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RESEARCH PAPER

Humic substances induce lateral root formation and expression of the early auxin-responsive *IAA19* gene and DR5 synthetic element in *Arabidopsis*

S. Trevisan[1], D. Pizzeghello[1], B. Ruperti[2], O. Francioso[3], A. Sassi [4], K. Palme[5], S. Quaggiotti[1] & S. Nardi[1]

1 Department of Agricultural Biotechnology, University of Padua, Agripolis, Legnaro (Padua), Italy
2 Department of Environmental Agronomy and Crop Science, University of Padua, Agripolis, Legnaro (Padua), Italy
3 Department of Agroenvironmental Science and Technology, University of Bologna, Bologna, Italy
4 ISTM-PADOVA, Department of Chemical Science, University of Padua, Padua, Italy
5 Institute for Biology II/Botany, Faculty of Biology, Albert-Ludwigs-University of Freiburg, Freiburg, Germany

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Correspondence
S. Quaggiotti, Department of Agricultural Biotechnology, University of Padua, Agripolis, Viale dell'Università, 16, 35020 Legnaro (Padua), Italy.
E-mail: silvia.quaggiotti@unipd.it

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ABSTRACT

Humic substances (HS) have positive effects on plant physiology, but the molecular mechanisms underlying these events are only partially understood. HS exert auxin-like activity, but data supporting this hypothesis are under debate. To investigate the auxin-like activity of HS, we studied their biological effect on lateral root initiation in *Arabidopsis thaliana*. To this aim we characterised HS by means of DRIFT and ^{13}C CP/MAS NMR spectroscopy, and measured their endogenous content of IAA. We then utilised a combination of genetic and molecular approaches to unravel HS auxin activity in the initiation of lateral roots. The data obtained using specific inhibitors of auxin transport or action showed that HS induce lateral root formation mostly through their 'auxin activity'. These findings were further supported by the fact that HS used in this study activated the auxin synthetic reporter DR5::GUS and enhanced transcription of the early auxin responsive gene *IAA19*.

INTRODUCTION

Humic substances (HS) are recognised as a key component of soil fertility properties, since they control chemical and biological properties of the rhizosphere (Nardi *et al.* 2005). According to some authors, HS present a random polymeric, amorphous structure formed by polyaromatic building blocks bridged to each other by ester, ether and C links and carrying variable proportions of carboxyl, hydroxyl, amino and other hydrophilic groups (Andreux 1996). Other authors support a new theory, which considers HS to be a supramolecular association of heterogeneous molecules held together by hydrophobic interactions (van der Waals, π-π, ion-dipole) and hydrogen bonds (Piccolo 2001; Sutton & Sposito 2005).

Besides controversy about the structure of HS there is also a debate on their functional properties in plant development and physiology. Since the 1980s it has been hypothesised that the positive effects of HS on plant metabolism may depend on uptake of some macro- and micronutrients (Vaughan & Ord 1981; Clapp *et al.* 2001; Chen *et al.* 2004; Pinton *et al.* 2007). However, the hypothesis that they may interact with root cells by inducing endogenous activities, without being taken up by the plant, cannot be excluded. Furthermore, several studies have hypothesised that physiological mechanisms through which HS exert their effects may depend on hormones and, in particular, on the presence of auxin or auxin-like components in their structure (Nardi *et al.* 2002 and references therein). This hypothesis is partially based on results that allowed immunological or spectrometric identification of indoleacetic acid (IAA) in a number of HS isolated from different sources: earthworm faeces (Muscolo *et al.* 1998), forest soils (Pizzeghello *et al.* 2001) and compost (Canellas *et al.* 2002), and on data obtained in *Nicotiana plumbaginifolia* using two inhibitors of auxin (TIBA and PCIB) (Nardi *et al.* 1994).

The hypothesis is also supported by other reports showing a positive effect of HS on specific targets of auxin action. This is the case for the Mha2 maize isoform of H^+-ATPase that appears to be strongly stimulated at the transcriptional level in response to auxin (Frias *et al.* 1996) and shows a significant up-regulation of its mRNA abundance in roots of maize seedlings treated for 48 h with earthworm low-molecular size HS (Quaggiotti *et al.* 2004). Furthermore, another study (Russell *et al.* 2006) conducted on pea using two different molecular weight fractions of earthworm faeces HS, highlighted an auxin-like effect of both fractions on stomatal opening as influenced by phospholipase A2, which is considered to be involved in auxin-mediated signalling (reviewed

by Macdonald 1997; Scherer 2002). However, the measured effects did not always correlate with the amount of IAA detected in the humic acids, but seem to also be connected to the structural complexity of the HS utilised (Muscolo *et al.* 2007). Although there has been a noteworthy interest in different auxin compounds or molecules that might either mimic action or stimulate *in planta* endogenous metabolism of auxin, very little is known of how HS induce lateral root formation.

In order to shed light on the auxin-like activity of HS, we studied their biological effect on lateral root initiation and showed that the HS utilised induced lateral root formation in *Arabidopsis thaliana*. Root development is a complex process under regulatory control of a number of exogenous (*i.e.* nutrient availability, soil characteristics) and/or endogenous (developmental and hormonal) factors. However, lateral root formation has been shown to rely on auxins as a primary dominant signal in promoting mitotic activity of pericycle cells in the process of primordia initiation (reviewed by Casimiro *et al.* 2003; De Smet *et al.* 2006). In fact, mutations in genes involved in auxin polar transport or action, such as *AUX1* (Marchant *et al.* 2002) and *TIR1* (Ruegger *et al.* 1998), or treatment with chemicals inhibiting the same processes, such as 1-NOA (Parry *et al.* 2001) and PCIB (Oono *et al.* 2003), result in a reduced number of lateral root primordia (LRP).

HS, extracted from worm coprolytes, were analysed by means of diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy and cross polarisation magic angle spinning ^{13}C nuclear magnetic resonance (^{13}C CP/MAS NMR) spectroscopy, and their endogenous content of IAA was quantified by using an immunoassay method. To further unravel auxin activity of HS in the initiation of lateral roots we employed the widely used auxin reporter DR5::GUS (Ulmasov *et al.* 1997) to visualise auxin responses in roots (Sabatini *et al.* 1999; Benkova *et al.* 2003) and to characterise the distribution of LRP stages in both wild type (Col-0) and *aux1* mutant background. In addition, we evaluated transcription of the known early auxin responsive genes *IAA5* and *IAA19* (Goda *et al.* 2002; Nakamura *et al.* 2003; Oono *et al.* 2003; Yamazoe *et al.* 2005) in parallel treatments with HS and comparable IAA concentrations.

