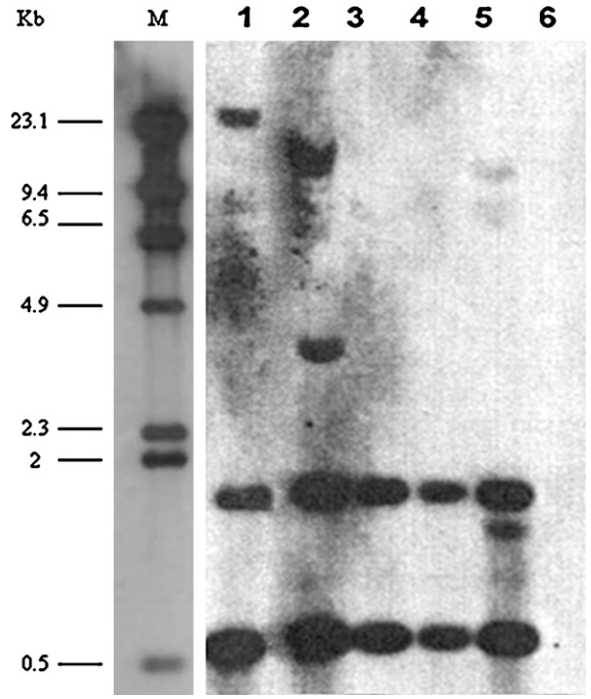

Fig. 3. DNA blot of total DNAs from transformants Kel4, Kel10, Kel14-1, Kel14-2 and Kel15 (lanes 1–5), and from *T. harzianum* T34 (lane 6). Genomic DNAs were *Sal*1 and *Sac*I-digested and hybridized with a probe of 1.3 kb, which contains the *phe* gene. On the left, molecular size standards of *Hind*III-digested λ DNA are indicated in kilobases.

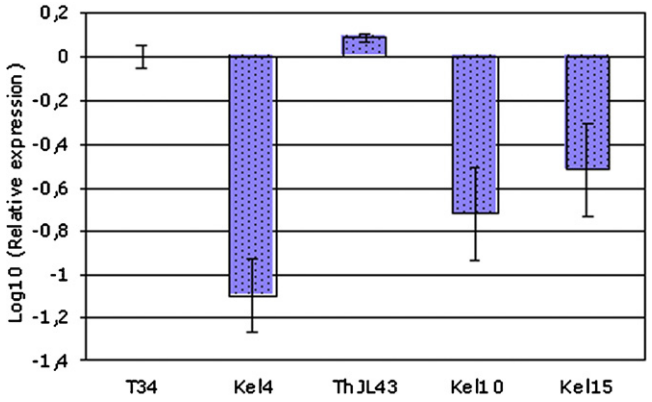

Fig. 4. qRT-PCR quantification of *Thkel1* transcript in the silenced transformants *T. harzianum* Kel4, Kel10 and Kel15, and in the non-silenced transformant ThJL43. Values correspond to relative measures against the *Thkel1* transcript in *T. harzianum* T34 wild-type (endogenous gene). The experiment was carried out with mycelia grown for 4 h in MM (Penttilä et al., 1987), without glucose, at 4 °C. *T. harzianum* T34 *β-tubulin* was used as an internal reference gene.

Table 1
Glucosidase activity measured in extracellular protein extracts from *T. harzianum* T34, *Thkel1*-silenced (Kel4, Kel10 and Kel15) and non silenced-transformant (ThJL43) strains after 24 h growth in MM at 4 or 28 °C. The activity was expressed as μmol of *p*-nitrophenol released per min per mg of protein. Results represent the average of 3 independent experiments ± standard deviation.

