The Response of the Root Proteome to the Synthetic Strigolactone GR24 in *Arabidopsis*  

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Strigolactones are plant metabolites that act as phytohormones and rhizosphere signals. Whereas most research on unraveling the action mechanisms of strigolactones is focused on plant shoots, we investigated proteome adaptation during strigolactone signaling in roots of *Arabidopsis thaliana*. Through large-scale, time-resolved, and quantitative proteomics, the impact of the strigolactone analog rac-GR24 was elucidated on the root proteome of the wild type and the signaling mutant *more axillary growth 2* (*max2*). Our study revealed a clear MAX2-dependent *rac-GR24* response: an increase in abundance of enzymes involved in flavonol biosynthesis, which was reduced in the *max2* mutant. Mass spectrometry-driven metabolite profiling and thin-layer chromatography experiments demonstrated that these changes in protein expression lead to the accumulation of specific flavonols. Moreover, quantitative RT-PCR revealed that the flavonol-related protein expression profile was caused by *rac-GR24*-induced changes in transcript levels of the corresponding genes. This induction of flavonol production was shown to be activated by the two pure enantiomers that together make up rac-GR24. Finally, our data provide much needed clues concerning the multiple roles played by MAX2 in the roots and a comprehensive view of the *rac-GR24*-induced response in the root proteome. *Molecular & Cellular Proteomics* 15: 10.1074/mcp.M115.050062, 2744–2755, 2016.  

Root development is pivotal for plant survival, providing anchorage, ensuring water and nutrient uptake, and allowing the plant to engage in beneficial interactions with soil microorganisms. Root growth is modulated in response to numerous abiotic and biotic environmental cues, which are interpreted and transduced by hormonal pathways. Besides the well-known regulators of root development, such as auxin and cytokinin, a group of carotenoid-derived terpenoid lactones, coined strigolactones, have been described to play a role in the regulation of root architecture. The influence of strigolactones on the lateral root density (LRD), adventitious root formation, and induction of root hair elongation has been demonstrated, but the molecular networks ruling these belowground effects are still not well understood (1–7).  

Multiple research teams have contributed to a better understanding of the strigolactone biosynthesis pathway, early signaling processes, and transport mechanisms (8–14). Early signaling occurs mainly through the action of an *α/β*-hydrodrolase DWARF14 (D14)/DECREASED APICAL DOMINANCE2 (DAD2) that interacts with an F-box protein, MORE AXILLARY GROWTH2 (MAX2) (15). MAX2 together with an additional *α/β*-hydrodrolase and a D14 paralog, KARRIKIN INSENSITIVE2 (16).  

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1 The abbreviations used are: C4H, cinnamate-4-hydroxylase; CFI, chalcone flavone isomerase; CHS, chalcone synthase; D14, DWARF14; DAD, DECREASED APICAL DOMINANCE; DPBA, diphenylboric acid 2-amino ethyl ester; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; GO, gene ontology; HPLC, high-performance thin-layer chromatography; hpt, hours post treatment; HTL, HYPOSENSITIVE TO LIGHT; KAI, KARRIKIN INSENSITIVE; LRD, lateral root density; LTQ, linear trap quadrupole; MAX, MORE AXILLARY GROWTH; MS, Murashige and Skoog; NAP, nonintrinsic ABC protein; PAL, phenyl ammonia-lyase; PN, PIN-FORMED; rac-GR24, strigolactone analog; UGT, UDP-glucosyl transferase; UPLC, ultra-performance liquid chromatography; WT, wild type.
vonol accumulation in the root. We used this knowledge to further dissect the link biased metabolite profiling experiment, revealed that MAX2–response to altered shoot branching (35, 37). That results in modified auxin flows in the stem and, finally, recycling at the plasma membrane in xylem parenchyma cells by the direct effect of strigolactones on PIN-FORMED1 (PIN1) max2–1 To this end, we adopted a mass spectrometry-driven, quan- into the intricate strigolactone signaling network in the roots. tion Columbia-0) plants were surface sterilized with consecutive (34). On the whole, only a few differen- tial expression data sets (30–33), strigolactone-regulated tran- scription factors and strigolactone-responsive genes are rare, of which BRANCHED1 (BRC1) is one of the best known in Arabidopsis thaliana (34). On the whole, only a few differen- tially expressed genes, often with low differences in expres- sion levels, were identified upon rac-GR24 treatment, a syn- thetic strigolactone analog (31–34). Of last, several studies have emerged that support strigolactone signaling occurring to a large extent at the protein level (29, 35, 36), as illustrated by the direct effect of strigolactones on PIN-FORMED1 (PIN1) recycling at the plasma membrane in xylem parenchyma cells that results in modified auxin flows in the stem and, finally, altered shoot branching (35, 37).

