A new mutant of *Arabidopsis* disturbed in its roots, right-handed slanting, and gravitropism defines a gene that encodes a heat-shock factor

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Abstract

A new mutant of *Arabidopsis* named *rha1* is characterized and the gene involved cloned. In roots, the mutant shows minimal right-handed slanting, reduced gravitropic response, notable resistance to 2,4-D, but scarce resistance to IAA and NAA. The roots also show a clear resistance to the auxin transport inhibitors TIBA and NPA, and to ethylene. Other characteristics are a reduced number of lateral roots and reduced size of shoot and root in the seedlings. The gene, cloned through TAIL-PCR, was found to be a heat-shock factor that maps on chromosome 5, close to and above the RFLP marker m61. The *rha1* structure, mRNA, and translation product are reported. Since, so far, no other gravitropic mutant has been described as mutated in a heat-shock factor, *rha1* belongs to a new group of mutants disturbed in slanting, gravitropism, and auxin physiology. As shown through the RT-PCR analyses of its expression, the gene retains the function connected with heat shock. If the characteristics connected with auxin physiology are considered, however, it is also likely that the gene, as a transcription factor, could be involved in root circumnutation, gravitropic response, and hormonal control of differentiation. Since GUS staining under the gene promoter was localized mainly in the mature tissues, *rha1* does not seem to be involved in the first steps of gravitropism, but is rather related to the general response to auxin. The alterations in slanting (mainly due to reduced chiral circumnutation) and gravitropism lead to the supposition that the two processes may have, at least in part, common origins.

Key words: auxin, ethylene, gravitropism, HSFs, slanting.

Introduction

When grown on a hard-agar plate, especially if tilted on the vertical, *Arabidopsis* roots do not elongate straight down, but make different kinds of movements. In the wild type they wave almost regularly, slant preferentially toward one side, which has been considered the right hand because of the right-handedness of the root helix, and at the same time twist on themselves. In addition, they frequently make strict right-handed coils. After Darwin (1880), who observed the process in different plants, the wavy pattern in *Arabidopsis* was reported by Okada and Shimura (1990), who interpreted it as the consequence of an interaction between thigmotropism and positive gravitropism. In the same paper, Okada and Shimura also reported the torsion (cell file rotation or CFR) seen in the waves alternatively to the left and to the right hand, concomitantly, respectively, with the right- and left-hand waves. The right-handed slanting was first reported by Simmons *et al.* (1995), who observed it when working on the mutant *rgr1*, which shows reduced gravitropic response. Slanting mutants were subsequently reported by Rutherford and Masson (1996), who named them *sku*. Later, Migliaccio and Piconese (2001) gave a general interpretation of the movements of *Arabidopsis* roots growing on tilted agar plates. The process was described as the result of an interaction between right-handed...
circumnutation, positive gravitropism, and negative thigmotropism, whereas the CFR was described as a secondary effect, due to the adjustment of the three-dimensional right-handed root helix to the flat surface of the agar in the Petri dish. Following a different interpretation and excluding circumnutation, Thompson and Hollbrook (2004) described the waving and CFR as a consequence of the constraint that the growing root tip encounters in moving down a 45° tilted agar plate. This would be a possible alternative to the thigmotropic effect, if it explained the chiral slanting and the frequently observed waving and coiling on vertically set plates, processes that seem to depend on the circumnutations of the activity of the roots. Two mutants, i.e. spir1 and spir2, which show a slanting opposite to that seen in the wild type were reported by Furutani et al. (2000). In their paper and in others that followed (Nakajima et al., 2004; Ishida et al., 2007), the authors showed that the genes involved in the mutations encode proteins that are constituents of the microtubules, and suggested that the helical handedness of microtubules in the epidermal root cells was opposite to the torsion seen in the cell files. A little earlier a left-handed mutant was described by Marinelli et al. (1997) and simply named 1–6, a probable allele of spir1.

Other significant isolated mutants were wvd2 and rhd3. wvd2 shows constitutive root and shoot right-handed CFR, and left-handed root slanting on agar surfaces (Yuen et al., 2005). The mutant also presents a modified arrangement of cortical microtubules in the elongation zone and root cap cortical cells. rhd3, which almost totally lacks waving, slanting, and CFR, probably because of the thickness of its roots, was previously implicated in vesicle trafficking between the endoplasmic reticulum and Golgi apparatus, but was later found to be involved in anisotropic cell expansion, and again in microtubule synthesis (Yuen et al., 2003). In addition, another mutant named adk1, which lacked waving, was recently found to be mutated in a gene for adenosine kinase (Young et al., 2006). This finding added the enzyme to the factors affecting root movements.

