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RESEARCH PAPER

Root hydrotropism and thigmotropism in *Arabidopsis thaliana* are differentially controlled by redox status

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ABSTRACT

Factors that affect the direction of root growth in response to environmental signals influence crop productivity. We analyzed the root tropic responses of thioredoxin (*trxs)*, thigmotropic (*wav2-1*), and hydrotropic (*ahr1* and *nhr1) Arabidopsis thaliana* mutants treated with low concentrations of paraquat (PQ), which induces mild oxidative stress, and established a new method for evaluating root waviness (root bending effort, RBE). This method estimates root bending by measuring and summing local curvature over the whole length of the root, regardless of the asymmetry of the wavy pattern under thigmostimulation. In roots of the *wav2-1* mutant, but not in those of the *trxs* and *ahr1* mutants, RBE was significantly inhibited under mild oxidative stress. Thigmotropic stimulation of *wav2-1* mutant roots, with or without PQ treatment, showed high levels of reactive oxygen species fluorescence, in contrast to roots of the *ahr1* mutant. Furthermore, PQ inhibited root growth in all genotypes tested, except in the *wav2-1* mutant. In a hydrotropism assay of the *trxs* and *wav2-1* mutants, root growth behavior was similar to the wild type with and without PQ, while the root growth of *ahr1* and *nhr1* mutants was diminished with PQ. These results indicate that hydrotropic and thigmotropic mutants respond differently to exogenous PQ, depending on the tropic stimulus perceived. Therefore, the mechanisms underlying hydrotropism and thigmotropism may differ.

Abbreviations: PQ, paraquat; RBE, root bending effort; ROS, reactive oxygen species

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Introduction

Post-embryonic plant development is largely regulated by differential growth movements, or tropisms, which alter the direction and extent of growth in response to environmental cues such as gravity, light, water gradient, obstacles, pathogens, or temperature. The combined effects of stress-specific phenotypes and stimulus-induced morphogenesis define the shape of an adult plant. Changes in root architecture control productivity in crops such as *Oryza sativa* (rice)[1] *Zea mays L.* (maize),[2] and *Phaseolus vulgaris* (bean)[3]; thus, studies of root tropic responses are of great interest.

In *Arabidopsis thaliana*, roots integrate different stimuli such as the gravity vector, moisture gradients, and touch to modulate their growth, avoid drought and obstacles, and fix the plant in the soil.[4] Three major forces can redirect root growth: the gravity vector, source of nutrients such as N and P, and a water source. Terrestrial plants direct their root systems downwards into the soil, mainly in response to the gravity vector; however, as the roots search for water and nutrients, they frequently change direction to avoid obstacles (thigmotropism).

Root hydrotropism is a mechanism found in many species to avoid water stress. To date, four hydrotropic response mutants have been isolated in Arabidopsis: *nhr1* (*no hydrotropic response 1*),[5] *ahr1* (*altered hydrotropic response* 1),[6] *miz1* (*mizu-kussei 1*), and *miz2*.[7] Only two loci (*MIZ1* and *MIZ2*) affecting root hydrotropism have been identified. Genetic redundancy underlying this tropism may have concealed other loci.

Okada and Shimura (1990) reported a gravity-induced touch response in seedlings grown on hard agar in a tilted plate, which resulted in 'wavy' roots.[8] They isolated a set of mutants with abnormal wavy growth, and found that one of these, *wav6*, was allelic to *pin2*.[9] PIN2 is an important regulator of gravitropism as it regulates the redistribution of auxin from the stele to the elongation zone of roots.[13]

In a study of hydrotropic responses using two *wav* mutants (*wav2-1* and *wav3-1*), increased sensitivity to the moisture gradient was observed after hydrostimulation,[10] indicating that common auxin signaling mechanisms might account for the perception and signaling of these two tropisms. Cloning the *WAV2* gene revealed that it encodes a protein belonging to the *BUD EMERGENCE 46* family. This has an N-terminus transmembrane domain and an α/β-hydrolase domain at the C terminus, which regulates stimulus-induced root bending through inhibition of root tip rotation; however, its molecular function remains elusive.[11]

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Root responses to gravity are well studied in Arabidopsis.[12] Amyloplasts are considered key in gravity sensing. Gravitropic responses are mainly regulated by auxin gradients, cytokinin, reactive oxygen species (ROS), pH, and Ca^{2+} for the transmission of the signal(s), which results in differential growth responses.[13-16] There is some evidence to suggest that the mechanism governing root gravitropism is different from that governing hydrotropism; although crosstalk between these two responses has made the elucidation of root tropic responses much more complex than expected.[17,18] Nevertheless, when the root perceives a water source or an obstacle, curvature is induced in the root elongation zone.

ROS are also important regulators of differential growth elongation during the development of gravitropic and hydrotropic responses.[19,20] However, we are just beginning to understand the functions of ROS during this process. Thus, their participation in tropic responses constitutes an open area for research. Superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are generally considered to be metabolic byproducts that participate in the gravitropic response of maize roots.[21] However, after 1 h of gravistimulation, the intracellular ROS concentration increased threefold in the primary root tip, indicating that it functions as a second messenger (and/or modulator) in this tropic response.[21]

The enzymatic pathway for ROS generation probably contributes most of the ROS required for signaling mechanisms. Indeed, in root apical meristems, where cells rapidly proliferate, superoxide anions are found in a greatly enriched zone. The ratio of $O_2^{\cdot-}$ to H_2O_2 determines where cells in the root tip transition from the proliferation to elongation zone, where differentiation occurs.[22] Therefore, ROS are important plant growth regulators, and as such, are comparable to a plant hormone.[22,23]

Accumulation of glutathione and thioredoxins (Trx) in the meristem region is also involved in root development,[24] supporting genetic evidence that links thioredoxins, glutathione (GSH), and auxin to the control of shoot and root development.[21] Plant Trxs are present in the main cell compartments, including the cytosol and plastids. Trxs act in an antioxidant network, supplying the reducing power necessary for detoxifying lipid hydroperoxides, repairing proteins, regulating enzyme activity, directly detoxifying active ROS, and modulating the redox status of components involved in pathways linked to oxidative stress and the control of gene expression.[25]

The formation of carbonyl groups on protein amino acid residues as a result of free radical-initiated reactions is also well documented.[26] Oxidized proteins are partially denatured, and some hydrophobic regions are exposed to the action of chaperones and the ubiquitin/proteasome system (UPS).[26,27] Several reports implicate the proteasome in the degradation of oxidized proteins.[29-31] Increased levels of ubiquitin (Ub) conjugates can be detected before changes in other classic oxidative stress markers, such as glutathione disulfide/glutathione and NAD(P)/NAD(P)H ratios, and protein carbonyl content. Thus, the accumulation of Ub conjugates is a sensitive indicator of cellular oxidative stress.[32]

Root waviness is caused by periodic rotation of the root tip.[8] Two methods have been described for estimating root waviness in Arabidopsis. In the first one, the mean wavelength of a root wave and the wave tangent angles are estimated to infer root waviness.[11,33] In the second, root waviness is inferred by spectral analysis.[34] The first method is best for analyzing roots that have a largely symmetric and periodic wave pattern, while the second is more suitable for randomly wavy roots, and for brief and interrupted time series.

We established a new method to evaluate the root bending effort (RBE) of Arabidopsis seedlings with different root wave intensities. Assuming that root waviness is not symmetrical or sinusoidal, we developed a simple method to obtain an index that describes the effort a root makes for bending under thigmotropic stimulation. Thus, RBE estimates the global waves of a root by measuring and summing its local curvature over the whole length of the root, providing a clear measurement of the thigmotropic phenotype.

The involvement of ROS in thigmotropism has not yet been described; therefore, we compared the effect of ROS produced by paraquat (PQ) on root growth, as well as the effect of PQ on hydrotropic and thigmotropic stimulation in different mutant genotypes of Arabidopsis (i.e., *nhr1, ahr1, wav2-1*, and several *trx* mutants). We also analyzed the levels of endogenous Ub conjugates in hydrostimulated roots in response to mild oxidative stress.

Since changes in redox state affect the growth and elongation of plant organs, we hypothesized that, in the presence of PQ, some *trx* mutants might show altered hydrotropic root curvature and altered waviness compared to hydrotropic and wavy mutants. We found that the mechanisms underlying hydrotropism and thigmotropism differ.