Here we executed a proteome-wide study to gain insight into the intricate strigolactone signaling network in the roots. To this end, we adopted a mass spectrometry-driven, quantitative proteomics approach to compare the profiles of the max2–1 mutant and wild-type (WT) Arabidopsis roots in response to rac-GR24. This procedure, in concert with an un- biased metabolite profiling experiment, revealed that MAX2- dependent and rac-GR24-induced changes in protein abundance give rise to specific changes in the root metabo- lome. We used this knowledge to further dissect the link between signaling pathways stimulated by rac-GR24 and flavonol accumulation in the root.

**EXPERIMENTAL PROCEDURES**

**Plant Material—** Seeds of Arabidopsis thaliana (L.) Heynh. (accession Columbia-0) plants were surface sterilized with consecutive treatments of 70% (v/v) ethanol with 0.05% (w/v) sodium dodecyl sulfate (SDS), and then washed with 95% (v/v) ethanol. For material destined to proteomics experiments or RNA preparation, seeds were sown on nylon meshes (20 μm) placed on half-strength Murashige and Skoog (MS) medium containing 1% (w/v) sucrose. Fifty seeds were sown per plate in two rows of 25 and were stratified for 2 days at 4 °C, whereafter the plantlets were grown for 5 days, before being transferred to mock-treated medium or medium containing 1 μM rac-GR24. For high-performance thin-layer chromatography (HPTLC) analysis, seeds were stratified for 2 days at 4 °C, whereafter the plantlets were grown for 5 days either on mock or rac-GR24-containing medium before methanol extraction. All plants were grown at 21 °C under permanent light conditions.

The rac-GR24 that was used for the proteome and the metabolite profiling contained both the GR24DS (GR24+) and GR245DS (GR24−) enantiomers (18). In experiments designed to test the effect of the stereochemistry on the flavonol response, purified enantiomers, GR24+ and GR24−, were applied separately.

**Time-Resolved Quantitative Proteomics—** The roots of 5-day-old Arabidopsis WT and max2–1 plants were transferred to MS medium containing 1% (w/v) sucrose and either 1 μM rac-GR24 or 100 μl of the acetone carrier, harvested, and snap-frozen in liquid nitrogen at given time points. Tissues were thawed in 1.5 ml extraction buffer (1% (v/v) CHAPS, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS, 5 mM EDTA, 10% (v/v) glycerol in phosphate buffered saline, pH 7.5) and a protease inhibitor mixture according to the manufacturer’s instructions (Roche Diagnostics, Vilvoorde, Belgium). Lysates were incubated for 30 min on ice before centrifugation at 16,000 × g for 20 min at 4 °C to remove any debris. Samples were desalted over a NAP-10 column (GE-Healthcare, Little Chalfont, UK) with 1 ml of 20 mM triethylammonium bicarbonate buffer. Protein concentrations were measured with the Bradford DC assay (Bio-Rad, Hercules, CA, USA) to keep 400 μg of protein material for the following steps. Samples were digested with endoproteinase-LysC (Sigma-Aldrich, St. Louis, MO) and incubated overnight at 37 °C with gentle agitation.

Because of the reference pool design, samples were divided into two equal parts. One half of each sample was pooled together to produce a reference sample and the other half was maintained to represent the sample itself. The samples were labeled differentially: the reference pool with heavy 13C3-propionate and the individual samples with light 12C3-propionate as described (38). Labeling was followed by quenching of N-hydroxysuccinimide esters with 40 mM glycine to remove excess NHS esters, followed by 80 mM hydroxyl-amine (NH2OH) to revert O-propionylation of Ser (S), Thr (T), and Tyr (Y). Individual samples were mixed in a one-to-one ratio with the reference pool (checked on a single shot pre-run on a XL linear trap quadrupole (LTQ) Orbitrap (Thermo Fisher Scientific, Waltham, MA)).