The involvement of microtubules in the CFR was also supported by experiments made with drugs which interfere with their formation, since these substances can increase or reverse the torsion processes (Furutani et al., 2000). The relationship between microtubules, cellulose microfibrils, and CFR in epidermal cells is, however, still not clear and data contradicting the currently accepted picture were reported. For instance, roots of the mutant saku6/spir1 maintain a transverse orientation of the microtubules in spite of the right-handed CFR of the roots (Sedbrook et al., 2002), and CFR also occurs in the mutant mor1-1, whose microtubules do not show biased orientation. On the basis of these results, Wasteneys recently proposed that CFR could be independent of microtubule and cellulose microfibril arrangements in the cells, and simply depend on the loss of anisotropy in the radial tissues or inherent twisting handedness of the roots (Wasteneys, 2004; Wasteneys and Collings, 2004). Further investigation may be necessary to settle the argument.

Other research regarded the effect of environmental and growing conditions on root movements, such as the tape used to seal the Petri dishes, the composition of the growing medium, or the production of ethylene by the root waving and twisting (Buer et al., 2000, 2003). It was shown that root slanting, waving, and CFR can be modified by these conditions. Salt stress was also recently added to the environmental factors that can influence root movements (Shoji et al., 2006).

The present paper reports on the characterization of a new root mutant of right-handed slanting and gravitropism, which also shows resistance to 2,4-dichlorophenoxy-acetic acid (2,4-D), some auxin transport inhibitors, and ethylene. The gene involved was cloned, and shown to encode an Arabidopsis heat-shock factor (HSF), which could be involved, through the control of auxin-mediated processes, in the regulation of root circumnutation and gravitropic movements.

Materials and methods

Plant material and growth conditions

The Feldmann–Du Pont collection of T-DNA mutagenized seeds from the ecotype Ws (Wassilewskija), as well as the mutants axr1-7, axr1-3, eir1, and axr2 were provided by the Nottingham Arabidopsis thaliana Stock Centre (NASC, Nottingham, UK). Seeds of Arabidopsis thaliana (L.) Heynh., ecotype Ws, as well as the rgr1 and wav6 mutants were obtained from the laboratory of D Söll (Yale University, New Haven, CT, USA). Before plating, seeds were sterilized in 50% commercial bleach, 0.01% SDS, plus four sterile distilled water washes. Seeds were plated in horizontal rows, in Petri dishes, on a medium made up (except in some cases where 0.8% and 1% agar were used) of 1.5% agar, 1% sucrose, and 0.5× MS basal medium, enriched with Gamborg’s vitamins (Sigma, St Louis, MO, USA), adjusted to pH 5.7 with NaOH. To synchronize germination, dishes were left in a cold room (4 °C) for 2 d before moving them to the growth chamber.

Plants were at first grown on dishes for 10–14 d in an Arabidopsis growth chamber (Percival Scientific, USA), in white fluorescent light at 150 μmol m⁻² s⁻¹ light intensity, and 23 °C air temperature, and then transferred to Arasystem pots (7 cm) on a medium made up of 40% sand, 35% turf, and 25% soil. The plants were grown, keeping them at first in short days (8/16 h light/dark photoperiod) to promote rosette growth, and, after 3 weeks, in continuous light to promote flowering. Humidity in the growth chamber was ~65%.

Mutant selection

Forty-eight out of the 49 pools of the 100 lines that constitute the original T-DNA-tagged Feldmann–Du Pont collection, for a total of 20 758 individuals, were submitted to a first screening. This consisted of the visual examination of the growth direction (slanting and gravitropism) and of the general behaviour of the primary root, looking for modifications in the above characters.
Seeds were germinated and grown in Petri dishes, set in horizontal rows, on agar medium containing kanamycin (50 mg 1\(^{-1}\)). Dishes were kept for 3 weeks inclined at 60\(^\circ\) on the horizontal plane to check for waving, slanting, and any deviation from the vertical. Plants resistant to kanamycin, showing an anomalous root growth pattern were selected, grown to maturity, and selfed. The second generation was submitted to a new screening with the same procedure on a medium deprived of kanamycin and compared with the wild type as a control. As a result of the screening, a few interesting root mutants were selected. Of these the one that was named *rha1* (Fig. 1), for its characteristic of showing reduced or inverted root slanting (handedness) on agar plates, was selected for analysis as presented in this paper.

**Mutant characterization**

Shoot and root lengths were determined on 6-d-old seedlings, lateral root numbers on 15-d-old seedlings, cotyledons length on 8-d-old seedlings, and silique length and silique stalk distance at the time of fruit ripening on 35-d-old plants. Measurements were done under a stereomicroscope. Plants were grown in an *Arabidopsis* growth chamber at 150 \(\mu\)mol m\(^{-2}\)s\(^{-1}\) light intensity, 65% relative humidity, and 23 °C air temperature, on day/night cycles of 8/16 h photoperiod up to the third week, and 16/8 h thereafter.