MATERIAL AND METHODS

Earthworm culture conditions

The faeces of *Nicodrilus* [= *Allolobophora* (Eisen) = *Aporrectodea* (Oerley)] *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) were collected from the surface of uncultivated couchgrass (*Agropyron repens* L.) at the College of Agriculture farm (Padua University) as described in Muscolo *et al.* (1999). The soil was classified as calcaric cambisol (FAO-ISSDS 1999).

HS extraction

Humic compounds were extracted from the faeces of *Nicodrilus* and *A. rosea* using 0.1 N KOH, as described in Muscolo *et al.* (1999). The extract was dialyzed using 14-kDa cut-off Visking membrane tubing (Medicell, UK) against distilled water. Subsequently, the extract was desalted on ion exchange Amberlite IR-120 (H+ form) (Stevenson 1994) and freeze-dried. The nominal molecular size of HS corresponded to the high-molecular fraction. The C and N contents were measured using an elemental analyser (Thermo Electron mod. EA 1110, Waltham, MA, USA).

The concentration of COOH groups was determined by dissolving 12 mg of freeze-dried HS in 20 ml Milli-Q Millipore water containing 0.05 M NaCl, to keep the ionic strength constant. pH was adjusted to 3.0 by adding 0.05 M HCl. The solutions were titrated to pH 10.5 with 0.05 M NaOH using a VIT 90 titrator radiometer (Radiometer, Copenhagen, Denmark). Potentiometric titrations were carried out in triplicate at 25 °C under N_2 flow, and delivery range was 10 $\mu l \cdot min^{-1}$ (± 0.01). The first derivative method was used to determine the concentration of COOH groups.

DRIFT spectroscopy

The spectrum was recorded with a Bruker TENSOR series FT-IR spectrophotometer (Ettlingen, Germany) equipped with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT, USA). The spectrum was collected as Kubelka-Munk units using KBr (Sigma-Aldrich, Milwaukee, WI, USA) as background reference, from 4000 to 400 cm^{-1} and averaged over 100 scans (resolution ± 4 cm^{-1}). Analysis of spectral data was performed with Grams/386 software (Galactic Industries, Salem, NH, USA). Spectral sections from 1850 to 500 cm^{-1} were baseline-corrected to an absorbance value of 0.00 at 1850 cm^{-1}.

CP-MAS ^{13}C NMR spectroscopy

The solid-state ^{13}C NMR spectrum was obtained using a Bruker AC200 spectrometer equipped for solid-state analysis and operating at 50.26 MHz. Samples were spun in the range 3000–7000 Hz in 7-mm diameter zirconia rotors with Kel-F caps. The ^{13}C SPE MAS NMR (SPE = single pulse experiment) spectrum was obtained with high-power proton decoupling during acquisition, 30 s relaxing delay, and processed with a 10 Hz exponential line broadening. ^{13}C chemical shifts were externally referenced to solid sodium 3-(trimethyl-silyl)-1-propane sulfonate at 0 ppm. Magic angle conditions were adjusted by observing ^{79}Br spinning side band patterns in a rotor containing 5% KBr (Frye & Maciel 1982). The following resonance intervals are generally attributed to different carbons (Conte *et al.* 2007): 220–162 ppm (carbonyls of ketones, quinones, aldehydes and carboxyls), 162–108 ppm (aromatic carbons), 108–80 ppm (anomeric carbons), 80–50 ppm (C–O systems, such as alcohols and ethers), 50–35 ppm (C–N groups and complex aliphatic carbons), 35–0 ppm (alkyl carbons).

IAA determination in HS

The quantitative determination of IAA in HS was made by an enzyme linked immunosorbent assay (ELISA) (Phytodetek-IAA, Sigma-Aldrich, St Louis, MO, USA). For ELISA, an anti-IAA monoclonal antibody was utilised that allowed sensitive detection of IAA in the range 0.05–100.0 pmol. The competitive antibody binding method was adopted to

measure concentrations of IAA. IAA labelled with alkaline phosphatase (tracer) was added to antibody-coated microwells together with the sample, which had been pretreated with diazomethane to convert the acid to its methyl ester form competing with the tracer for antibody binding sites.

Tracer and standard solutions were prepared following the manufacturer's instructions (Phytodetek-IAA, Sigma). To each well, 100 µl of standard IAA concentration or serial dilutions of HS and 100 µl diluted tracer were added. For the standard curve, a progression of 500, 100, 50, 20, 5, 1, 0.1 and 0.02 pmol methylated IAA 100 μl^{-1} was used, whereas for HS the progression was 15, 25, 35, 50 and 75 µgC 100 μl^{-1}. After incubation at 4 °C for 3 h, the wells were decanted and unbound tracer was washed out by adding 200 µl of wash solution before adding 200 µl of substrate solution for colorimetric detection. After 60 min at 37 °C, 50 µl of stop reagent were added to each well and the colour absorbance read at 405 nm using a 450 Biorad microplate reader (Biorad, Hercules, CA, USA). Both the standard curve and the progression of HS dilutions were repeated twice.

Plant growth and treatments

Seeds of *Arabidopsis thaliana* Columbia ecotype (Col-0) and DR5::GUS transgenic plants (Ulmasov *et al.* 1997) were surface sterilised, sown on solid AM medium, and germinated as described by Muller *et al.* (1998). Usually, 50 seedlings were used per sample in each experiment, which was repeated three times.

The number of lateral roots per mm of main root length was scored in all experiments on 1-week-old DR5::GUS transgenic plants, for easier visualisation of primordia at all developmental stages, after the following treatments. For the HS dose–response curve, seedlings were treated for 24 h with 0.1, 0.5 and 1.0 $mgC \cdot l^{-1}$ HS. For time-course experiments, plants were incubated for 16, 24 or 48 h, with 1.0 $mgC \cdot l^{-1}$ HS or with 34 nM IAA, corresponding to the content of IAA measured in this humic fraction, or with sterile mQ water as a control. For inhibitor treatments, plants were kept for 24 h in 1.0 $mgC \cdot l^{-1}$ HS, 34 nM IAA or mQ water in the presence or absence of 50 µM 1-NOA, 50 µM TIBA or 50 µM PCIB. Treatment of *AUX1*-DR5::GUS 1-week-old seedlings was performed with 1.0 $mgC \cdot l^{-1}$ HS or 34 nM IAA for 24 h. Histochemical GUS staining was performed as described by Jefferson *et al.* (1987).