Strain	Glucosidase activity at 4 °C	Glucosidase activity at 28 °C
T34	0.20 ± 0.06a	2.67 ± 0.08c
Kel4	0.05 ± 0.02b	1.25 ± 0.07d
ThJL43	0.29 ± 0.03a	4.25 ± 0.16e
Kel10	0.17 ± 0.02a	1.70 ± 0.02d
Kel15	0.29 ± 0.02a	2.12 ± 0.11cd

Numbers followed by different letters represent statistically significant differences ($P < 0.05$; Fisher's test).

vector transformant ThJL43 to that of wild-type expression levels after growing at 4 °C (Fig. 4). Similar results were observed when fungi were grown at 28 °C (data not shown). The results showed that the silenced-transformants, especially Kel4, exhibited statistically significant lower *Thkel1* transcript levels than the wild-type strain T34. In addition, no difference was observed between the empty vector transformant and wild-type. The silenced strains exhibited statistically significant lower glucosidase activity values than those of T34 strain after growing at 28 °C for 24 h (Table 1) whereas ThJL43 strain exhibited the highest levels of glucosidase activity.

Statistically significant lower colony diameters were observed in the silenced transformants (Kel4 and Kel15) under the four stress conditions assayed (1 and 5% NaCl, and 0.5 and 1 M mannitol), compared to T34 (Table 2). Greater differences between wild-type and Kel4 and Kel15 were measured when higher concentrations of NaCl or mannitol were present in the medium. No statistically significant differences were observed between empty vector transformant and T34, except in the presence of 5% NaCl. When the growth of wild-type and silenced-transformant strains was tested after 5 weeks of incubation at 4 °C, only slightly lower colony diameters were observed in the silenced-transformant Kel4 than those in the wild-type.

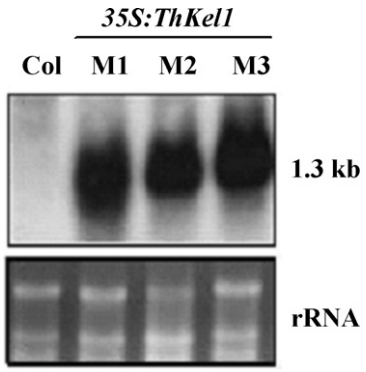

Fig. 5. RNA blot analysis of transgenic lines overexpressing *Thkel1* transgene. Total RNA (10 μg/line) from wild-type plants and M1–M3 transgenic plants was isolated and hybridized with a specific *Thkel1* probe. Bottom, ethidium bromide-stained gel showing rRNAs.

Table 3
Glucosidase activity measured in protein extracts from four week old Col-0 seedlings and M1, M2 and M3 transgenic lines, without or with a salt stress (5 ml of 100 mM NaCl for 10 days). The activity was expressed as μmol of *p*-nitrophenol released per min per mg of protein. Results represent the average of 3 independent experiments ± standard deviation.

Line	Activity without stress	Activity under salt stress
Col-0	1.29 ± 0.03a	1.30 ± 0.01a
M1	1.62 ± 0.04b	1.76 ± 0.04b
M2	1.64 ± 0.02b	1.87 ± 0.01b
M3	1.41 ± 0.04c	1.72 ± 0.06b

Numbers followed by different letters represent statistically significant differences ($P < 0.05$; Fisher's test).

Generation and characterization of 35S:Thkel1 transgenic lines

Thkel1 transgenic plants were selected in the presence of kanamycin and, subsequently three independent T_3 homozygous lines showing high levels of the transgene were selected (Fig. 5). DNA blot analysis of these lines revealed that M1 line contained one insertion of the transgene, M2 two insertions and M3 line three insertions (data not shown).

Glucosidase activity was analyzed in four-week-old transgenic and Col-0 plants under normal and salt stress conditions. As shown in Table 3, the activity values were significantly higher in the three transgenic lines than those of wild-type plants, the highest differences being observed under salt stress conditions.

Seed germination of *Thkel1* transgenic lines was analyzed in the presence of 150 mM NaCl and 350 mM mannitol and compared with that of wild-type plants (Table 4). After 10 days under these conditions, approximately 80% of all transgenic plants were able to germinate and develop green cotyledons, compared with 40%

Table 2
Mycelial growth diameters of *T. harzianum* T34, silenced-transformant (Kel4 and Kel15) and empty vector transformant (ThJL43) strains measured on MM, without glucose, supplemented with 1 or 5% NaCl, and 0.5 or 1 M mannitol, after incubation at 28 °C for 2 or 3 (NaCl) days. Data of 8 independent experiments are shown including ±standard deviations.