**Gravitropism and slanting determination**

Root slanting was measured by growing seedlings on 1.5% MS medium, and by keeping the dishes vertical for 3 d at first, and then inclined 60° over an additional 7 d to produce the slanting (Simmons *et al.*, 1995). The slanting was evaluated by measuring the deviation of the root tips from a vertical line parallel to the gravitational vector, passing through the shoot base, traced on the bottom of the plates.

To quantify the gravitropic response of the roots, *rha1* seedlings were grown in square Petri dishes on 1.5% agar, kept vertical for the first 10 d, then rotated 90° anticlockwise, and kept in this position over 24 h. Digital pictures were taken at 2 h intervals and up to the 24th hour. Angles of deviation were measured using the Scion image analysis program (Scion Corporation, Frederick, MD, USA). Both the slanting and the graviresponse experiments were done in a growth chamber especially built to grow *Arabidopsis* from Percival under the same conditions as reported for the general characterization.

**Response to growth substances**

Seeds were germinated and grown for 5 d in the light, in vertically set Petri dishes. Thereafter, seedlings of the same size were transferred to a new medium containing the growth substances, and grown for 7 d in the dark. Root elongation was measured at the end of 7 d, and from the measurements the growth relative to controls was evaluated. Data are mean values of at least 30 samples ± standard error. Growth substances, i.e. indole-3-acetic acid (IAA), naphthalenecarboxylic acid (NAA), 2,4-D, 2,3,5-tri-iodobenzoic acid (TIBA), and naphthalenemethanolic acid (NPA) were administered by dissolving them in ethyl alcohol, and by dispersing a minimal amount of the substance in the media [alcohol <0.1% (w/v), added to controls in same amount]. Ethylene was administered as 1-aminocyclopropane-1-carboxylic acid (ACC) dissolved in water. All data were submitted to Student’s t-test, to check for consistency of the mean value differences. Environmental conditions were the same as those applied in the gravitropic and slanting experiments.

**TAIL-PCR**

The TAIL-PCR (thermal asymmetric interlaced PCR) technique was utilized to clone the gene *RHA1*. It was applied following the methodology given by Liu *et al.* (1995). As primers (Table 1), three nested oligonucleotides complementary to sequences of the T-DNA left-border (TL-1, TL-2, and TL-3) and one degenerate (DEG-2) were used.

**RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses**

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad CA, USA) as described by the manufacturer. First-strand cDNA was made from 2.5 μg of total RNA from different *Arabidopsis* tissues (roots, leaves, and total seedlings). The RNA was incubated at 65 °C for 5 min in 10 mM dNTP, 100 pmol oligo(dT) (Promega), and water to 12 μl. Samples were chilled on ice, then incubated at 42 °C for 50 min in 1× first-strand buffer, 10 mM DTT, and 200 U RT-Super Script II (Invitrogen) in a total volume of 20 μl, followed by incubation at 70 °C for 15 min. Two microliters of the reaction fluid were used for each PCR. PCR was carried out under standard conditions using 10 μmol of each gene-specific primer (Rha1F5 and Rha1R3) and 30 cycles of 95 °C for 45 s, 50–62 °C for 30 s, and 72 °C for 30–60 s in GeneAmp PCR System 2400 (Perkin Elmer). Products were separated on 1.0% agarose gels and revealed by ethidium bromide staining. RT-PCR products were sequenced by Genelab (ENEA-Casaccia, Rome, Italy).

The expression of *RHA1* in the different parts of the plants, the effect of heat and cold shocks at different times, the action of auxins, and the microgravity stress were determined through semiquantitative RT-PCR, by utilizing pairs of primers homologous to sequences of the cDNA (Table 1). Plant material was collected and treated by a standard technique to extract total RNA. Heat shock was at 37 °C, cold shock at 4 °C, and auxins were administered dissolved in a minimum volume of alcohol (<0.1%).