Results

Root growth of Trx and hydrotropic response mutants in the presence of PQ

Root growth is a dynamic process that is modulated by several signaling pathways, including Ca^{2+} signaling; auxin polar transport; and H^+-ATPase activity. Since ROS have emerged as important signaling molecules in roots,[39] we applied low concentrations of PQ, a widely used herbicide that induces oxidative stress,[36] to Arabidopsis roots; this did not completely block growth of wild-type roots, but increased intracellular ROS levels.

We hypothesized that plants deficient in redox signaling would show altered patterns in tropic responses such as root hydrotropism and thigmotropism. To test this hypothesis, we compared the root growth of *trx* mutants grown on normal medium versus a medium containing PQ. Seeds were germinated on normal medium containing 0.03 μM PQ and root growth was evaluated after 7 d. Under the PQ treatment, seedling root growth was diminished compared to those grown on normal medium without PQ. In the *nhr1, ahr1*, and *trx h9, trx h1, trx h3, trx h4, trx o*, and *trx m3* mutants, and in the Col-0 and L*er* ecotypes, average root growth was reduced by approximately 50% (Fig. 1). However, root growth of the thigmotropic mutant *wav2-1* was inhibited by only 20–30% compared to its respective wild type, suggesting that in this thigmotropic mutant root growth might be negatively influenced by the presence of ROS (Fig. 1).

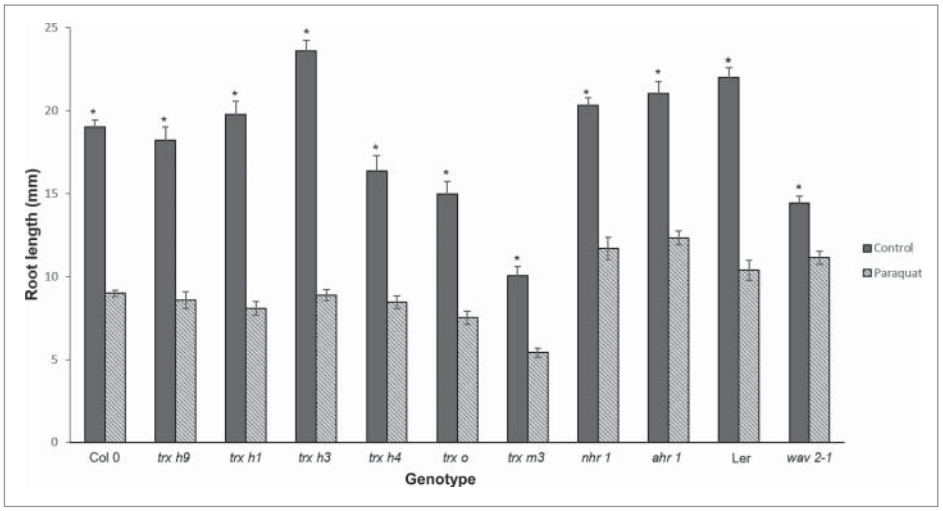

Figure 1. Effect of paraquat on the root growth of *trx mutants (trx h9, trx h1, trx h3, trx h4, trx o, trx m3*), root hydrotropic response (*ahr1* and *nhr1*), and root waviness (*wav2-1*) mutants. Seven-day-old seedlings were grown in normal MS medium (without paraquat [PQ], solid bars) or in the presence of 0.03 μM PQ (shaded bars). Root length of PQ-treated roots was compared with controls. PQ reduced root growth by 50% in all genotypes tested, except *wav2-1*. Vertical bars show mean $\pm$ standard error (SE). *Indicates significant differences as determined by two-way ANOVA and Tukey's *post-hoc* ($P < 0.05$), $n = 50$. Data represent one of three biological replicates.

ROS affect root thigmotropism

We then calculated the root bending effort (RBE) for each root before and after applying a thigmotropic stimulus to the roots of PQ-treated and control (without PQ) seedlings. The mean RBE of PQ-treated roots was subtracted from the mean RBE of control roots. After applying the thigmotropic stimulus, PQ caused an increase in waviness in the root patterns of *ahr1* and *trx o, trx h1, trx h9, trx m3*, and *trx h3* mutants compared to the wild type, *nhr1*, and *trx h4* (Fig. 2C). By contrast, the waviness of *wav2-1* roots was significantly decreased under the same conditions (Fig. 2). These observations indicate that thigmotropic behavior requires an appropriate redox state in which thioredoxins participate in root elongation or in differential growth responses.

In this study, in the presence of PQ, the roots of all *trx* mutants (independently of its localization: cytosolic or plastidic) had more waves. This effect was specific to thigmotropism because it was only detected when the root encounters an obstacle (in this case, an impenetrable, inclined barrier of hard

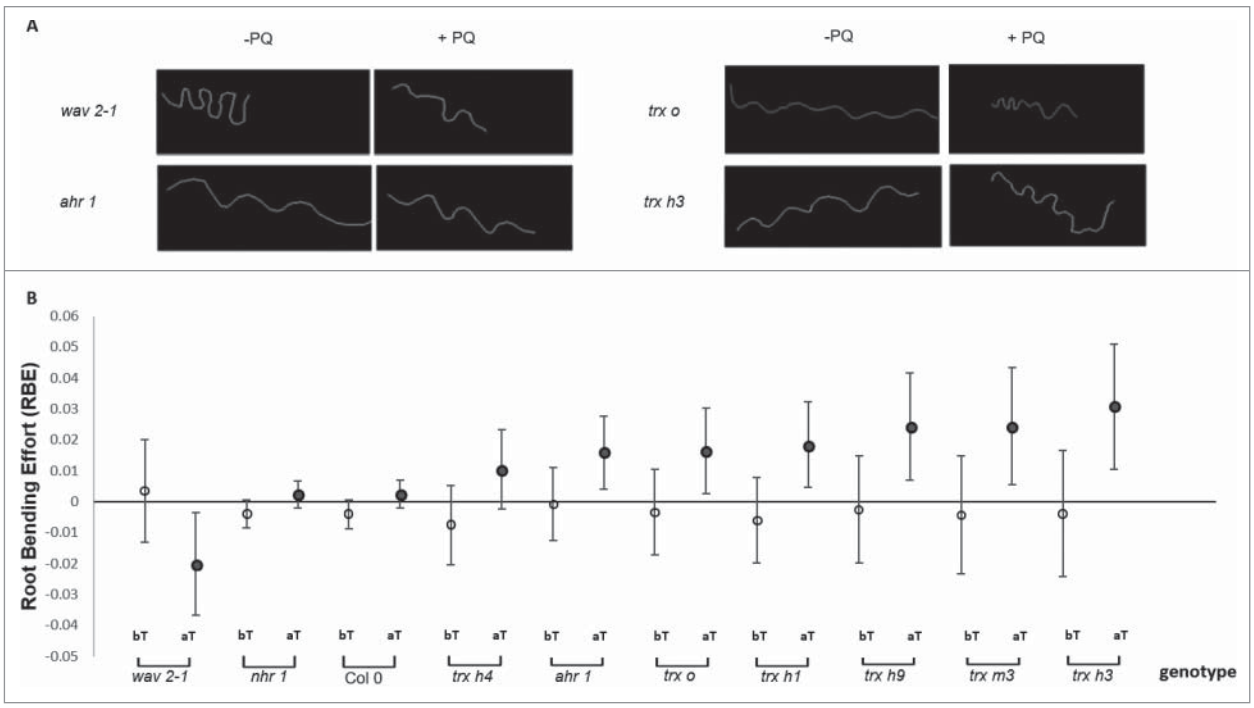

Figure 2. Root waviness of different genotypes under oxidative stress. (A) Root images showed the wavy pattern of *wav2-1, ahr1, trx o*, and *trx h3* mutant roots after thigmotropic stimulation in the presence and absence of PQ. *wav2-1* roots were less wavy than the other genotypes tested. (B) The mean root bending effort (RBE) of PQ-treated roots was subtracted from the mean RBE of roots not treated with PQ before (bT) and after (aT) thigmotropic stimulation. The RBE values of *ahr1, trx o, trx h1, trx h9, trx m3* and *trx h3* were higher after exposure to thigmotropic stimuli compared with *wav2-1*. The RBE of the Columbia-0 (Col-0) genotype was designated as 0 and was calculated as described in Materials and Methods.