Lateral root primordia at different stages of development (Malamy & Benfey 1997; Casimiro *et al.* 2003) were observed with an optical microscope (Olympus BX60, Tokyo, Japan) equipped with a C-3040 Olympus digital camera (Olympus). Differences among means were calculated using the Kruskal–Wallis H-test (Gibbons 1976), and a P value of less than 0.05 was considered statistically significant. Regression analysis was used to analyse the HS dose–response curve. All statistical analyses were conducted using SPSS for Windows software, version 11.0 (SPSS, Chicago, IL, USA).

RNA extraction and cDNA synthesis

Total RNA was isolated using the NucleoSpin RNA Plant kit (Macherey-Nagel, Dure, Germany) following the protocol provided by the manufacturer. The concentration of RNA isolated was calculated from the A_{260} in distilled water, and the quality assayed by electrophoresis of a 1-µg aliquot on Tris-acetate/agarose gels. First-strand cDNA was synthesised from 1 µg total RNA, after Dnase I treatment (Promega, Milano, Italy), using 200 U MMLV reverse transcriptase (Promega) and oligodT as primer, in 20 µl reactions, as described in Sambrook *et al.* (1989). Following the same procedure, RNA samples were also processed, in which the RT step was omitted to rule out amplification from contaminating genomic DNA.

Real-time PCR

Real-time PCR relative quantification was performed in a total volume of 10 µl using Power SYBR® Green PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA) with 3 pmol of each primer and 2 µl of a 1 : 10 dilution of cDNA. The gene-specific primers for *IAA5* (At1g15580) and *IAA19* (At3g15540), listed in Table 1, were designed with Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the instructions in the SYBR® Green PCR Master Mix protocol (Applied Biosystems). The specificity of amplification was assessed by subsequent subcloning and sequencing of the PCR products obtained under the same conditions adopted in the real-time experiments. The amplification was performed in a 7500 real-time PCR system (Applied Biosystems) as described in Botton *et al.* (2008). After every PCR cycle, a data acquisition step was introduced to record the fluorescence signals at the optimum temperature, previously determined by melting point analysis of every specific amplification product. Data were acquired, elaborated and exported using the software SDS Sequence Detection System v1.2 (Applied Biosystems), whereas all final calculations were carried out with the automated spreadsheet Q-Gene designed by Simon (2003), using

Table 1. Target genes for analysis of expression profile.

gene ID	primer	sequence (5′–3′)	T annealing (°C)	cycles	bp
At1g15580	IAA5	for AGATATCGTCGTCTCCGGTG rev GCCGAAGCAAGATCTTGGTA	61	27	251
At3g15540	IAA19	for GAGCATGGATGGTGTGCCTTAT rev TTCGCAGTTGTCACCATCTTTC	61	27	141
At3g18780	ACT2	for AACATTGTGCTCAGTGGTGG rev TCATCATACTCGGCCTTGG	58	25	206
At2g01010	18S	for CGGCTACCACATCCAAGGAA rev GCTGGAATTACCGCGGCT	77.4	25	186

the modifications of the ΔCt method suggested by Pfaffl (2001). Two genes (*ACT2*, At3g18780 and *18S*, At2g01010), giving similar expression values, were selected as housekeeping genes. Gene expression values were normalised to the *18S* gene and reported as arbitrary units (AU) of mean normalised expression (Pfaffl 2001), using equation 2 of Q-Gene. The correct size of the amplification products was checked by running each reaction in a 1.5% agarose gel stained with ethidium bromide and viewed under UV light.

RESULTS

Characterisation of HS

Elemental composition of HS showed a total carbon content of 55.97% ± 0.10, a nitrogen content of 4.40% ± 0.16, and total acidity of 424 ± 10 mmol/100 g dry weight.

The DRIFT spectrum (Fig. 1A) was dominated by two strong and sharp bands around 1720 cm^{-1} and 1650 cm^{-1}, characteristic of undissociated carboxyl groups stretching vibrations (Niemeyer *et al.* 1992; Francioso *et al.* 1998, 2002) and to stretching vibration (C=O) in amide I, respectively. Moreover, a band at 1530 cm^{-1} indicated the presence of C=C in aromatic ring vibrations from a lignin derivative. The intense band at 1230 cm^{-1} confirmed the presence of C–O stretching vibrations in phenols and carboxyl groups. Furthermore, a strong signal in the region of 1100–800 cm^{-1} was unequivocally assigned to C–O stretching in carbohydrates (Bellamy 1975; Stevenson 1994).

The CP-MAS NMR spectrum of HS (Fig. 1B) showed features very similar to those extracted from earthworm compost (Canellas *et al.* 2002). The 0–40 ppm region assigned to CH_2 in alkyl chains and terminal methyl groups was not well resolved, indicating a low content of aliphatic substances. In our sample, the resonance between 40–60 ppm, assigned to C bound to N in amino acids appeared higher that that of Canellas *et al.* (2002). Presence of C–N groups was also visible in the vibration of amide I (Fig. 1B). The prominent signal around 70 ppm can be mainly attributed to C–O in polysaccharides, while the weak resonance around 100 ppm suggested the presence of anomeric C. The aromatic C resonating between 130 and 160 ppm was not well resolved. A signal at around 170 ppm was assigned to quaternary C in carboxylic groups. The potentiometric titration supported the high amount of COOH groups found in this sample. Finally, a broad and weak resonance at 240 ppm also suggested the presence of C in ketone.

IAA content of HS

Quantitative determination of IAA in HS was made using an ELISA assay. An IAA standard curve obtained by plotting the percentage binding ($\%B/B_0$) *versus* the nmol methyl-indole-3-acetate concentration was used for quantification (Fig. 2A). The HS response curve for IAA content determination was made in a range from 15 to 75 μgC HS (Fig. 2B). Fitting the percentage binding of each point of the HS curve into the standards equation, an IAA content of 34 ± 0.31 nM was calculated to be present in 1 mgC HS l^{-1}. As far as auxin and conjugated forms of auxin are concerned, molecules such as indole-3-acetylglycine, indole-3-acetone, indole-3-acetylalanine, indole-3-butyric acid, indole-3-acetonitrile, indole-3-acetamide, indole-3-propionic acid, indole-3-aldehyde and indole-3-acetaldehyde display a low affinity and do not cross-react significantly with the monoclonal IAA antibody used in this study. Except for indole-3-acetylglycine showing a cross reactivity of 57%, the cross reaction for other molecules ranged from 5.0% to <0.01%. Therefore the IAA content determined on HS mainly reflects their content of free IAA.