The expression of heat and cold shocks were evaluated on 15-d-old seedlings at 30, 60, 120, and 240 min and 24 h after exposure at 37 °C or 4 °C. Auxin effects were evaluated on seedlings germinated and grown for 12 d on simple agar, and thereafter transferred and grown for 3 d on a new agar medium containing the hormones. Gravitational stress was determined on 15-d-old seedlings after a run of 8 h on a two-axes clinostat (a random positioning machine from Dutch Space, The Netherlands, located at the University of Milan, Italy; setting 60° s\(^{-1}\)). Every experiment was performed both

<table>
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<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Tm (°C)</th>
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<tr>
<td>AAP</td>
<td>GGC CAC AGC GTC GAC TAG TACT</td>
<td>66.1</td>
</tr>
<tr>
<td>AUAP</td>
<td>GGC CAC GCG TCG ACT AGT AC</td>
<td>63.4</td>
</tr>
<tr>
<td>DEG-2</td>
<td>(AGCT)TC GA(GC) T(AT) (GCG)ATGTT</td>
<td>43.7</td>
</tr>
<tr>
<td>TL-1</td>
<td>CAG CCA ATT TTA GAC AAG TAT CA</td>
<td>55.3</td>
</tr>
<tr>
<td>TL-2</td>
<td>AAC TGT AAT GAC TCG GCG CAA TA</td>
<td>58.9</td>
</tr>
<tr>
<td>TL-3</td>
<td>TCT GGG AAT GGC GTA ACA AAG GC</td>
<td>62.4</td>
</tr>
<tr>
<td>ARHA1 F1</td>
<td>AGC TTT AAT GGA GGC AGC TCA AGA</td>
<td>62.7</td>
</tr>
<tr>
<td>ARHA1 F3</td>
<td>AGC TTT GAG ACT ACA TAA TGG ATC TA</td>
<td>58.9</td>
</tr>
<tr>
<td>ARHA1 F5</td>
<td>TAA TGG AGG TTC AAG CTC ACT TCC</td>
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<td>GCG TTA ACG ATG ACT TTT GGG AAC</td>
<td>61.0</td>
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<tr>
<td>ARHA1 F8</td>
<td>GAA ATC A AA GGA CTT ATT GGT GG</td>
<td>58.9</td>
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<td>AAG CAT CAA GAA GGA GCT CTC GCA</td>
<td>62.7</td>
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<td>CCA TGG GTT GTG TCT AAA AAG CGG</td>
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<td>TCCCAA AAC GTC AAG GAA GAT TC</td>
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<td>ARHA1 R4</td>
<td>ACC TTA ACC ACT TCG AAT CCA AG</td>
<td>58.9</td>
</tr>
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<td>TGA ACC TTC ATT TTC ATC CA</td>
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<tr>
<td>ARHA1 R6</td>
<td>GGT TCG TTG AAA ACT CGT CGT GAT</td>
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on rha1 and on the wild-type Ws. Rha1R3 and Rha1F5 were used as primers to check the expression of the RHA1 gene. ACTIN8 gene was used as a control for the cDNA. All the RT-PCR were normalized with the actin gene, utilizing the primers ACTS-Fw (5'-ATGAA-GATTAAGGGTGCGA-3') and ACTS-Bw (5'-TCCGAGTTTG- GAAGAGCTAC-3'). For each experiment the relative optical intensity was calculated by means of the Kodak Digital Science ID software (Kodak), and corresponds to the ratio of the mean intensity of each spot to that of the related actin spot. The experiments were repeated in triplicate with the same results, one of which is shown in Fig. 6.

5′-RACE and 3′-RACE

The 5′-RACE was performed using the Kit version 2 (Life Technologies) according to the manufacturer’s protocol, on 1 µg of total RNA extracted from wild-type plants. First-strand cDNA synthesis was primed with a gene-specific primer (RHA1 R6). After the second-strand synthesis, the cDNA was amplified using an anchor oligo- and a gene-specific primer (RHA1 R5). 5′-RACE amplification was performed with a nested gene-specific primer (RHA1 R4).

The 3′-RACE was performed using 1 µg RNA from wild-type plants and 200 U of M-MLV RT (Gibco BRL). First-strand cDNA synthesis was primed with a poly(dT) primer with anchor sequences. After second-strand synthesis, the cDNA was amplified using an anchor oligo- and gene-specific primer (RHA1 F7). 3′-RACE amplification was performed with a nested gene-specific primer (RHA1 F8). The PCR products from both 5′- and 3′-RACE analysis were subcloned in pGEM-T Easy vector (Promega), according to the manufacturer’s protocol, and sequenced by GeneLab (ENEA-Casaccia Rome, Italy). All the sequences produced were analysed using the NCBI-BLAST server network.