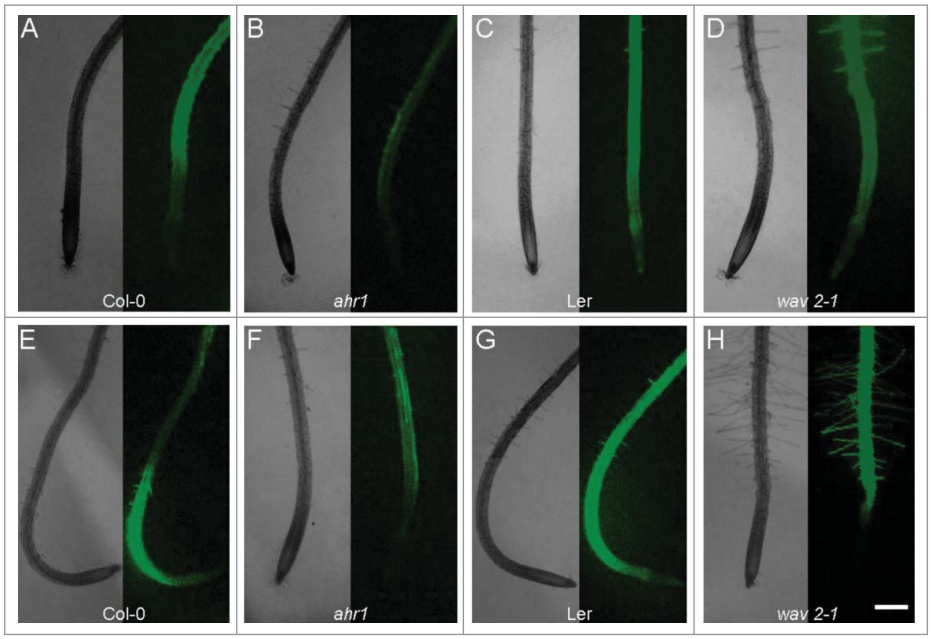

Figure 3. The differential distribution of reactive oxygen species in *ahr1* and *wav2-1* roots under thigmotropic stimulus. Using a hydrogen peroxide-sensitive fluorescent probe (CM-H2DCFDA), reactive oxygen species (ROS) were localized in roots of *ahr1* and *wav2-1* mutants and their respective wild-type (Col-0) seedlings in a vertical position (A–D) and under thigmotropic stimulation (E–H). Each panel shows an optical bright field image (left) and a fluorescent image (right). Roots of *ahr1* mutant seedlings showed reduced fluorescence in the vertical position compared to Col-0 (A–B). Under thigmotropic stimulus, Col-0 seedling roots (E) showed higher fluorescence in the elongation zone and root tip, while *ahr1* roots were less curved and showed less fluorescence in the elongation zone and the root tip. Roots of the *wav2-1* mutant and its respective wild type (L*er*) had elevated fluorescence in both the vertical position and under thigmotropic stimulus, indicating that the ROS levels in these genotypes are higher (C–D, G–H) compared to Col-0 and the *ahr1* mutant (A–B, E–F). Scale bar = 200 μm.

agar in angled plates (Fig. 2B–C). Since PQ inhibited root growth in wild-type plants and all *trx* mutants (Fig. 1), differential growth responses of the root are most likely affected by ROS-regulated cell cycle progression.[30] Thus, thigmotropic curvature might depend not only on meristem activity and the cell proliferation rate of each genotype, but also on the differential speed of growth elongation in the external or internal curvature of the wave.

Thigmotropism and PQ treatment induce a differential distribution of ROS in the roots

To determine the role of ROS during the thigmotropic response and correlate this with RBE, we compared the intracellular ROS levels in the first root turn or wave of the root tip in roots grown vertically, and in thigmotropic assays of the hydrotropic mutant *ahr1* and the wavy mutant *wav2-1*. The latter two mutants were chosen because they display opposite RBE behaviors (Fig. 2).

Vertically positioned *ahr1* roots had very low ROS levels in the elongation zone; however, wild-type roots (Col 0 and L*er*) clearly displayed higher ROS levels in this zone (Fig. 3A, C). Wild-type roots in the inclined position had the highest ROS levels in the first root wave (at the root tip) (Fig. 3E, G). This is a noteworthy observation because root tips grown in the vertical position have no physical obstacle, whereas they do (hard agar) in the inclined position, and might require higher ROS levels to develop a root wave.

When thigmotropically stimulated, the roots of *ahr1* seedlings exhibited very low levels of ROS in the elongation zone compared to Col-0 (Fig. 3E–F). In the vertical position, *wav2-1* roots and their respective wild type (L*er*) had higher fluorescence in the elongation zone. Under thigmotropic stimulus, L*er* had higher levels of fluorescence in the whole root, while *wav2-1* lacked fluorescence at the root tip, indicating that in these genotypes, ROS were also differentially localized (Fig. 3C–D, G–H) compared to Col-0 and *ahr1* (Fig. 3A–B, E–F).

We also tested the effect of PQ on the distribution of ROS fluorescence in *ahr1* and *wav2-1* root seedlings grown in the vertical position compared to their respective wild types (Col-0 and L*er*). Col-0 and *ahr1* roots both displayed considerably higher fluorescence compared to *wav2-1* and L*er* (Fig. 4). In addition, all genotypes tested showed significantly decreased fluorescence when under the thigmotropic stimulus in the presence of PQ (Table S1).

Trx mutants displayed hydrotropic responses in the roots

Using square Petri dishes containing normal growth medium in the upper part and medium with a low water potential (the hydrotropic assay medium) in the lower part, 5 different *trx* mutants (*trx* h9, *trx* h1, *trx* h3, *trx* h4, *trx* o and *trx* m3) were grown on the hydrotropic part of the medium. This was designed to isolate the *nhr1* mutant.[37] Seedlings were classified as having no hydrotropic response when its root grew beyond the border between the two media when the Petri dish was placed vertically on edge.

The 9 *trx* mutants showed a similar root hydrotropic response to the wild type (data not shown); *trx h9* and *trx m3* were included to comparing their hydrotropic responses with the no-hydrotropism (*ahr1* and *nhr1*) and wavy (*wav2-1*) mutants (Fig. 5A, C). Only the roots of *nhr1* and *ahr1* grew

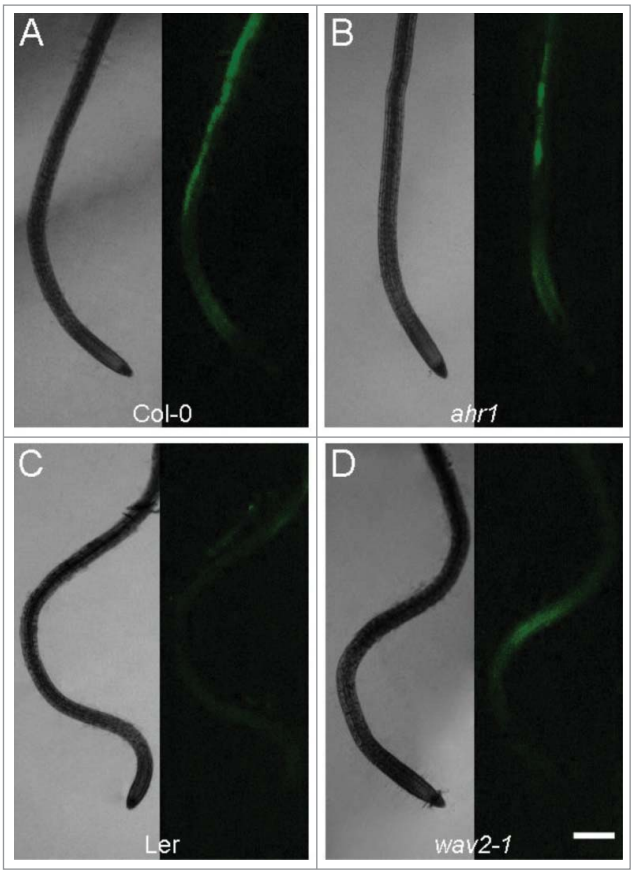

Figure 4. Paraquat treatment changes the distribution of reactive oxygen species in *ahr1* and *wav2-1* mutants. Root seedlings of Col-0 (A), *ahr1* (B), L*er* (C), and *wav2-1* (D) were grown in the vertical position in the presence of paraquat (PQ). Each panel shows root tips in optical bright field (left) and fluorescent images (right). Roots of the *ahr1* mutant and Col-0 had considerably higher fluorescence than the *wav2-1* mutant and L*er* (A-B). These genotypes (L*er* and *wav2-1*) had a significantly reduced fluorescence signals under thigmotropic stimulus when in the presence of PQ (C-D) (see data in Supplemental Table 1). Scale bar = 200 μm.

into the hydrotropic assay medium, indicating a lack of hydrotropic response (Fig. 5A, B).