**RP-HPLC Fractionation of Peptide Mixtures—** Peptides were separated on a 2.1 mm internal diameter (I.D.)×150 mm column (Zor- bax®, 300 SB-C18 Narrowbore, Agilent Technologies, Santa Clara, CA) preceded by a C8 pre-column. A 140-min gradient was used with HPLC solvent A, consisting of 10 mM ammonium acetate (pH 5.5) in a 2% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid. The obtained peptide mixtures were intro- duced into the Ultimate 3000 RSLC nano LC-MS/MS system (Dionex, Sunnyvale, CA) connected in-line to a hybrid LTQ Orbitrap Velos (Thermo Fisher Scientific). The sample mixture was loaded on an in-house-made trapping column (100 μm I.D. × 20 mm, 5-μm C18 Reprosil-HD beads (Dr. Maischi)). After back-flushing from the trap-
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The sample was loaded on an in-house-made analytical column (75 μm I.D. × 150 mm, 5-μm C18 Reprosil-HD beads (Dr. Maisch, Ammerbuch-Entringen, Germany)). Of the peptide mixture, 6 μL was loaded with solvent A' and separated with a linear gradient from 2% (v/v) solvent A' (0.1% (v/v) formic acid) to 50% (v/v) solvent B' (0.1% (v/v) formic acid and 80% (v/v) acetonitrile) at a flow rate of 300 nL/min followed by a wash with 100% solvent B'.

LC-MS/MS Analysis and Peptide Identification—The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the 10 most abundant peaks in a given MS spectrum. In the LTQ-Orbitrap Velos (Thermo Fischer Scientific), full-scan MS spectra were acquired at a target value of 1E6 with a resolution of 60,000. The 10 most intense ions were isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 20 s. Peptides were fragmented after filling the ion trap at a target value of 1E4 ion counts. The MS/MS spectra were searched with the MaxQuant software (version 1.4.0.3) (39, 40) against The Arabidopsis Information Resource (TAIR10_pep_20101214 containing 27,416 protein coding genes) database, with a precursor mass tolerance set at 10 ppm for the first search (used for nonlinear mass recalibration) and at 4.5 ppm for the main search. Methionine oxidation was searched as fixed modification, whereas variable modifications were set for pyroglutamate formation of amino-terminal glutamine and acetylation of the protein N terminus. Mass tolerance on peptide precursor ions was set at 10 ppm and on fragment ions at 0.5 Da. The peptide charge was set to 2+–3+.

Endoprotease-LysC was the selected protease, with one missed cleavage allowed; cleavage was accepted as well when lysine was followed by proline. Only peptides were withheld that ranked first and scored above the 99% confidence threshold score. 13C3-proline and 12C3-propionate were used as heavy and light labels, respectively, with specificity for lysines and peptide N termini. The feature “matching between runs” was activated. The false discovery rate (FDR) for peptide and protein was set to 1% and the minimum peptide length was 7. All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the PRIDE accession PXD003879. The results can be accessed through MS-Viewer (41) on the Protein Prospector website (http://prospector2.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mview) with the Search Key: lsiutdkzh.

Statistical Analysis of the Shotgun Proteomics Data—We first applied a stringent filter to the data set, keeping only protein for which at least two valid values were available from the four biological repeats for every condition tested. The remaining 1,968 proteins were analyzed by fitting a linear mixed model of the following form:

\[ Y_{ijkl} = \mu + \alpha_i + \gamma_j + m_{ki} + t_{ij} + g_{mk} + g_{lj} + m_{ki} + g_{mk} + e_{ijkl} \]  

(1) partitioning the variation in protein abundances \( Y_{ijkl} \) into fixed genotype effects (WT and max2 represented by \( g \)), treatment effects (mock and strigolactone represented by \( m \)), time effects (0 h, 9 h and 24 h for mock, 9 h and 24 h for strigolactone represented by \( t \)) and all interaction effects, and random block effects, referring to the biological replicates. The genotype*treatment interaction effect is one of the highest importance because it assesses whether the difference in response between the two genotypes is affected by the treatment (averaged over the time series). Random block effects in the model were assumed to be independent and normally distributed with means zero and variance \( \sigma^2 \). The linear mixed model was fitted by the residual maximum likelihood (REML) approach as implemented in Genstat v17 (For details see Payne, R.W. (2013) Genstat Release 17 Reference Manual, Part 3: Procedure library PL24. Oxford: VSN International, Hemel Hempstead, UK). Significance of the fixed main and interaction effects was assessed by an F-test.