Complementation, generation, and analysis of Arabidopsis transgenic plants

A 4836 bp fragment (RHA1 gene with its promoter region) was amplified by PCR using Arabidopsis genomic DNA as template, the oligonucleotides RHA1 F1 and RHA1 R1 as primers, and high fidelity Tth DNA polymerase XL (Perkin Elmer). The amplification product was cloned in pGEM-T Easy vector (Promega) and sequenced. The insert was recovered as an ApaI–SacI fragment, and blunt and cloned in pCAMBIA 1300 vector in the Smal site. Agrobacterium tumefaciens strain GV3101(pMP90) carrying pCAMBIA 1300-RHA1 vector was grown to stationary phase in liquid culture at 28–30 °C, 200 rpm in sterilized LB (10 g tryptone, 5 g yeast extract, 5 g NaCl 1 l⁻¹) with added kanamycin (50 mg l⁻¹), rifampicin (50 mg l⁻¹), and gentamycin sulphate (25 mg l⁻¹). rha1 plants were dipped in this liquid culture after most of the secondary cells from a 150 ml overnight culture, 0.03% (300 µl l⁻¹) of the surfactant Silwet L-77. Vacuum was applied for 8 min at a pressure close to 600 mmHg. After the inoculation, the plants were left in a low-light location, and covered with a transparent plastic dome to maintain humidity. Then, the dome was removed, and the plants returned to the growth chamber for 24 h.

To select for transformed plants, sterilized seeds were plated on hygromycin B selection plates (24 mg l⁻¹). Siliques from transformed plants were collected individually in 2 ml tubes.

GUS staining

The RHA1::GUS reporter construct was made by amplifying through PCR (primers ArRHA1 F3 and R2) a 1164 bp genomic fragment, supposedly containing the RHA1 promoter region. The DNA was cloned between the HindIII and NcoI sites of the pGEM-T Easy Vector, and subsequently subcloned into the HindIII–NcoI sites of the pCAMBIA 3301 vector. The vector was then introduced into the A. tumefaciens strain GV3101(pMP90) by electroporation. Wild-type Arabidopsis plants, ecotype Wassilewskija, were transformed by vacuum infiltration (Bechthold et al., 1998). T₃ seedlings homozygous for the insertion were identified by segregation analyses, and were used for GUS staining. For the experiments, four independent transformed lines were used. Seedlings containing the RHA1::GUS construct were grown on agar plates for 3 d. Four plates were rotated 90° anticlockwise from the vertical for 3 d to produce gravistimulation, four plates were run on a two-axes clinostat (RPM) for 6 h at 60° s⁻¹, and seedlings of four plates were transferred to a medium containing either the auxin IAA, NAA, or 2,4-D at a concentration of 10⁻³ M, and grown for 3 d. Subsequently, the seedlings were immersed in the X-gluc solution (0.1 M NaPO₄ buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM NaEDTA pH 8.0, and 1 mg ml⁻¹ X-glucuronide), and incubated in the dark at 37 °C overnight, followed by washing with 70% ethanol. Microscopy was performed with a Zeiss stereomicroscope (Jena, Germany). Images were captured using a Kodak digital Science apparatus, and transferred to a computer running the Adobe Photoshop CS2 program (Mountain View, CA, USA). The experiments were repeated three times with similar results. The most representative are shown in Fig. 7.

Results

Mutant isolation and genetic analyses

rha1 (for root handedness 1) was isolated from the Feldmann–Du Pont collection of T-DNA-tagged Arabidopsis mutants (Fig. 1) through a screening procedure directed to select seedlings disturbed in the right-handed slanting of the primary root (as defined in Migliaccio and Piconese, 2001), following the procedure reported in Materials and methods. The mutant was selected for its lack of a strong right-handed slanting, which characterizes the wild-type Ws. Its roots grow mostly parallel to the gravitational vector down a 60° tilted agar plate, or by moderately and randomly slanting to the right or the left hand, with a slight preference for the right.

In addition, the mutant presents other variations with respect to the wild type (Table 2), i.e. both the shoot (−17%) and the root (−8.0%) are shorter in the rha1 seedlings and the production of lateral roots is notably lower in the mutant (−39.8%). The siliques in rha1 are shorter, as are the spaces between the flowering stalks. All these characteristics are in line with the mutant being resistant to the auxinic substances as reported later. The number of statolysts is normal in the root cap cells but, when the mutant seedlings are run on a clinostat, their roots mainly produce random movements (Piconese et al., 2003). This differs from the wild type which makes clockwise loops.
The self-pollination of *rha1* produced in both F1 and F2 (Table 3) kanamycin (kan)-resistant and mutant individuals. The cross with the wild type produced in the F1, 82 wild-type kan-resistant individuals, and in the F2, 60 wild-type kan-resistant, 26 wild-type kan-sensitive, 25 *rha1* kan-resistant, and zero *rha1* kan-sensitive individuals, corresponding to a ratio of 2:1:1 ($\chi^2=0.75$, $P=0.95$).