The effect of adding PQ to each medium in the hydrotropic assay was also tested. Roots of *nhr1* and *ahr1* elongated and crossed beyond the border between the two media in the presence of the oxidant, whereas roots of other genotypes did not. However, PQ inhibited *nhr1* root elongation to a lesser extent (27%) than *ahr1* (46%) (Fig. 5A). As expected, wild-type roots did not grow in this assay; neither did the *trx* mutants *trx h9* or *trx m3* (Fig. 5). The other *trx* mutants responded in a similar way to *trx h9* and *trx m3* (data not shown). Nonetheless, compared to *nhr1, ahr1* roots were the most sensitive to PQ-induced oxidation; this might suggest that in these mutants, ROS are handled in different pathways compared to the wild type (Fig. 5A–B). Roots of the *wav2-1* mutant did not grow much beyond the border of the two media in the hydrotropic assay medium (Fig. 5A–B).

The gravitropic responses of hydrotropic mutants were unaffected by oxidative stress

ROS positively regulate the gravitropic response of maize roots.[21] We examined the root curvature of mutant roots after reorienting them at 90° for 24 h. Under control conditions (without PQ), most *trx* mutants produced root angles similar to those of the wild type. PQ did not affect the gravitropic curvature of *trx h9, trx h3, trx h4, trx o*, or *trx m3* roots (Fig. 6), suggesting that, in the absence of Trx products, ROS may not be involved in the gravitropic response.

PQ treatment reduced the angle of root curvature of wild-type, *txr h1, nhr1*, and *ahr1* roots (Fig. 6). This is different to observations made in maize roots (*Zea mays* L. cv. Golden Cross Bantam),[21] indicating that PQ-generated ROS have a different effect on the root gravitropic response of these Arabidopsis mutants, and that in Arabidopsis, gravitropism is promoted by ROS (most probably from the dissociation of H_2O_2).

Lateral root formation in the Trx, wavy, and hydrotropic mutants in the presence of PQ

The branching process required for roots to acquire water and nutrients during root development is sensitive to stress conditions, and is also regulated by the circadian clock and auxin signaling.[38] In our hydrotropic assay medium, PQ treatment reduced lateral root development by ~80% in the wild type, *trx m3* and *trx h9* mutants, and *ahr1*. In *nhr1*, lateral root emergence was repressed by only 53% (Fig. 7). While PQ inhibited the development of lateral roots in almost all seedlings tested, roots of the wild type, *trx h9, trx m3*, and *ahr1* were more sensitive than those of *nhr1*, indicating a slight resistance of *nhr1* to PQ treatment and a lesser susceptibility to ROS in terms of lateral root emergence.

Surprisingly, *wav2-1* was unaffected by PQ-induced oxidative stress, and in fact produced 21% more lateral roots in the hydrotropic assay medium than the wild type (Fig. 7). This suggests that, in *wav2-1*, root branching may have a different mechanism to overcome PQ-induced oxidative stress. The mechanism by which ROS affects lateral root emergence has not been elucidated, although there is evidence to suggest that redox signaling regulates lateral root formation.[39]

The emergence of lateral roots in both *nhr1* and *wav2-1* was less sensitive to PQ in the low water potential conditions of the hydrotropism assay than the wild-type, indicating that these mutants might possess a common mechanism to cope with water deficit and elevated ROS.

Protein analysis: Protein carbonylation, protein ubiquitination, and 20S analysis

In plant cells, ROS are both emergency signals and cytotoxic molecules. Terrestrial plants have evolved scavenging mechanisms to use these signals to acclimatize to stress conditions. In response to cellular stress, such as mild oxidative stress, levels of endogenous Ub conjugates are increased, which can be detected before changes in other 'classic' oxidative stress markers, such as glutathione disulfide/glutathione and NAD(P)/NAD(P)H ratios, and protein carbonyl content. Accumulation of Ub conjugates is therefore one of the most sensitive indicators of cellular oxidative stress.[32]

The formation of carbonyl groups on protein amino acid residues as a result of free radical-initiated reactions is another good marker of redox imbalance.[26,40] The relationship between

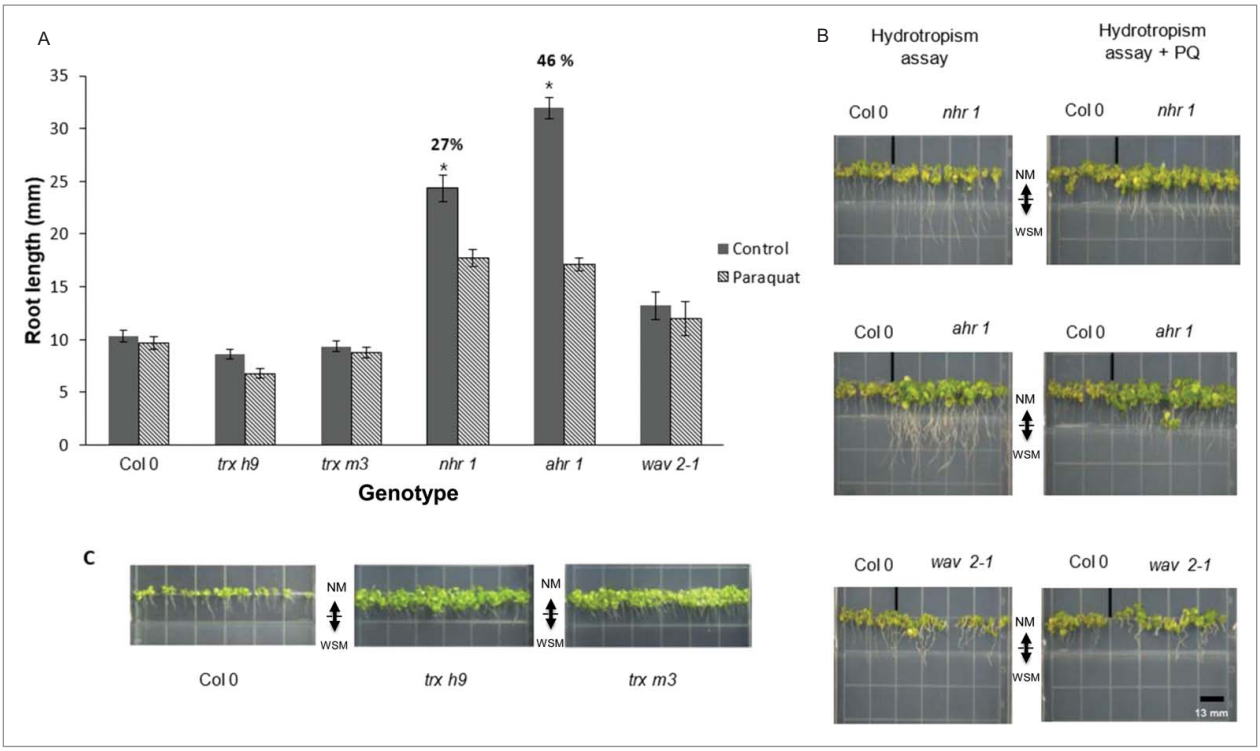

Figure 5. Roots of *trxs* mutants display a hydrotropic response in the assay medium with or without paraquat. (A–B) Seedlings were grown on hydrotropism assay medium for 11 d, and root length was compared in plates treated (shaded bars) or untreated (solid bars) with paraquat (PQ). Roots of *nhr1* and *ahr1* mutants showed similar hydrotropic responses in the assay medium with and without PQ (roots crossed the border between the two media); however, growth of *nhr1* roots was less inhibited in the presence of PQ. (C) Representative images of wild-type Col-0, *trx h9* and *trx m3* seedlings are shown in the hydrotropic assay medium only in the presence of PQ. Indicates significant differences compared with its own genotype when treated with PQ (as determined by two-way ANOVA and Tukey's *post hoc*, $P < 0.05$, from three biological replicates), $n = 50$. NM, normal medium; WSM, water stress medium. Arrowhead depicts the border between the NM and WSM. Scale bar = 13 mm.

oxidatively damaged proteins and their degradation via the UPS has been extensively studied. Proteins are partially denatured by oxidation (i.e., carbonylation), exposing some hydrophobic regions to the action of chaperones and the UPS.[29-31]

To explore the role of oxidative stress on root hydrotropism, we determined the contents of protein carbonyls, Ub conjugates, and proteasomes in a set of tropic mutants (*ahr1, nhr1, wav2-1*) grown under four conditions: control medium, control medium with PQ, hydrotropism assay medium, and hydrotropism assay medium with PQ (Fig. 8). All data were standardized to wild-type growth under control conditions (wild type = 1). We determined the reacting carbonyl groups of protein carbonylation with 2,4-dinitro phenylhydrazine (DNPH) that formed protein-bound 2,4-dinitrophenyl hydrazones.