Strain	Control	1% NaCl	5% NaCl	0.5 M mannitol	1 M mannitol
T34	3.30 ± 0.09a	2.11 ± 0.06a	1.20 ± 0.05a	2.23 ± 0.05a	1.37 ± 0.04a
Kel4	3.20 ± 0.05a	1.82 ± 0.07b	0.92 ± 0.04b	1.93 ± 0.05b	1.07 ± 0.04b
ThJL43	3.65 ± 0.10b	2.12 ± 0.07a	1.05 ± 0.05b	2.28 ± 0.06a	1.35 ± 0.05a
Kel15	3.23 ± 0.05a	1.92 ± 0.04b	0.93 ± 0.05b	1.95 ± 0.05b	1.03 ± 0.05b

For a same column, numbers followed by different letters represent statistically significant differences ($P < 0.05$; Fisher's test).

Table 4
Percentage of *Arabidopsis* Col-0 and M1, M2 and M3 transgenic seeds that germinated and developed green cotyledons in A. MS medium supplemented with 150 mM NaCl. (B) MS medium supplemented with 350 mM mannitol. Data followed by an asterisk differ significantly with respect to Col-0 ($P < 0.05$; Fisher's test).

	4 d	6 d	8 d	10 d
A				
Col-0	0	30.2 ± 3.1	33.9 ± 4.0	34.8 ± 2.9
M1	10.1 ± 0.5*	60.4 ± 6.8*	68.6 ± 7.1*	84.8 ± 4.7*
M2	10.8 ± 0.6*	30.3 ± 4.2	59.5 ± 4.9*	85.0 ± 3.8*
M3	11.8 ± 0.5*	80.3 ± 7.9*	82.7 ± 8.9*	85.1 ± 3.6*
B				
Col-0	0	14.2 ± 1.7	18.7 ± 2.1	37.6 ± 4.0
M1	8.3 ± 0.7*	38.2 ± 3.6*	57.9 ± 4.3*	76.7 ± 3.7*
M2	0	37.5 ± 3.0*	40.0 ± 5.3*	59.9 ± 2.1*
M3	0	60.7 ± 8.8*	79.2 ± 7.5*	80.1 ± 3.2*

Table 5
Percentage of bleaching plants in four-week-old *Arabidopsis*, Col-0 and *Thkel1* transgenic line (M1, M2 and M3) plants, after daily washing with 5 ml 100 mM NaCl for 10 days. Mean values obtained from four different experiments.

Line	% bleaching plant
Col-0	53.1 ± 21.7a
M1	23.5 ± 8.4b
M2	27.8 ± 7.6b
M3	21.6 ± 12.9b

Numbers followed by different letters represent statistically significant differences ($P < 0.05$; Fisher's test).

observed in wild-type seeds, indicating that these transgenic seeds were more tolerant to the adverse conditions used in this study. When the seeds of wild-type and *Thkel1* overexpressing plants were incubated at the low temperature of 12 °C, the wild-type and the three transgenic lines initiated germination on the 5th day and no significant changes in germination rate and seedling growth were observed among different plant lines. Furthermore, under normal conditions almost 100% of both wild-type and transgenic seeds were able to germinate (data not shown).

To check the tolerance of *Thkel1*-overexpressing plants to salt stress, wild type and transgenic seeds were sown on MS medium for one week and after that, plants were transferred to soil, grown for 3 weeks and then treated with NaCl or water for 10 days. The percentages of bleaching of Col-0 plant, in four different experiments, were always higher than those observed in transgenic lines (Table 5).

Because transgenic plants showed more resistance to several abiotic stress conditions, ABA levels were analyzed in four-week-old plants, Col-0 and transgenic lines, under normal and salt stress conditions. As shown in Table 6, the levels of ABA were significantly higher in wild-type plants than those in the transgenic ones. ABA levels were increased under salt stress conditions and this fact was more evident in Col-0 plants. In addition, JA, SA and IAA levels were also measured under normal and salt stress conditions; however, the amount of these hormones was very different among the three transgenic lines (data not shown) and conclusions could not be established.