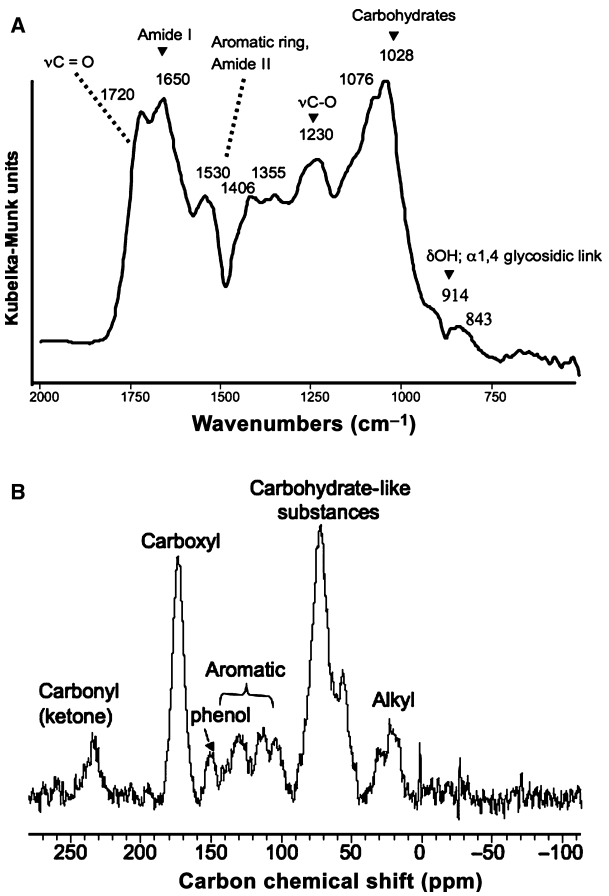

Fig. 1. DRIFT spectrum of HS fraction extracted from earthworm faeces (A). The bands correspond to undissociated carboxylic groups (1720 cm^{-1}); stretching vibration in amide I (1650 cm^{-1}); amide II and C=C vibrations in aromatic rings (1514 cm^{-1}); C–O stretching vibrations of alcohol and carboxyl groups (1230 cm^{-1}); and C–O stretching in carbohydrates (1100–800 cm^{-1}). In CP-MAS NMR spectrum (B), the main resonance intervals are attributed to different carbons: 220–162 ppm (carbonyls of ketones, quinones, aldehydes and carboxyls), 162–108 ppm (aromatic carbons), 108–80 ppm (anomeric carbons), 80–50 ppm (C–O systems, such as alcohols and ethers), 50–35 ppm (C–N groups and complex aliphatic carbons), 35–0 ppm (alkyl carbons) (Conte *et al.* 2007)

HS induce expression of the synthetic auxin reporter DR5 and lateral root development in *Arabidopsis*, and rescued the lateral root phenotype of the *aux1* mutant

The effect of different concentrations of HS was estimated on development of lateral roots in wild-type plants of *Arabidopsis thaliana* and on induction of the auxin responsive synthetic reporter DR5::GUS (Ulmasov *et al.* 1997). In particular, DR5::GUS has been used as a tool to visualise

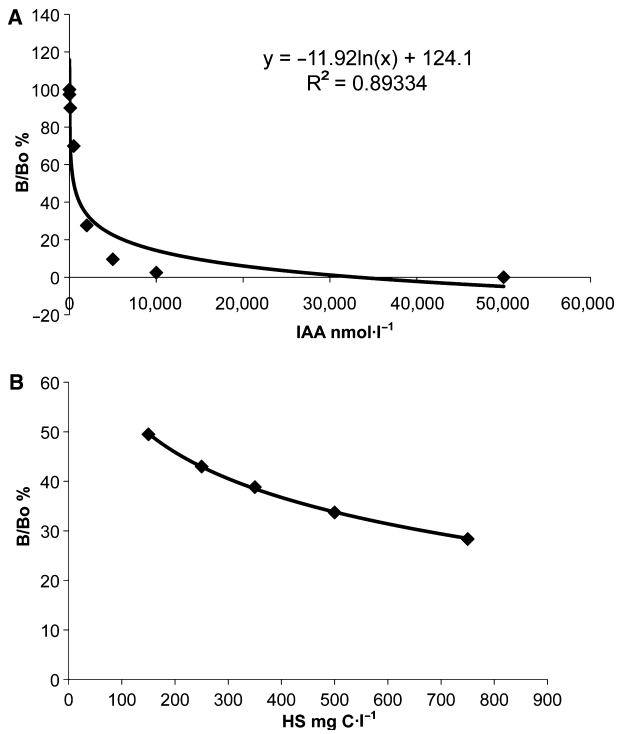

Fig. 2. Determination of IAA in HS by ELISA assay. IAA standard curve (A) and HS dose response (B). The intensity of the colour produced was related to the sample IAA concentration by means of a standard curve

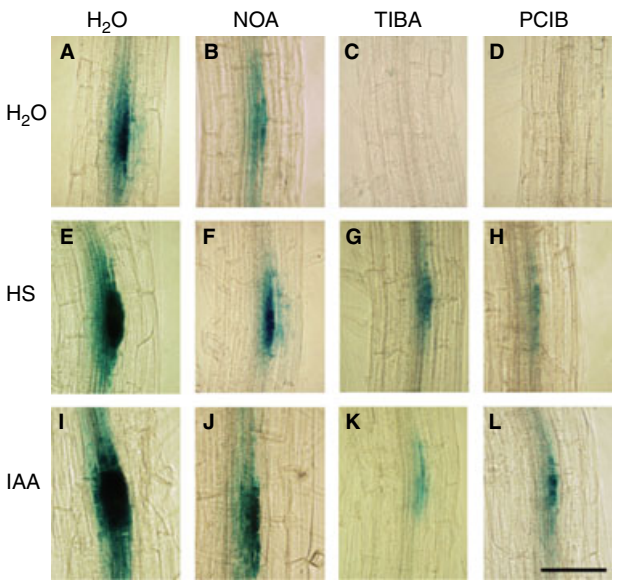

Fig. 3. Visualisation of Gus activity in root of DR5::GUS transgenic plants treated with different auxin inhibitors. Plants were grown for 4 days in MS medium plates and then transferred for 24 h into: water, CTR (A), 50 μM NOA (B), 50 μM TIBA (C), 50 μM PCIB (D), 1 mgC·l^{-1} HS (E), 50 μM NOA + 1 mgC·l^{-1} HS (F), 50 μM TIBA + 1 mgC·l^{-1} HS (G), 50 μM PCIB + 1 mgC l^{-1} HS (H), 34 nM IAA (I), 50 μM NOA + IAA 34 nM (J), 50 μM TIBA + IAA 34 nM (K), 50 μM PCIB + 34 nM IAA (L). Histochemical GUS staining was performed as described by Jefferson *et al.* (1987). Lateral root primordia are represented at different developmental stages: I (B, G, H, K and L), II (A, F and J) and III (E and I). Scale bar is 50 μm.