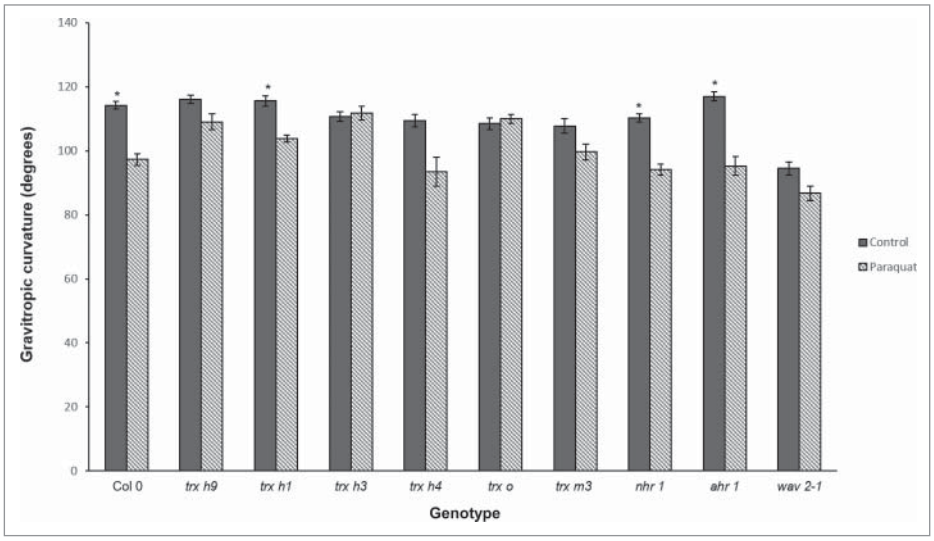

Figure 6. Root gravitropic responses of different genotypes under oxidative stress. Four-day-old seedlings were reoriented by 90° and the root curvature (angle) was measured after 24 h. Gravitropic curvature was diminished by paraquat (PQ) treatment in all genotypes except for *trx h3, trx o* and *wav2-1*, in which the angle of curvature was similar in seedlings treated and untreated with PQ. The *trx h1*, wild type (Col-0), *nhr1* and *ahr1* genotypes showed significant differences in their gravitropic curvatures when treated with PQ (as determined by two-way ANOVA and Tukey's *post-hoc*, $P < 0.05$, from three biological replicates). $n = 30$

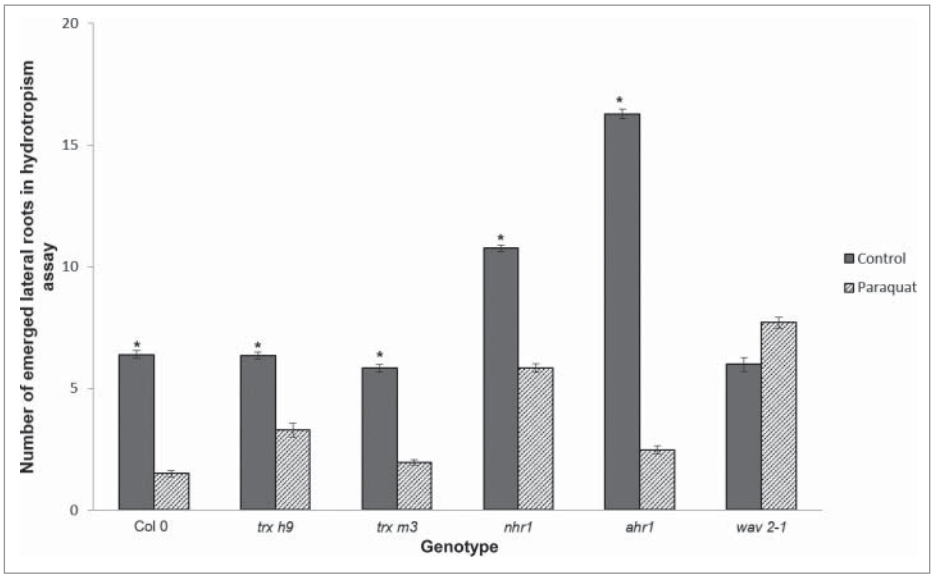

Figure 7. Oxidative stress promotes lateral root emergence in the *wav2-1* mutant. Lateral roots of 11-day-old seedlings grown in hydrotropism assay medium were counted. Paraquat (PQ)-inhibited lateral root emergence was observed in all mutants tested, except the *wav2-1* mutant. Bars indicate means ± standard error (SE) from three biological replicates. *Indicates significant differences compared to controls and PQ-treated medium using Dunn's multiple comparison test, Kruskal-Wallis test, and Mann-Whitney U test, from three biological replicates ($P < 0.05$). $n = 30$.

Hydrazones were quantified with an anti-hydrazone antibody. Under control conditions, the proportion of oxidized proteins in the three mutants was greater than 1 (approximately 1.2–1.8), and only the wild type had more oxidized proteins in the presence of PQ (1.46) (Fig. 8B).

In *wav2-1* mutants grown in the hydrotropic assay medium, the carbonyl group content was nearly 50% lower than that of the wild type; similar results were observed in this assay medium in the presence of PQ (Fig. 8C–D). Roots of the *ahr1* mutant showed a comparable reduction in carbonyl groups, but only when PQ was present in the hydrotropic assay medium (Fig. 8D). Other genotypes tested showed no changes in carbonyl group content in the hydrotropism assay medium, in both the presence and absence of PQ (Fig. 8C–D). These observations indicate that the change in the level of oxidized proteins might be related to the hydrotropic or thigmotropic phenotypes of these mutants.

Several studies indicate the influence of oxidative stress on the capacity of the UPS to degrade proteins.[41] An increase in substrate availability can increase intracellular protein degradation in response to oxidative stress. The activities of the E1 and E2 components of the Ub conjugation system increased in response to mild oxidative stress.[42] The different sensitivities of the Ub conjugation machinery and the proteasome to mild oxidative stress may account for the accumulation of Ub conjugates; such accumulation may be a useful indicator of cellular oxidative stress. By contrast, inactivation of the Ub-conjugating enzymes may reduce Ub conjugate levels in cells under severe oxidative stress. In control medium, the basal level of ubiquitination in *ahr1* was similar to that of Col-0, while *nhr1* and *wav2-1* had high levels of Ub conjugates (8- and 7-fold, respectively) (Fig. 8A). In the presence of PQ, this response was reduced in *nhr1* and *wav2*-1 by threefold and twofold, respectively, while in Col-0 and *ahr1* the quantity of Ub proteins doubled (Fig. 8B). In the hydrotropism assay medium, the roots of Col-0, *nhr1*, and *wav2-1* seedlings exhibited fewer Ub conjugate levels, though this decrease was fivefold more pronounced in *nhr1* and *wav2-1* (Fig. 8C), while protein ubiquitination was fivefold greater in *ahr1* roots, and was slightly lower in the presence of PQ (Fig. 8C–D). This indicates a clear difference in redox regulation in *ahr1, nhr1*, and *wav2-1*.