Table 6
Amount of abscisic acid (ng g^{-1} dry weight) in four week old *Arabidopsis* plants from Col-0 and *Thkel1* transgenic lines (M1, M2 and M3). Values are averages of three replicates ± standard deviation.

Line	ABA under control conditions	ABA under salt stress conditions	Ratio ABA stress/ABA control
Col-0	49.46 ± 3.02a	80.06 ± 2.05c	1.62
M1	43.09 ± 2.28a	49.83 ± 2.52a	1.16
M2	34.04 ± 3.99b	37.09 ± 1.38ab	1.09
M3	33.92 ± 3.78b	44.94 ± 4.79ab	1.32

Numbers followed by different letters represent statistically significant differences ($P < 0.05$; Fisher's test).

Table 7
Stomatal conductance measured as μmol m^{-2} s^{-1} from four week old Col-0 and M1, M2 and M3 *Thkel*-transgenic line plants, without or with a salt stress (5 ml of 100 mM NaCl for 10 days). Values are means from 6 replications ± standard deviation.

Line	Stomatal conductance under control conditions	Stomatal conductance under salt stress conditions
Col-0	0.053 ± 0.012a	0.012 ± 0.002c
M1	0.087 ± 0.007b	0.032 ± 0.009d
M2	0.072 ± 0.013ab	0.017 ± 0.002cd
M3	0.085 ± 0.008b	0.022 ± 0.002cd

Numbers followed by different letters represent statistically significant differences ($P < 0.05$; Fisher's test).

Finally, stomatal conductance was measured in all plant lines and under both conditions, with or without salt stress. As shown in Table 7, the highest values were observed in *Thkel1*-overexpressing plants and after maintaining them under normal growth conditions.

Discussion

Since the *Trichoderma Thkel1* gene did not show nucleotide identity with genes deposited in public databases and only displayed a low level of homology with hypothetical fungal proteins and plant nitrile ESPs, which contain kelch domains, the biological role of this fungal gene is not easily determined. We analyzed the expression of this gene in *T. harzianum* T34 under a plethora of growth conditions (data not shown), and the highest transcript levels were detected at 4 °C and with 10% NaCl. *Thkel1* gene expression was not increased under glucose starvation but was repressed in the presence of glucose. The functional analysis of *Thkel1* was carried out by a silencing approach in *T. harzianum* T34 and as could be expected *Thkel1* transformants showed lower qRT-PCR transcript levels than the wild-type strain, grown under conditions of maximum gene expression: 4 °C for 4 h (Fig. 4).

While absent in prokaryotes, kelch proteins are ubiquitous in eukaryotes. They are thought to be involved in the formation of protein complexes but their role is not completely well understood (Adams et al., 2000; Schumann et al., 2010). Fungal proteins with kelch domains have been related to G protein-mediated pathways in *Saccharomyces cerevisiae* (Niranjan et al., 2007) or galactose oxidase activity in phytopathogenic species from *Botrytis*, *Gibberella*, *Magnaporthe* and *Stagonospora* (Soanes et al., 2008). ThKEL1 did not show such amino acid identity but shared homology with *Arabidopsis* ESPs involved in the complex regulatory glucosinolate/myrosinase defense system (Morant et al., 2008). Furthermore, one of the 6 ESPs identified in *Arabidopsis* has been considered to be the ancestor of proteins with kelch motifs (Burow et al., 2009), also present in hypothetical proteins from *Aspergillus* and *Gibberella*, and shows the highest identity with ThKEL1. Some ESPs are supposed to be involved in the formation of protein complexes with β-glucosidases, playing an important role in plant defense (Grubb

and Abel, 2006; Yan and Chen, 2007). For instance, glucosidase PYK10 and its partner PBP1 (PYK10 binding protein), a molecular chaperone that allows the correct folding of PYK10 when *Arabidopsis* subcellular structures are damaged (Nagano et al., 2005, 2008). Another example of interaction between a β-glucosidase, Glu1, and its partner β-glucosidase aggregation factor (BGAF), has been reported in maize (Blanchard et al., 2001). However, the biological role of these complexes is not well understood, although it has been suggested that the formation of these aggregates between a β-glucosidase and its interacting protein may be the way to protect the enzyme molecule (Nagano et al., 2005).