The distributions of the \( p \) values for the treatment effect, the genotype effect and the genotype*treatment effect were assessed. Only for the genotype effect we have estimated the FDR to correct for multiple hypotheses testing, considering the large number of proteins significant for this term.

Metabolite Profiling: LC-MS Conditions—For the LC-MS analysis, an Acquity Ultra-Performance Liquid Chromatography (UPLC) system was used connected to a Synapt Q-TOF high-definition MS system (Waters, Milford, MA). Chromatographic separation was done by injecting a 15-μL aliquot on an Acquity BEH C18 column (2.1 mm I.D. × 150 mm, 1.7 μm beads; Waters) with a gradient elution. Mobile phases consisted of water containing 1% (v/v) acetonitrile and 0.1% (v/v) formic acid (A) and acetonitrile containing 1% (v/v) water and 0.1% (v/v) formic acid (B). All solvents used were ULC/MS grade (Biosolve Chemie, Dieuze, France). Water was produced by a Direct-Q-UV water purification system (Millipore). The column temperature was maintained at 40 °C and the autosampler temperature at 10 °C. A flow rate of 350 μL/min was applied during the gradient elution starting at time 0 min 5% (B), 30 min 50% (B), and 33 min 100% (B). The eluant was directed to the mass spectrometer equipped with an electrospray ionization source and lock spray interface for accurate mass measurements. MS source parameters were: capillary voltage 2.5 kV, sampling cone 37 V, extraction cone 3.5 V, source temperature 120 °C, desolvation temperature 400 °C, cone gas flow 50 L/h, desolvation gas 550 L/h. The collision energy for trap and transfer cells was set at 4 V and 3 V, respectively. For data acquisition, the dynamic range enhancement mode was activated. Full-scan data were recorded in negative centroid V-mode with a mass range between \( m/z \) 100–1000 and a scan speed of 0.2 s/scan by means of the Masslynx software (Waters). Leu-enkephalin (400 pg/μL solubilized in water/acetonitrile (1:1, v/v)) acidified with 0.1% (v/v) formic acid) was used for the lock mass calibration by scanning every 10 s with a scan time of 0.5 s; three scans were averaged. For MS/MS purposes, the same settings were applied, except that the trap collision energy was ramped from 10 V to 45 V.

For the LC-MS data processing, the Progenesis QI software v 2.0 (Nonlinear Dynamics, Durham, NC) was used to align all chromatograms and to analyze statistically the ArcSinh-transformed compound intensities (normalized to dry weight) through principal component analysis and analysis of variance (p value threshold = 0.01). Descriptive statistics were calculated by EZInfo extension (v 3.0) (Umetrics, San José, CA) on Pareto-scaled compound intensities.

RNA Extraction and Quantity (q)RT-PCR—Roots from WT and max2–1 plants were harvested and snap-frozen in liquid nitrogen 24 h post treatment (hpt). Cell walls were disrupted by 3-mm metal beads in 2-ml tubes (Eppendorf, Hamburg, Germany) with a mixer mill 400 (Retsch, Haan, Germany) for 2 min at 20 Hz. RNA was extracted and purified with the RNAeasy mini kit (Qiagen, Hilden, Germany). Genomic DNA was removed by DNase treatment and the samples were purified by ammonium acetate (2.5 M final concentration) precipitation. Concentrations were measured with a ND-1000 Spectrophotometer (Thermo Fisher Scientific Nanodrop). The iScript cDNA synthesis kit (Bio-Rad) was used to reverse transcribe RNA. qRT-PCR primers were designed with the Quant Prime website software. SYBR Green detection was used during qRT-PCR run on a Light Cycler 480 (Roche Diagnostics). Reactions were done in triplicate in a 384-multwell plate, in a total volume of 5 μL and cDNA fraction of 10%. Cycle threshold values were obtained and analyzed with the 2−ΔΔCT method (42). The values from four biological repeats and three technical repeats were normalized against those of ACTIN2 (ACT2, AT3G18780) that was used as an internal standard. Normalized val-
ues were analyzed according to the published model (43) with the mixed model procedure, Kenilworth, NJ (SAS Institute, Cary, NC).