Available data indicate that the proteasome is important in selectively degrading oxidized proteins, in either a Ub-dependent or Ub-independent manner. The exposure of hydrophobic patches may be a molecular basis for the recognition of oxidized proteins by chaperones, or by the proteasome itself.[43,44] Proteasome activity increased upon adaptation to mild oxidative stress. Mild to moderate oxidative stress increases the susceptibility of proteins to degradation and enhances proteolytic capacity, therefore promoting intracellular protein degradation. By contrast, extensive but non-lethal oxidative stress impairs the proteolytic system function, reducing intracellular protein degradation and inducing intracellular accumulation and aggregation of damaged proteins. The proteasome is a target of oxidative stress, and is more susceptible to oxidative inactivation than ubiquitination enzymes.[45,46] In many cell types, when UPS function was impaired or inhibited, redox sensitive transcription factors enhanced the expression of proteasome subunits and increased proteasome activity.[47-49]

In this study, we used slot blot hybridization to compare the proteasome of hydrotropic mutants and *wav2-1* by tracing the presence of total 20S core particles. All root seedlings grown in the control medium had similar quantities of 20S; however, the addition of PQ caused a slight increase in *ahr1* and *nhr1* roots (Fig. 8A, B). Roots of *nhr1* and *wav2-1* showed a sevenfold higher level of Ub conjugates under control conditions (Fig. 8A), indicating that in the presence of PQ, they degrade more proteins than both Col-0 and *ahr1* (Fig. 8B). In the hydrotropism assay medium, 20S levels of wild-type and *ahr1* roots doubled, while *nhr1* and *wav2-1* roots exhibited similar levels to those observed in the control medium. Total 20S was unaltered in all genotypes, despite the presence of PQ in the

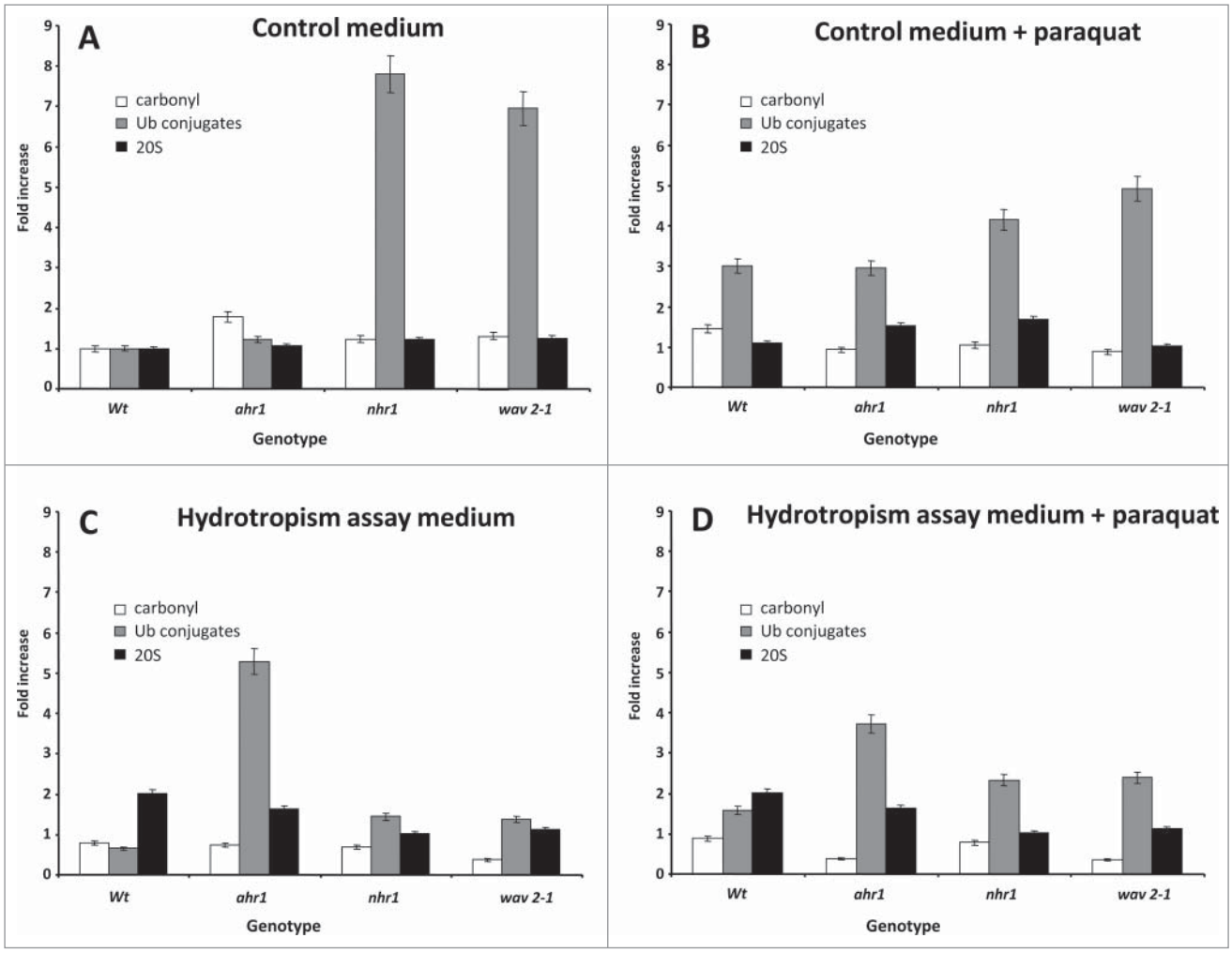

Figure 8. Carbonyl, ubiquitin conjugates and 20S content of total proteins extracted from Arabidopsis root seedlings. The roots of eleven-day-old seedlings grown in control medium without (A) and with paraquat (PQ; B), or in the hydrotropic assay medium without (C) or with PQ (D). Equal amounts of total protein (5 mg) were used to measure protein carbonyl, ubiquitin (Ub) conjugates and 20S proteasome content by slot blot, as described in Materials and Methods. Ub conjugate content was the most responsive and sensitive parameter (A–D). Root seedlings of the *nhr1* and *wav2-1* mutants had higher Ub conjugate content under control conditions (A), while in the presence of paraquat (PQ) or in the hydroptropism assay medium, the same mutants showed a clear decrements (B–C), indicative of more active Ub conjugate-degrading machinery. The *ahr1* mutant had the highest Ub conjugate content in the hydrotropism assay medium, whether with or without PQ. Carbonyl and 20S content showed minor but significant variations. Values are expressed as fold increases, where the wild type (Wt) in control medium is considered as 1. Means and standard deviations (SD) were calculated from three biological replicates.

hydrotropism assay medium (Fig. 8D). In the hydrotropic assay medium, *ahr1* roots had higher levels of Ub conjugates, indicating its normal growth pattern in this medium. In the PQ-treated hydrotropic assay plates, *ahr1, nhr1*, and *wav2-1* roots had slightly higher Ub conjugate levels than Col-0. The four mutants tested showed no considerable change in protein carbonylation (Fig. 8D).

Discussion

There is increasing interest in the area of oxidative stress signaling in plants. A recent study reported that ROS are important in integrating stimulus responses, tuning root tropisms by promoting gravitropism and negatively regulating hydrotropism.[20] Here, we present evidence to suggest that ROS signaling is involved in the root thigmotropic and hydrotropic responses of different *trxs* mutants, and hydrotropism (*ahr1* and *nhr1*) and thigmotropism (*wav2-1*) mutants. In both the wild type and the tested mutants, root growth was diminished in the presence of mild oxidative stress caused by the presence of PQ, indicating that the tested mutants are PQ sensitive despite different percentages of observed growth inhibition. Interestingly, growth of *wav2-1* roots was less sensitive to the presence of ROS, suggesting that they are either resistant to the inhibitory effect of PQ, or they have accelerated ROS scavenging mechanisms to maintain growth under oxidative stress (Fig. 1).