From these crossings, and from the fact that a kan-sensitive *rha1* individual was never observed in the present experiments, it was established that the gene causing the mutation was, with high probability, tagged for a single T-DNA insert. More genetic tests investigated the possibility of absence of complementation with other mutants already known to be disturbed in gravitropism and auxin physiology. The test involved *axr1*, *axr2*, *aux1*, *eir1/pin2*, and *rgr1*, all of which complemented with *rha1*.

The presence of a T-DNA tag in the mutant was also demonstrated through Southern analysis performed with a T-DNA probe (data not shown), and by RT-PCR, showing that the *RHA1* gene is expressed in the wild type, but not in the mutant (Fig. 6E). Sequencing data indicated that the T-DNA tag is made up of at least two head to tail concatemers.

**Physiological analyses**

The physiological analyses tested the root slanting, the gravitropic response, and the reduced sensitivity of the roots to the auxins 2,4-D, IAA, and NAA, to the auxin transport inhibitors TIBA and NPA, and to ethylene, given as ACC. All these substances are known to alter the gravitropic response.

Analysis of the slanting showed that the deviation from the vertical of the *rha1* roots, notably to the right hand in the wild-type Ws, is clearly reduced and almost random (Fig. 2, top), even though a slight preference for the right hand is still apparent (the slanting values of *rha1* towards the left were considered negative in preparing the graph). Analysis of gravitropism (Fig. 2, bottom) showed that the roots have a significantly reduced gravitropic response, which is about 20% less than that seen in the wild type or in the complemented plants. Analysis of the response to the growth factors revealed that the mutant roots have a reduced sensitivity to the auxin 2,4-D, to the inhibitors of auxin transport TIBA and NPA, and to ethylene, whereas the sensitivity to IAA and NAA was close to that of the wild type (Fig. 3). *rha1* can therefore be considered
a new slanting, gravitropism, and 2,4-D mutant belonging to a new group, since, as reported later in this paper, the gene involved in the mutation is an HSF, whose knock out results in the observed disturbances. So far no gravitropic mutant has been shown to be mutated in an HSF.

Cloning of the gene

The cloning of the gene involved was performed by the TAIL-PCR technique, described in the Materials and methods. The acquired data, analysed through the online NCBI-BLAST program, showed that the T-DNA was inserted in a gene encoding an HSF from Arabidopsis, which was named in accordance with the mutant rha1. In particular, the gene was part of the contig MRA 19, located on chromosome 5 of A. thaliana, close to and above the RFLP marker m61 (Kazusa, Japan; physical map coordinates 18558082–18560081). By RT-PCR the core part of the mRNA was isolated and sequenced, and through RACE-PCR the 5'- and 3'-untranslated regions were also isolated and sequenced, furnishing the whole mRNA sequence of the gene.

The comparison between the genomic DNA and the mRNA (Fig. 4) showed that the gene is made up of three exons, 242, 290, and 961 bp long, and two introns, 405 and 90 bp long. The cDNA is 1490 bp, and the open reading frame 1038 bp. The protein sequence is 345 aa long (Fig. 5), translated from the second and third exon, the first exon being transcribed, but not translated. RT-PCR analysis showed that the messenger in the mutant is not transcribed at all, and thus the mutation in rha1 can be considered null. The full gene and putative protein sequence were deposited in Gen Bank with accession number AY350739. Comparison of the putative RHA1 protein sequence (Fig. 5) with other HSFs from Arabidopsis showed that the protein, as is usual for the HSFs, has a high degree of homology with them, especially in the DNA-binding domain. The HSFs are small proteins that function as transcription factors, being involved in the activation of the genes encoding heat-shock proteins (HSPs), but also in different metabolic and development-related processes. The HSFs of Arabidopsis have recently been divided into three groups, A, B, and C (Nover et al., 1996, 2001) on the basis of their structure. According to the present results, RHA1 belongs to the A group, and was recently and independently named HsfA4c by the above authors.

The mutation was then complemented by transformation of the full gene in the mutant, and reproduction of the wild-type phenotype (Figs 1–3) in the slanting, the graviresponse, and the sensitivity to growth substances. By RT-PCR it was also shown that the complemented plants expressed the gene RHA1 (data not shown).

Expression of the RHA1 gene

In Fig. 6, through an RT-PCR analysis, the expression of the RHA1 gene under various environmental conditions is reported. The experiments showed that the expression is increased under a heat shock of 37 °C (Fig. 6A) throughout the 24 h of the experiment. A cold shock of 4 °C (Fig. 6B) produced a similar effect, even though more moderate, which appears to vanish after 24 h. The growth of the plants with the auxin 10⁻⁵ M 2,4-D clearly increased the expression (Fig. 6C), whereas the auxins IAA and NAA had no effect. These data seem to show that rha1 is a 2,4-D, but not an IAA and NAA mutant. Figure 6D shows also that a gravitational stress induced through clinorotation in a random positioning machine (at 60 ° s⁻¹) over 8 h was ineffective in modifying the expression of the gene. Figure 6E shows the expression of RHA1 in whole seedlings and in seedling roots and leaves.
A reduced expression in leaves appears significant. On the other hand, the lack of expression of the gene in rha1 shows that the mutant is null.