auxin responses in tissues and mark auxin signalling in lateral root primordia at all developmental stages (Sabatini *et al.* 1999; Benkova *et al.* 2003). It has also been reported to be activated by auxins in a dose-dependent manner (Ulmasov *et al.* 1997). Treatment with HS caused activation of DR5::GUS expression in lateral roots in a way comparable to exogenously applied auxins, as shown in Fig. 3E and I, respectively. Consistently, this induction was sensitive to treatment with inhibitors such as PCIB (Oono *et al.* 2003) (Fig. 3D, H, L), auxin uptake or transport obtained by treating plants with NOA (Parry *et al.* 2001) (Fig. 3B, F, J) or TIBA (Fig. 3C, G, K). In fact, the auxin inhibitors, even though not fully preventing DR5::GUS expression, delayed it significantly and determined a reduction in both number and intensity of positive signals in roots in response to HS or IAA treatment, as highlighted by representative pictures in Fig. 3. DR5::GUS expression was also activated in control plants (Fig. 3A), but to a significantly lower extent. This finding reflects the action/signalling of endogenous auxin. Pictures in Fig. 3 also evidence different stages of development of lateral root primordia for different treatments, with HS and IAA inducing earlier lateral root initiation in comparison to the control and inhibitor treatments. DR5::GUS staining was also detected at the root apex level, but no significant differences were seen among samples, with the exception of TIBA treatment, which inhibited DR5::GUS induction in most, but not all, apices (Fig. S1). Scoring of the overall DR5::GUS-positive lateral root primordia showed a significant effect of HS on the number of lateral roots formed in 7-day-old *Arabidopsis* plants. A strong and positive linear relationship (Y = a + bX) was found between number of lateral roots and HS concentration (r = 0.97, $P < 0.05$). The high value of R^2 indicates that the model as fitted explains 93.51% of variability in the dependent variable (Y) (Fig. 4). A value of 1 mgC·l^{-1} was chosen as the concentration for later experiments, since it corresponds to a relatively low IAA content (34 nM). In addition, this concentration for both treatments with HS and IAA caused no significant inhibition of primary root elongation in comparison to control plants (Fig. S2). In contrast, auxin inhibitors induced a limited but statistically significant reduction in root elongation in both the presence and absence of treatments with HS and IAA (Fig. S2).

In order to evaluate the dynamics of lateral root induction by HS in comparison to equal concentrations of IAA (34 nM) present in 1 mgC·l^{-1} HS, a time-course experiment was carried out. Results reported in Fig. 5 showed statistically ($P < 0.05$) higher and faster induction of lateral root development in HS- and IAA-treated plants already after 16 h in comparison to control plants. The positive effect of HS appeared to be significantly higher than that of IAA after 24 h, while after 48 h no significant difference between treatments was found (Fig. 5). Interestingly, while HS treatment induced a reproducibly higher number of LRP than both control and IAA-treated plants after 24 h, the addition of NOA at saturating concentrations (50 μM) led to a reduction of the number of LRP to comparable levels in both treated and control plants (Fig. 6A). A similar inhibitory effect was exerted by PCIB, with no significant differences between treatments, on both HS- and IAA-treated plants and in

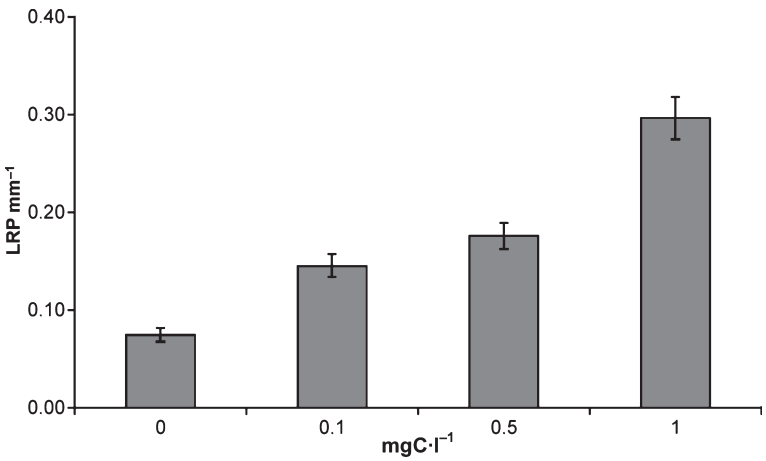

Fig. 4. LRP per mm of primary root in 1-week-old DR5::GUS *Arabidopsis* seedlings after treatment for 48 h with increasing concentrations of HS (0, 0.1, 0.5, 1.0 mgC·l^{-1}). Bars indicate standard error of means. A significant linear model Y = 0.094 + 0.197X ($P < 0.05$) described the relationship between variables ($r = 0.97$)

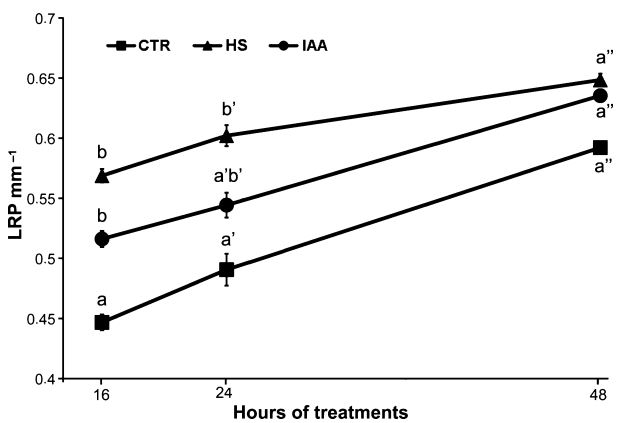

Fig. 5. Time course of LRP formation mm^{-1} of primary root in response to IAA (34 nM) or HS (1 mgC·l^{-1}). Four-day-old DR5::GUS seedlings were treated for different lengths of time (16, 24 and 48 h) with HS or IAA. The letters on bars represent non-significant ranges according to the SNK test ($P \leq 0.05$). Bars indicate SE.

comparison to untreated plants (Fig. 6A). Use of the auxin transport inhibitor TIBA appeared to be the most effective in preventing formation of LRP. Its action led to a significantly different reduction of the number of LRP among treatments, resulting in a 92% decrease in control plants, 86% in IAA-treated plants, and only 74% in HS-treated plants (Fig. 6A).