We established a new method for evaluating the root bending effort (RBE) of Arabidopsis seedlings that, when grown on a hard agar surface, produce different root wave patterns. Using this method, we analyzed the root waviness of different Arabidopsis genotypes in the presence or absence of PQ. According to Okada and Shimura (1990),[8] waviness is quantified by measuring the tangent angle (the angle between a tangent to the direction of root growth and a hypothetical axis at each intersection between that axis and the root), the wavelength, and the root growth rate. Roots of *wav2-1* have larger wave tangent angles and shorter wavelengths than the roots of wild-type seedlings. However, their method does not take into account

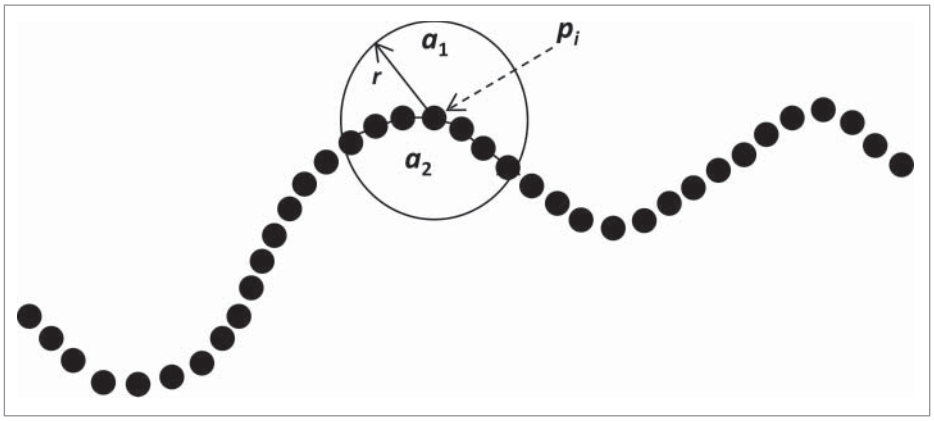

Figure 9. Root bending effort. For each point P_i along the root, mean root bending effort (RBE) is calculated by averaging the ratio of the surfaces a1/a2 are enclosed by the circular mask with radius r, centered sequentially over points p_i.

the fact that in some Arabidopsis genotypes exhibited root waves even in the absence of thigmostimulation (data not shown).

The RBE method is not restricted to sinusoidal or symmetrical waves, and considers the accumulated waves in a root, divided by total root length (see equations 1 and 2 in Materials and Methods). Using this method, the RBE of wild-type roots was considered as control value. The waviness of *wav2-1* roots was reduced in the presence of PQ (Fig. 2A, C), but was promoted in *ahr1* and the *trxs h4, trx o, trx h1, trx h9, trx m3 and trx h3*, but not *nhr1* (Fig. 2C). Roots of *nhr1* exhibited no differences in waviness after thigmostimulation in the presence of PQ. These results indicate that the sensitivity of *wav2-1* to mild oxidative stress is sufficient to decrease its wavy phenotype. In other words, regulation of the ROS detoxifying system might be different in root thigmotropism.

Using the $H_2O_2^-$sensitive fluorescent probe H2DCF-DA, we compared the spatial distribution of root ROS levels in seedlings grown in a vertical position or under thigmotropic stimuli in *ahr1, wav2-1* and their respective wild types. In seedlings grown vertically, the spatial distribution of ROS fluorescence was considerably diminished in *ahr1* compared to *wav2-1*, L*er* and Col-0 (Fig. 3A–D). This pattern was also observed in thigmostimulated roots (Fig. 3E–H). Elevated fluorescence was seen in the roots of *wav2-1* and L*er* grown in the vertical position, showing that the ROS levels in these genotypes are increased compared to Col-0 and *ahr1* (Fig. 3C–D). Roots of *ahr1* accumulated significantly less $O_2^{\bullet -}$ and proline,[50] thus it is not surprising that they also had reduced ROS fluorescence.

The addition of PQ to plates positioned vertically or under thigmotropic stimulation substantially decreased ROS fluorescence and distribution in Col-0, L*er* and *wav2-1*, but not *ahr1* roots (Supplemental Table 1), indicating that *ahr1* is mostly insensitive to ROS when submitted to thigmotropic stimuli but is sensitive to gravitropic stimuli (Figs. 2, 4, and 6). On the other hand, *wav2-1* roots were insensitive to PQ under thigmostimulation, hydrostimulation and gravistimulation (Figs 2, 5, and 6). This indicates that hydrotropic and thigmotropic mutants respond differently to PQ depending upon the tropic stimuli perceived. Endogenous ROS signals might regulate which tropic stimulus is more important to the root for maintaining its explorative function.

The phenotypes of *nhr1* and *ahr1* was affected by addition of PQ to the hydrotropic assay medium; nonetheless, there were marked differences in the inhibition of their respective root growth (27% and 46%, respectively) compared to the untreated assay medium (Fig. 5). The reduced sensitivity to mild oxidative stress of these hydrotropic response mutants might be related to their differential levels of Ub conjugates (Fig. 8C–D). In the hydrotropic assay medium, root growth of *wav2-1* was very similar in both untreated and PQ-treated plates, indicating that *wav2-1* is hydrotropic (Fig. 5). All *trx* mutants tested displayed a hydrotropic response, as did the wild type (data not shown), suggesting that the function of *TRX* genes is dispensable in the hydrotropic response.

In roots of genotypes tested after being reoriented at 90° for 24 h, gravitropic responses indicated that only *trx h3, trx o*, and *wav2-1* were insensitive to PQ, while wild-type, *trx* mutants (*trx h1, trx h9, trx h4* and *trx m3)*, and the hydrotropic mutants *ahr1* and *nhr1* were sensitive (Fig. 6). This variability might reflect differences in the normal redox status and redox resistance and/or sensitivity of these seedlings when gravitropically stimulated in the presence of PQ. Hence, it is difficult to generalize how mild oxidative stress regulates the root gravitropic response of different Arabidopsis genotypes. These results differed from those reported recently by Krieger et al. (2016),[20] since we applied mild oxidative stress to seedling roots using PQ, while they utilized ROS scavengers such as ascorbate and diphenyliodonium.

Most studies exploring the regulation of lateral root emergence have focused on the role of auxin synthesis or signaling processes. Recent reports shed light on circadian clock-related gene regulation in the formation of lateral roots.[38] However, an earlier report on lateral root formation supported the idea that manipulating auxin levels or signaling was insufficient to induce ectopic root branching, and that periodic branching and bending of primary roots required an endogenous mechanism.[51] In our study, the number of emerged lateral roots in PQ-treated seedlings was compared with those grown under normal conditions. We found that *wav2-1* had more emerged lateral roots in the presence of PQ (Fig. 7), indicating that is stimulated by ROS, and thus might be a good model for studying ROS-regulated lateral root formation.

To determine whether oxidative stress participates in the root hydrotropic response, we examined protein carbonylation, ubiquitin conjugate and proteasome content of two hydrotropic

mutants (*ahr1* and *nhr1)* and a wavy mutant (*wav2-1*) grown under four different conditions (Fig. 8). Under control conditions, the oxidized protein content of the three mutants considerably increased; however, in the presence of PQ only the wild type had increased levels of these proteins (Fig. 8A, B). This suggests that the three mutants are insensitive to PQ.

In the hydrotropic assay medium without PQ (Fig. 8C), the quantity of oxidized proteins slightly decreased in the wild type, and in *ahr1* and *nhr1*, while in the wavy mutant *wav2-1*, levels of these proteins were significantly diminished (Fig. 8C). In the hydrotropic assay medium with PQ, the levels of these proteins decreased substantially in *ahr1* roots only (Fig. 8D).

The hydrotropic mutants and *wav2-1* displayed different protein degradation patterns upon application of a hydrotropic stimulus, and also during mild oxidative stress, which is indicative of the complex interactions between root tropisms, ROS, and protein degradation. Future studies should consider these complex interactions in more detail to understand how roots respond to different simultaneous environmental cues.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana seeds of the wild-type ecotypes Columbia-0 (Col-0) and *Landsberg erecta* (L*er*), and *wav2-1* and *trx* mutants, were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA). Thioredoxin (*trx)* mutants were from the homozygous Salk collection: 006237C (*trx h1*), 018261C (*trx o*), z061968C (*trx m3*), 081049C (*trx h9*), 111160 (*trx h3*), and 151722 (*trx h4*). The hydrotropic mutants *nhr1* and *ahr1* were isolated in our lab from the Col-0 background.

Seeds were germinated on 'normal' medium (half-strength Murashige and Skoog [MS] medium with 0.5% [*w/v*] sucrose, at pH 5.7), or hydrotropic assay medium containing two media layers (upper: normal medium, lower: water stress medium containing half-strength MS, 0.5% [*w/v*] sucrose, 0.5% [*w/v*] alginic acid and 2.5% [*w/v*] glycerol, as described by Eapen et al., 2003).[37] To generate ROS, 0.03 μM paraquat (PQ) (Anaquat, Agricultura Nacional de Jalisco S.A de C.V.) was added to the media. Arabidopsis seedlings were grown for the lengths of time indicated under long day conditions (16 h light/8 h dark) cycle, 80 μM photons m^{-2} s^{-1} at 24°C.