Localization of the GUS staining in RHA1::GUS transgenic plants

To study the possible localization of the RHA1 gene expression, wild-type Ws plants were transformed with the reporter gene GUS under the control of the RHA1 promoter region (about 1000 bp). Figure 7 shows the GUS staining in plants grown vertically (Fig. 7B), and after some of the above-reported shock conditions. One of these was gravitropic stimulation that was produced either by placing the plants in a horizontal position (Fig. 7C) or through clinorotation (Fig. 7D), and by transfer of the seedlings for 3 d to a medium containing $10^{-5}$ M of the auxins 2,4-D, IAA, or NAA (Fig. 7E, F, and G, respectively). The results showed localization of the GUS staining only in the shoot of the vertically set plants, whereas gravistimulation produced a notable spread of the expression in the primary root. In the clinorotated plants, the RHA1 expression is also localized in the leaves. On the other hand, the 2,4-D treatment localized the expression in the leaves, the basal part of the shoot, and the root, whereas scarce localization of the expression was visible in the IAA- and NAA-treated plants. In the IAA-treated

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Fig. 3. Sensitivity of rha1 roots to plant hormones and their inhibitors. Root elongation of wild-type Ws, rha1, and complemented rha1 was measured to quantify their sensitivities to indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,3,5-tri-iodobenzoic acid (TIBA), naphthylphthalamic acid (NPA), and 1-aminoacyclopropane-1-carboxylic acid (ACC). Seeds were germinated and grown vertically for 5 d on MS medium, then transferred to a medium supplemented with the indicated concentrations of the above substances (controls: media without hormones), kept in the dark, and their growth measured 7 d later. From the measurements of root elongation the growth relative to controls was evaluated. Error bars represent the standard error ($n$=about 30). The experiments were replicated at least three times with similar results. The measurements from one representative experiment are presented. *$P<0.05$. 
plants only patches along the base of the shoot and the mature root were visible, and, in the NAA-treated plants, patches appeared not only along the shoot and the roots but also in the leaves. Interestingly, the localization of the staining of the \textit{RHA1::GUS} gene was most clearly visible in the mature tissues of both root and shoot, but the reporter gene does not seem to be expressed in their elongation zone, apical meristems, and root cap (Fig. 7I). It is also notable that \textit{RHA1::GUS} plants did not show GUS staining in the lateral root primordia (Fig. 7J, white arrow).

\section*{Discussion}

This research describes a new \textit{Arabidopsis} mutant, isolated from the Feldmann–Du Pont collection of T-DNA-tagged Wassilewskjia lines, whose roots show very little slanting toward the right hand and sometimes also reversed symmetry. This condition is typical of the ecotype Columbia, but not of the ecotype Ws (Rutherford and Masson, 1996). Consequently, since the main factor that determines slanting is chiral circumnutation (Migliaccio and Piconese, 2001), \textit{rha1} can be considered a mutant of this process and, since the involved gene was shown to be an \textit{HSF}, and the complementation was successful, the modification should be ascribable to a lesion in this gene. As was mentioned in the Introduction, other mutants that show reduced slanting are known. These mutants have genetic alterations which lead to increased or reduced slanting, but none of them was mutated in an \textit{HSF}, or a gene connected with the activity of this class of transcription factors.

Furthermore, the primary roots of \textit{rha1} showed reduced gravitropism and reduced sensitivity to 2,4-D, to the auxin transport inhibitors TIBA and NPA, and to ethylene (given as ACC) in their physiological range of action. When looked at as a whole, these characteristics have been shown to be the consequence of a single recessive mutation in a gene named in accordance with the mutant \textit{RHA1}. The mutation seems to be the result of a total loss of function, because of the large T-DNA insertion in the first intron, and of the lack of expression of the gene in the mutant shown through RT-PCR.