When β-glucosidase activity was measured in *Trichoderma* strains, the three silenced transformants showed lower levels than those detected in strain T34 after incubation at 28 °C for 24 h. This result, also observed for the silenced transformants Kel4 and Kel10 at 4 °C, relates ThKEL1 to the modulation of β-glucosidase activity in *Trichoderma*. Taking into account that the highest *Thkel1* transcript level in the wild-type strain was observed in 4-h-old cultures and that glucose represses this gene (Fig. 2), it could be expected that this early transcript increase might lead to enhanced glucosidase activity. As shown by our experiments, glucose may also act as a catabolic repressor of *Thkel1*. Significant colony growth differences were observed between wild-type and silenced transformants when salt or mannitol was present in the medium (Table 2), being indicative of the involvement of ThKEL1 in fungal tolerance to salt and osmotic stresses. However, the absence of significant colony growth differences among *Trichoderma* strains after a 5-week thermal stress at 4 °C could be due to the scarce mycelia developed under this condition that could lead to low β-glucosidase activities (Table 1).

Some plant glucosidases have been related to plant responses to biotic (Burow et al., 2007; Mishina and Zeier, 2007; Minic, 2008) or abiotic stresses (Lee et al., 2006; Zhao et al., 2008). Thus, considering the behavior of *Thkel1* in *Trichoderma*, it should not be discarded that the overexpression of *Thkel1* in *Arabidopsis* may improve plant responses to abiotic stresses. In fact, transgenic *Thkel1* seeds showed higher percentages of germination in the presence of NaCl or mannitol than those from Col-0 *Arabidopsis* plants (Table 4). Furthermore, in later stages of development Col-0 plants had lower β-glucosidase activity and were more sensitive to salt stress than *Thkel1*-transgenic plants (Table 5).

It is known that osmotic shock blocks germination through ABA action (Leung and Giraudat, 1998) and, in order to elucidate the role of this hormone involved in plant responses to abiotic stress, ABA levels were analyzed in transgenic and Col-0 plants, under control and salt stress conditions. As shown in Table 6, except for line M1 under control conditions, ABA levels were significantly lower in *Thkel1* transgenic plants than those in wild-type plants under the two conditions tested. The highest differences were observed under salt stress conditions. ABA deficient mutants such as *aba2* are insensitive to salt and osmotic stress conditions and are able to germinate and develop green and fully expanded cotyledons compared with wild-type seeds, which showed a severe delay in germination (González-Guzmán et al., 2002). Thus, the lower levels of ABA detected in *Thkel1* overexpressing plants are consistent with the higher resistance to salt and osmotic stresses observed in *35S:Thkel1* transgenic seedlings (Table 4). Moreover, *aba2* mutants were more sensitive to salt stress at later stages of development, while this effect was not observed in *Thkel1* transgenic plants. Although ABA levels were lower in transgenic plants than those in Col-0 plants, *Thkel1* transgenic lines were more tolerant than wild-type to a salt stress (Table 5). It has been previously reported that the loss of function of AtBG1, a β-glucosidase of *Arabidopsis*, causes lower ABA levels, defective stomatal movements and abiotic stress-sensitive phenotypes. Furthermore, gain of function of this glucosidase provokes an increase in ABA levels and enhanced tolerance to abiotic stress through modulation of ABA metabolism (Lee et al., 2006). In the present work, we have observed that *Thkel* is able to modulate glucosidase activity and its overexpression in *Arabidopsis* causes a reduction of ABA levels accompanied by a reduction in the stomatal closure, data that are controversial with the higher tolerance to salt stress of adult transgenic plants compared with wild-type. Taking into account that a *Thkel1* heterologous overexpression in *Arabidopsis* was carried out instead of a homologous one, such as *AtBG1*, we hypothesize that this foreign gene may be also modulating in some way ABA biosynthesis/catabolism, affecting the plant responses to abiotic stresses. In any case, data presented along this manuscript point out the biotechnological value of *Thkel1*-overexpressing plants under salt and osmotic stress conditions, without an alteration of their phenotype, this fact being one of the main objectives of this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2011.01.027.

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