Root thigmotropism assay and RBE method

Thigmotropism was evaluated as described by Okada and Shimura (1990),[8] with modifications. Three or 6 days (control or PQ-treated plates, respectively) after sowing the seeds on hard MS medium (0.5 x and 1.5% [*w/v*] agar), the position of the root tip was marked on the Petri dishes (using a marker pen). Then, for 3 or 6 d (control or PQ plates, respectively), Petri dishes were tilted to an angle of 60°. After 3 d in this position, plates were photographed with a Nikon D1 Digital Camera equipped with an AF Nikon 70–300 mm lens, at a fixed distance between the camera and the different Petri dishes. Four digital photographs (2238 × 1468 pixels; 5 × 5 cm; Tiff format) were taken of each Petri dish containing Arabidopsis seedling roots with a wavy pattern, each covering one quarter of the whole square Petri dish. Each image was cropped into a rectangular sub-image (with a variable size fitting the root, but with a fixed resolution of 833 pixels/inch) and saved as a separate file. Using ImageJ software (version 1.48; http://rsb.info.nih.gov/ij/), each sub-image was binary-converted by assigning a white color to the root over a black background. Thus we obtained a 1 pixel-width skeleton of the root. A text file was created containing the x and y coordinates of all points along each root. The RBE analysis was made with at least $n = 100$ seedling roots from each genotype.

In this study, it was assumed that not all root waves are symmetrical or sinusoidal (Fig. 2). For this reason, we determined a new and simple way to obtain an index demonstrating the effort a root makes to bend under the influence of a thigmotropic stimulus. We defined this as RBE, which measures and cumulates each root wave or bend, and divided this by the total length of the root (see equations 1 and 2). In this way, a completely straight root would have a total normalized RBE of 0, while values increasing from 0 to 1 are applied to larger waveforms.

To measure the local bending effort of each root, we used the procedure described by Bullard et al. (1995)[52] to compute mean curvature in two or three dimensions (2D or 3D). In our 2D images, for each point p_i along the root, local root curvatures were calculated by computing the portion of the surface enclosed by a small circular template centered on p_i lying on one side of the interface (see Fig. 9). The local bending effort for a group of k points (enclosed by the circular template) centered over a point p_i within the root was defined as:

$$\mathrm{RBE}_{\mathrm{local}}(p_i) = 1 - \frac{\min(a_1, a_2)}{\max(a_1, a_2)} \tag{1}$$

where: 'p_i' is a point belonging to the root, on which the circle template is centered; 'a_1' and 'a_2' are the areas between the segment of the arc template and the root boundary.

For a certain root 'm' formed by n points, *RBEtotal* is then defined as:

$$RBE_{total}(m) = 1 - \frac{\sum_{i=1}^{n} \mathrm{RBE}_{\mathrm{local}}(p_i)}{RootLength} \tag{2}$$

where: n is the number of points of a root 'm'; $RootLength = k\,n$ (k = calibration constant). *RBEtotal* ranges from 0 (for a straight line) to value approaching to 1 (for maximal root bending) and shows the total bending effort of the root during its growth.

Treatment of root seedlings with a ROS sensitive dye (CM-H2DCFDA)

The ROS-sensitive probe CM-H2DCFDA [5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester] (catalog no. C6827, Molecular Probes), was dissolved in DMSO (catalog no. D8779, Sigma), and centrifuged for 2 min at 7000 rpm to remove non-dissolved particles. The dye solution

was then diluted with water to a final concentration of 30–50 μM and added to plates containing Arabidopsis root seedlings. After 10 min, the medium was replaced with free dye medium to discard any unincorporated dye, and measurements were taken. This procedure was done carefully to avoid any mechanical stress to cells that might otherwise result in the production of ROS.

Acquisition and processing of fluorescent images

All images were acquired with a CCD camera (Sensys, Roper Scientific) attached to a Nikon TE300 inverted microscope with a 40X/1 N.A. water immersion objective lens, and operated with MetaMorph/MetaFluor software (Universal Imaging, Molecular Devices). Dye-treated roots were excited using a xenon illumination source (DG-4, Sutter Instruments) containing a 175-Watt ozone-free xenon lamp (330–700 nm) and a galvanometer-driven wavelength switcher. Seedlings containing the CM-H2DCFDA probe were excited at 484 nm, and emission was collected at 530 nm (20 nm band pass). All filters used were from Chroma Technology, and image acquisition and analysis were carried out using MetaMorph/MetaFluor software. Relative fluorescence intensity levels were calculated using the line scan option from Image J in the region of interest; data were exported to Microsoft Excel to prepare graphs and conduct standard deviation analysis.

Localization of ROS in root seedlings grown in the root thigmotropic assay

In the thigmotropic assay, the final curve or wave produced by the growing root (that closest to the growing tip, which experiences the intracellular ROS required for wave formation) was selected. To collect data indicating fluorescence intensity, several lines were drawn from the internal side of the curve to the external region. This approach was useful to compare the fluorescence intensity at both the internal and external sides of the curve or wave of a root tip.

Analysis of root growth in the hydrotropism assay

Seedlings were grown on hydrotropism assay medium for 11 d, after being photographed with a Nikon D7000 digital camera (Nikon Co.). Lateral roots emerging through the root epidermis were counted under a light microscope (Nikon Eclipse E600; Nikon Co.). Images were analyzed using Adobe Photoshop CS 8.0 (Adobe Systems Inc., San Jose, CA, USA). Root growth was measured using ImageJ.

Root gravitropism

Root gravitropic curvature was measured using 4-day-old seedlings grown vertically on mock or 0.03 μM PQ medium. Petri dishes were reoriented to 90° for 24 h, and images were taken before and after reorientation. Images were analyzed as described above, and root angle was measured using ImageJ.

Analysis of protein carbonylation, protein ubiquitination and 20S content

Total proteins were extracted by adding one volume of 2 × Laemmli sample buffer to liquid nitrogen-frozen plant material. Samples were immediately heated to 95°C for 5 min, and centrifuged at 14000 *g* for 10 min. Proteins from recovered supernatants were precipitated with methanol/chloroform to remove waxes and pigments. Resultant pellets were re-suspended in 1 × Laemmli buffer for immunoblot or slot blot analysis. Protein content was estimated by Bradford assay. Equal amounts of total protein (5 mg) were loaded into each well for immunoblot or slot blot analysis. Nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences) were blocked in 5% low-fat milk in Tris-buffered saline 0.1% Tween 20 (TBS-T) for 30 min at room temperature. Incubation with primary antibody was for 1 h at room temperature. After washing three times with TBS-T, membranes were incubated with secondary anti-rabbit-HRP antibody or anti-mouse-HRP (Santa Cruz Biotechnology) at a 1:5000 dilution for 1 h at room temperature. Finally, the membrane was washed three times with TBS-T, developed with ECL reagent (Amersham cat. RPN2109), and exposed to X-ray films (Kodak cat. 6040331).

The following primary antibodies were used: rabbit-anti-ubiquitin antibody (cat. # sc-9133 Santa Cruz Biotechnology, Inc.) at 1:1000 dilution, and mouse-anti-proteasome 20S alpha+beta (Abcam ab22673) at 1:5000 dilution.

Protein carbonyl content was estimated using a protein slot blot procedure, followed by an immunochemical protocol (OxyBlot Protein Oxidation detection kit, from Chemicon International) that detects 2,4-dinitrophenylhidrazone after reacting samples with 2,4-dinitrophenylhydrazine (DNPH). Quantification of carbonyl content, 20S proteasome content and total Ub conjugates was made by densitometry of the autoradiograms using NIH ImageJ 1.48 software. All data were standardized to wild-type growth under control conditions (wild type = 1).

Statistical analysis

Data were analyzed using JMP® software, version 11.0 for Windows 7 Home Premium x64 (SAS Institute Inc., 1989–2007), and analyzed with two-way ANOVA and Tukey's *post hoc*, unpaired Student *t*-tests, Dunn's multiple comparison test, Kruskal-Wallis test, and Mann-Whitney U test. Graphs were prepared using Microsoft Excel. In all cases the difference was considered significant when $P < 0.05$. Images were digitalized for publication using Adobe Photoshop CS 8.0 (Adobe Systems Inc.). All experiments were repeated at least thrice.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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