The mode of HS enhancement of lateral root formation was further investigated by analysing the number of different stages (from I to VIII) (Malamy & Benfey 1997; Casimiro *et al.* 2003) of LRP in the DR5::GUS reporter line in the presence of the different inhibitors (Fig. 6B). Treatment with IAA and HS led to a general enhancement of the number of root primordia ranging from stage I to stage III, in comparison to control plants. No significant differences for later stages of development appeared between treatments. In contrast a strong induction of overall LRP formation was caused by HS, relative to a higher number of stage I primordia ($P < 0.05$) (Fig. 6B). In addition, stimulation of stage I LRP formation triggered by HS appeared to be less sensitive to TIBA inhibition (65% reduction in HS as opposed to 85% in IAA-treated plants), while inhibition of IAA loading by treatment with NOA or inhibition of IAA action by application of PCIB led to a comparable number of stage I primordia.

The auxin activity of HS was further investigated by analysing lateral root formation in the *aux1* mutant background. Mutation in the *AUX1* gene, encoding an auxin influx carrier, results in agravitropic roots, reduced ethylene sensitivity and a 50% reduction of LRP formation (Hobbie & Estelle 1995; Marchant *et al.* 2002). The *aux1* lateral root initiation phenotype has been shown to be due to suboptimal loading of auxin into founder cells of primordia that can be rescued by exogenous application of nM concentrations of auxin to plants (Marchant *et al.* 2002). In our study, a number of lateral roots in the *AUX1*-DR5::GUS plants treated with HS and IAA (34 nM) showed differences that were statistically significant with respect to the wild type for the same length of time (24 h) (Fig. 7A). Interestingly, a higher number of stage I LRP was induced by IAA and HS treatments in the *aux1* mutant background than in the control (Fig. 7B).

HS induce transcription of the auxin inducible gene *IAA19*

The AUX/IAA transcription factors IAA5 and IAA19 have been shown to be early transcriptionally induced by auxin and brassinosteroids (Nakamura *et al.* 2003; Oono *et al.* 2003). Detailed analysis of their time course of expression in response to brassinolide or IAA demonstrated faster down-regulation already after 2 h of auxin treatment as opposed to longer and steady up-regulation (up to 12 h) in the presence of brassinolide, allowing diagnosis of divergent molecular modes of action of these hormones (Nakamura *et al.* 2003). In an attempt to separate IAA-dependent effects from effects mediated by factors others than auxin present in HS, and to further characterise their molecular mode of action, we investigated the time course of expression of *IAA5* and *IAA19* following short parallel treatments with either HS or 34 nM auxin. Real-time quantitative RT-PCR showed that the *IAA19* gene was up-regulated already after 30 min of treatment of *Arabidopsis* Col-0 plants with either HS or 34 nM auxin, and subsequently displayed a decline in transcription after 2 h of treatment with IAA, while a steady increase was detected in the presence of HS and, to a lesser extent, in control plants (Fig. 8). *IAA5* transcript accumulation did not show significant stimulation by either IAA or HS (Fig. 8).

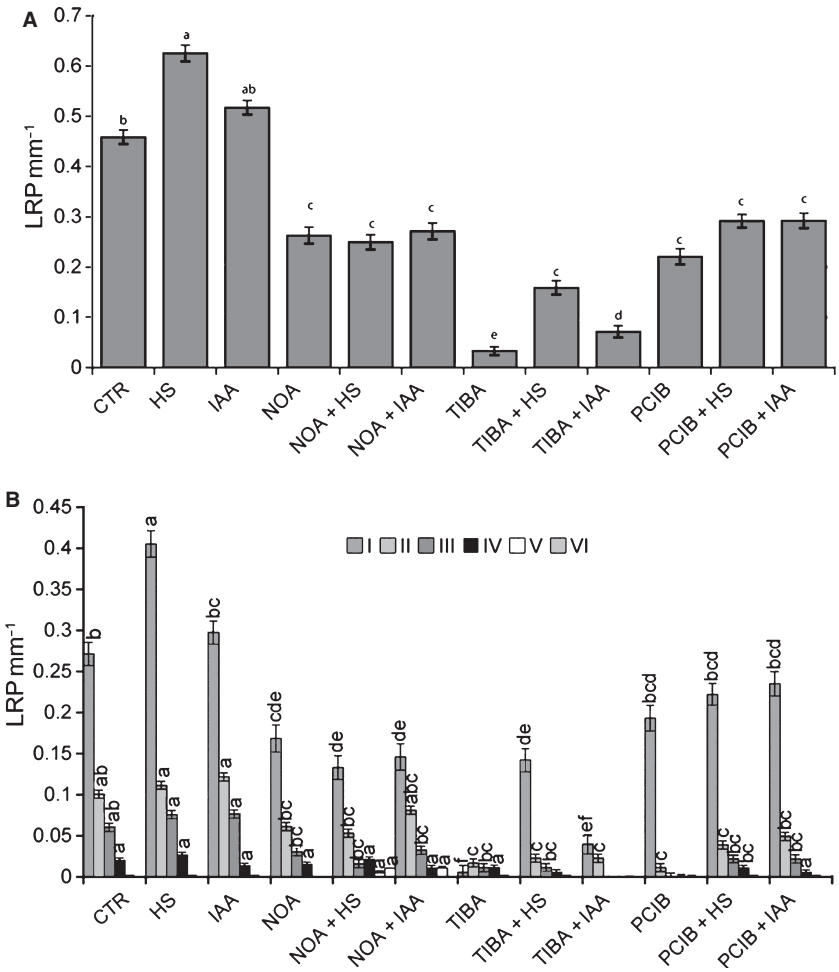

Fig. 6. Changes in density of LRP mm^{-1} in 4-day-old DR5::GUS *Arabidopsis* seedlings after 24 h of treatment with IAA (34 nM) or HS (1 $mgC \cdot l^{-1}$) and in response to different auxin inhibitors (A). Number of LRP mm^{-1} per stage of development (B). Bars indicate SE.