Until fairly recently the HSFs were thought to be involved only in the activation of the HSPs, through a mechanism only partly known, involving the formation
of a dimer or trimer, able to attach itself to the heat shock responsive element of the HSP DNA in the nucleus (Morimoto, 1998). If this were the only function of the HSFs, it would be difficult to explain RHA1 involvement in root symmetry, the reduced gravitropism, and root resistance to some hormones and their inhibitors. Today, however, this is no longer the case, since new functions have been found for the HSFs, which turned out to play a significant role in many different processes as transcription factors. For instance, in Drosophila (Jedlicka et al., 1997) it was shown that some of its HSFs are necessary for oogenesis and early larval development. In yeast, Gallo et al. (1993) showed that its unique HSF is necessary not only for the heat-shock response, but also for growth and fission at normal temperature. In tobacco and sugarcane, Harrington et al. (1994) showed that the HSPs (activated by HSFs) are involved in the phosphorylation process of proteins, and interact with calmodulin. In addition, recently it was also demonstrated that the HSFs and HSPs inhibit the expression of negative genetic characters that otherwise would affect the phenotype of different organisms (Pigliucci, 2002; Queitsch et al., 2002).

In particular, RHA1 seems to be involved in processes connected with auxin action and transport because of its resistance in the roots to 2,4-D, reduced gravitropism, reduced shoot and root length, inflorescence stalk distance, and lower secondary root number. These characteristics are similar to those seen in general in the mutants of auxin action and transport and, in particular, in aux1 and aux4 (Yamamoto and Yamamoto, 1998; Dharmasiri et al., 2006; Hobbie et al., 2006), whose roots, however, are also resistant to IAA, but not to NAA. In the latter case, the interpretation was that both aux4 and aux1 are involved in the active auxin transport inside the cells and that IAA and 2,4-D need active transport, whereas NAA diffuses...
passively. The same reasoning cannot be applied to \textit{rha1}, because its roots are not resistant to IAA, which seems also to enter the cells passively, which on the basis of the chemiosmotic theory would be the case. This fact seems to indicate that the difference in root resistance to the different auxins seen in some mutants cannot be ascribed only to differences of permeability, but also possibly to differences in the molecular structure of the receptors. This variation in the sensitivity to auxins has already been described for \textit{pin2} which shows greater sensitivity to auxins in the order NAA>IAA>2,4-D (Mueller \textit{et al.}, 1998). \textit{rha1} roots are also resistant to the auxin transport inhibitors NPA and TIBA and to the precursor of ethylene, ACC. These are substances that, at least in part, are considered to be actively transported inside the cells.

Because of its root resistance to NPA, \textit{rha1} also shares a similarity with the mutant \textit{rcn1}, which encodes a subunit of the 2A phosphatase and has been shown to be involved in the control of auxin transport (Garbers \textit{et al.}, 1996; Muday and De Long, 2001). Since the human HSF2 has been shown to interact with the 2A phosphatase, by substituting itself for the C unit of the protein (Hong and Sarge, 1999) a similar involvement of \textit{RHA1} can also be suggested.

The RT-PCR analysis, on the other hand, showed (Fig. 6) that the expression of the gene \textit{RHA1} is activated by heat and cold stresses and thus preserves its typical role, but also by the presence in the medium of the hormones 2,4-D, but not by IAA or NAA. This is in accordance with the data relative to the resistance of the roots to the auxinic substances (Fig. 3).

The rotation of the seedlings on the two-axes clinostat (the random positioning machine), however, was ineffective as concerns the data relative to the PCR expression, but, on the contrary, the \textit{GUS} gene expression clearly indicated activation by both gravistimulation and clinorotation. This could be explained by admitting that the general expression of the gene is not quantitatively changed by clinorotation or unilateral gravistimulation, whereas the localization of the mRNA is.

In addition, the GUS experiments showed that the gene is well expressed in the mature tissue of the seedlings in both the shoots and the roots, but not significantly in the elongation zone and meristems. The gene is therefore
probably not involved directly in the control of auxin redistribution at the apical meristems, but indirectly in gravitropism and the chiral aspect of circumnutation as shown by the reduction of the slanting (Fig. 2).

As usual, auxin appears also to be involved in circumnutation, but there is no indication of whether the cyclic variations of growth, which produce the helical elongation, could be ascribed to a form of redistribution of the hormone, nor is it known, as seems likely, if other factors are involved, and if active auxin transport is necessary. The mutant appears to be disturbed not in the circumnutation itself, but in the chiral aspect of it (slanting), as well as in gravitropism. This result leads one to think that the two processes could have a common basis, as Darwin (1880) thought. This possibility, in line with the hypothesis of involvement of gravitropism in circumnutation (Johnsson, 1997), has recently been strongly supported by some authors (Kitazawa et al., 2005; Kiss, 2006) who studied circumnutation and gravitropism in morning glory shoots. They suggested that it is the sensing of gravity that is necessary for both processes, because in a mutant not circumnuting and not responding to gravitropism in the shoot, the endodermis layer, considered the site of detection of gravity, is absent.

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