DISCUSSION

In the present paper, the HS deduced IAA concentration was estimated (34 nM) by both an immunological approach and on the basis of their similar effects compared to IAA. Due to the complexity of physiological responses to these substances, it seems that multiple signal cascades with different metabolic targets are involved. Only through a structural and molecular approach is it possible to clarify the signalling events governing the plant response to HS. In previous papers, HS biological activity was related to the lower molecular fraction characterised by a high content of aromatic and carboxyl groups in the acid (Muscolo *et al.* 1993, 1996, 2007; Nardi *et al.* 1994). In particular, the large content of carboxylic groups, as suggested by Rubery (1981) and Napier (2004), are needed for auxin binding and control the modulation of auxin bioavailability in relation to plant requirements. The IAA presence in HS from earthworm faeces and from a wide range of forest soils has been found in different studies (Muscolo *et al.* 1998; Nardi *et al.* 2000; Pizzeghello *et al.* 2001; Quaggiotti *et al.* 2004). How IAA is bound or is in association with HS is not well understood (Schulten & Schnitzer 1998), even though a high concentration of organic acids released by the roots into the rhizosphere is required to release IAA from HS (Nardi *et al.* 1988, 2002, 2005; Piccolo *et al.* 1996). Besides the organic acids released by plant roots, the pH gradient may also influence auxin availability in soil, interfering with the –COO:–COOH/IAA equilibrium. In this contest, plant ability to extrude H^+ thanks to ATPase-dependent mechanisms may significantly affect rhizosphere acidity. Therefore, auxin and other biologically active compounds may be entrapped in the complex molecular structure of HS and plants may modulate their bioavailability through extrusion of specific molecules. This hypothesis was also recently supported by results obtained in *Arabidopsis* seedlings by Dobbss *et al.* (2007). IAA and other plant growth promoting substances are produced by many bacteria (Lambrecht *et al.* 2000), so it is not surprising that they can be entrapped in the molecular structure of HS. Nevertheless, the presence of other biologically active compounds in HS cannot be excluded.

Our HS results highlight a high presence of carboxylic groups and sugar-like components, and a low content of aromatic substances, similar to those described by Canellas *et al.* (2002). Comparing the spectroscopic patterns of the high molecular size HS used in this work with the low molecular size (LMS) HS used in a previous study (Quaggiotti *et al.* 2004), some structural modifications can be seen. For instance, in our HS strong enhancement of the band around 1720 cm^{-1} could be due to a higher pK_a of the COOH groups, while in LMS (Quaggiotti *et al.* 2004) the contribution of carboxylate groups in acid dimers was dominant.

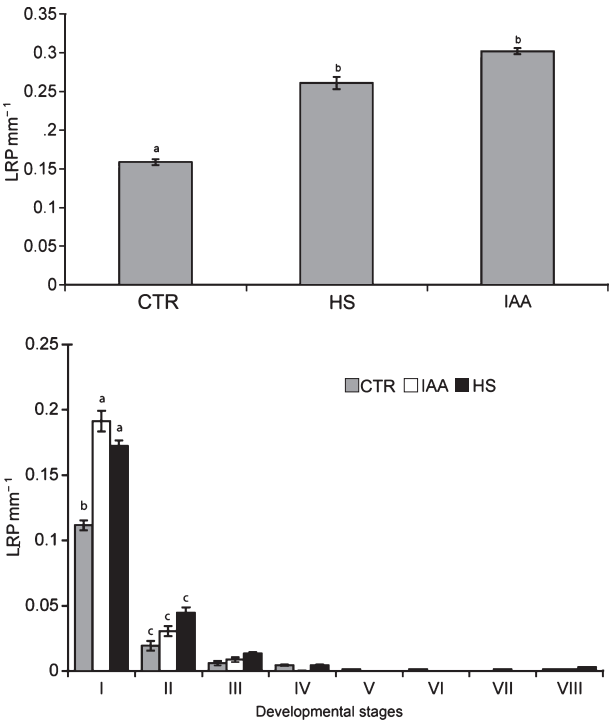

Fig. 7. Statistical analysis of total LRP mm^{-1} density in 4-day-old *AUX1*-DR5::GUS seedlings after 24 h of treatment (CTR, IAA, HS) (A) and per stage (B). Letters on bars represent non-significant ranges according to the LSD test ($P \leq 0.05$), whereas bars show the SE.

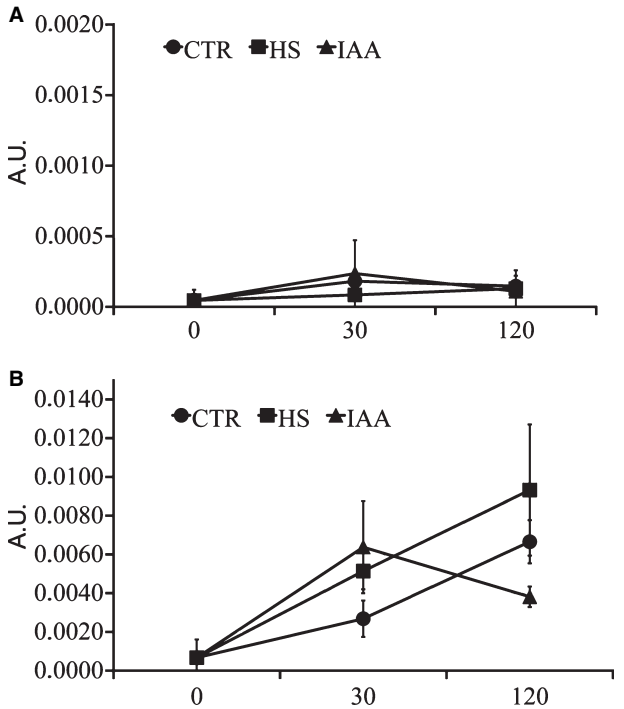

Fig. 8. Expression of IAA5 (A) and IAA19 (B) measured by real-time PCR using the *18S* gene as reference. *Arabidopsis* seedlings were grown for 4 days in MS plates and then treated with water (CTR), HS (1 $mgC \cdot l^{-1}$) or IAA (34 nM) for 30 min or 2 h. Analyses were carried out in triplicate for each cDNA obtained from two independent RNA extractions. Specific transcript levels are expressed as arbitrary units (AU) of mean normalised expression. Bars represent SE.

Since the DRIFT spectra were recorded at the same pH, this indicates that the COOH groups in HS were not as extensively dissociated, as is the case of benzoic acids containing a large number of OH or, in general, electron donor groups. A confirmation of the higher phenolic content in HS is given by the resonance at around 150 ppm, which may correspond to phenolic carbons that are close to the quinonoid groups. The combination of carboxyl and aromatic groups in HS is a confirmation of common characteristics found in HS from different origins (Muscolo *et al.* 2007). Moreover, the common structural characteristics arising from the results of Canellas *et al.* (2002) suggest that HS derived from earthworms are at an early stage of humification. On the basis of these results, chemical features are needed to explain the biological activity of HS. In fact, Schmidt *et al.* (2007), studying the effect of a HS on *Arabidopsis*, showed unchanged expression for some auxin-related genes, leading these authors to hypothesise an action of HS utilised independent of auxin. The lack of details on the chemical composition of HS makes it very difficult to interpret these conflicting results. Moreover, the authors treated plants with a very high amount of humic matter (50 $mgC \cdot l^{-1}$) and did not consider that the effect of HS on plant metabolism is selective and variable in relation to the concentration (Gumiński 1968; Vaughan & Malcolm 1985; Maggioni *et al.* 1987; Varanini & Pinton 2001; Nardi *et al.* 2002).

To gain a deeper understanding of auxin-like activity of HS, we investigated their inductive action on a well-defined auxin-dependent process, lateral root development, in the model plant *Arabidopsis thaliana*. The dominant inducing role played by auxins on the process of lateral root initiation has been widely demonstrated (reviewed by Casimiro *et al.* 2003; De Smet *et al.* 2006). Therefore, in this study, we undertook a comparative approach between auxin and HS, at a given known auxin equivalent concentration, for inducing the formation of lateral roots.

For easier visualisation of the auxin response and of LRP from very early stages of development, the auxin responsive reporter DR5::GUS was used (Ulmasov *et al.* 1997). Our data show that HS induce lateral root formation and activate the DR5 reporter already after 16 h of treatment. However, time-course experiments showed that HS appeared to induce lateral root formation faster and at higher rates than auxin after 24 h. This effect appeared to be reflected mainly by a higher number of primordia at early stages (stage I), suggesting that the faster and higher induction exerted by HS may depend on a stronger initiation process. Interestingly, these differences were abolished by treatment with the inhibitor of auxin influx, 1-NOA (Parry *et al.* 2001), or with the inhibitor of auxin signalling and action, PCIB (Oono *et al.* 2003), when both the total number of LRP and of stage I primordia were considered, suggesting that HS act on lateral root initiation mainly through auxin transport and signalling. This was supported by the finding that treatment with HS rescued the lateral root phenotype of the *aux1* mutant. Mutation of the *AUX1* gene has been reported to result in suboptimal auxin

unloading, where lateral roots are initiated, and has been shown to be rescued by exogenous feeding of auxin at nanomolar concentrations (Marchant *et al.* 2002). Here, we show that 24-h treatment with HS rescues the *aux1* lateral root initiation phenotype in a way that does not significantly differ from that of auxin. HS induce lateral root formation through auxin and, most probably, through uptake into root cells of auxin bio-available in their structure and, in particular, in the functional groups. In addition, the data suggest that an additive factor may be responsible for HS action on lateral root induction. In fact, unlike results obtained in the Col-0 background, treatment of the *aux1* mutant with humic acids did not result in higher induction of LPR or stage I LRP, in comparison to auxin. This is in agreement with data obtained with 1-NOA, which exerts a similar inhibition on lateral formation in both IAA- and HS-treated plants, and it may be speculated that the additive effect of HS on lateral root formation requires *AUX1* to be functional and relies on AUX1-dependent auxin transport. In addition, HS-induced lateral root formation appeared less sensitive than the auxin-induced process to treatment with the inhibitor TIBA. This was evident mostly in terms of induction of stage I LRP and may suggest that, besides auxin-dependent action, an additive factor may be present in HS. In a recent paper, Zandonadi *et al.* (2007) also hypothesised auxin-like activity of HS obtained from different soil sources, and demonstrated their positive effect on lateral root development in maize. Our data, taken together, demonstrate that this effect mainly relies on the action of auxin contained in HS.

In a first attempt to further explore the molecular mode of action of HS and shed light on common or divergent regulatory aspects with IAA, we studied HS effects on the time-course of expression of the auxin inducible genes *IAA5* and *IAA19* (Nakamura *et al.* 2003; Oono *et al.* 2003). In fact, both DR5::GUS and transcription of *IAA5* and *IAA19* has been shown to also be induced by brassinosteroid treatments (Nakamura *et al.* 2003; Oono *et al.* 2003; Bao *et al.* 2004; Yamazoe *et al.* 2005). However, AUX/IAA transcription is rapidly and transiently up-regulated by auxin and, in contrast, is enhanced in a more stable way by brassinosteroids (Nakamura *et al.* 2003; Oono *et al.* 2003; Yamazoe *et al.* 2005). This evidence suggests that transcription of these genes can be used as a diagnostic tool to dissect the molecular mode of action of different hormones or molecules in relation to the IAA signal transduction pathway. Our results show that *IAA19* transcript accumulation is induced after 30 min of either IAA or humic acid treatment, before returning to basal levels within 2 h in the presence of IAA but not of HS. This transient up-regulation in the presence of IAA is in agreement with previous reports, in terms of both induction and temporal regulation (Nakamura *et al.* 2003), and further strengthens the hypothesis that HS can induce auxin-inducible genes. However the lack of down-regulation of *IAA19* after 2 h by HS may favour the hypothesis on the presence of additional factors other than IAA in HS. The up-regulation of *IAA19* expression detected in control plants after 2 h of treatment suggests a positive action exerted by endogenous auxins on auxin signalling. In our experimental conditions, we could not show a significant induction of the *IAA5* gene in response to IAA or HS; however this is in agreement with data of Nakamura *et al.* (2003) showing that IAA significantly induced expression of this gene only at concentrations higher than 100 nM, unlike IAA19, which appeared to be already induced by IAA concentrations as low as 10 nM (Nakamura *et al.* 2003).

In summary, our data show that HS exert their action on lateral root development mostly through their auxin activity. This is based on independent experiments employing the induction, which is sensitive to different auxin inhibitors, of the DR5::GUS reporter and of lateral root initiation, on rescue of the lateral root phenotype of the *aux1* mutant and on early and transient transcriptional activation of an auxin responsive gene in *Arabidopsis*. At the same time, the presence of additional factors independent of auxin was evident based on *IAA5* and *IAA19* expression, suggesting that more systematic approaches are needed to unravel the molecular mode of action of HS. These aspects are currently under investigation in our laboratory.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Frequency of DR5::GUS staining in *Arabidopsis* apices.

Figure S2. *Arabidopsis* primary root elongation in response to applied water.

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