**HPTLC Diphenylboric Acid 2-Amino Ethyl Ester (DPBA) Staining**—For the HPTLC analysis of roots, methanol extracts from four biological repeats were prepared from 5-day-old Arabidopsis plants grown on 1% (v/v) MS medium containing either 1/30 M rac-GR24 or 100 μl of the acetone carrier. Roots were harvested. After a methanol extraction, samples were dried with a concentrator 5301 (Eppendorf). The dried samples were resuspended in 20 μl of an 80% (v/v) methanol solution. The concentrated extract was analyzed by HPTLC. Of the mixture, 2 μl was spotted onto a 20 cm × 10 cm silica-60 HPTLC glass plate (Merck) and placed in a glass tank with a Whatman paper wick of 18 cm by 9 cm (Thermo Fisher Scientific) and a mobile polar phase consisting of ethyl acetate, dichloromethane, acetic acid, formic acid, and water in a 100:25:10:10:11 ratio, respectively. After addition of the mobile phase, the glass tank was sealed with silicone grease and gels were run for 25 min. Gels were stained by spraying a methanol solution containing 1% (v/v) DPBA. Plates were placed into an HB-1000 Hybridizer (Thermo Fisher Scientific) at 100 °C for 10 min, whereafter the plates were sprayed with a 5% (v/v) methanol solution containing 4000-polyethylene glycol to stabilize the DPBA compound. Plates were observed after UV excitation at 350 nm. Pictures were taken with a D90 camera (Nikon, Tokyo, Japan).

**RESULTS**

**Proteome Profiling Reveals Differences Between WT and max2–1 Roots upon rac-GR24 Treatment**—To gain insight into the rac-GR24-induced signaling pathway and the role of MAX2 in the roots, we used max2–1 and WT Arabidopsis (accession Columbia-0) roots to study differences in protein abundance by means of a time-resolved, quantitative proteomics approach. Five-day-old plants were transferred to control (mock) medium or medium containing 1 μM rac-GR24 or 100 μl of the acetone carrier. This experiment was conducted in four biological replicates (Fig. 1).

A reference pool was created by mixing half of each digested proteome extract and labeling the resulting peptide pool with 13C3-propionate tags. The peptides of the individual samples were labeled with 12C3-propionate. Spectra were subsequently searched and analyzed with MaxQuant and Perseus. Quantified proteins were filtered and only those that had valid values for at least three of the four biological repeats of each sample were retained for final analysis.
Individual sample had been mixed with an equal amount of the reference pool, the peptides were prefractionated by RP-HPLC to reduce the sample complexity prior to LC-MS/MS analysis (Fig. 1). This set-up enabled the identification of proteins of which the abundances depended on MAX2, the rac-GR24 treatment, or both.

In total, 4,260 proteins were identified and quantified. To increase the stringency of our analysis, we kept only proteins that are significantly different with a p-value < 0.01 (red dots) in at least one of the terms are presented.

**FIG. 2.** Differential molecular responses in max2–1 and WT roots either mock grown or treated with rac-GR24. Heat map showing average log2 values of protein ratios of four biological repeats (z-scored for graphical representation). Levels are shown for WT and max2–1 plants grown under mock conditions (0, 9 h and 24 h) or after treatment (9 h and 24 h) in the presence of 1 μM rac-GR24. The columns labeled G, T, and T*G indicate whether the protein abundance was detected as statistically significantly different between genotypes, treatment, and interaction of both factors, respectively. Only the proteins that are significantly different with a p value < 0.01 (red dots) in at least one of the terms are presented.
with valid quantification values in at least two of the four biological replicates for every condition tested. As a result, a subset of 1,968 proteins was retained and subsequently a linear mixed model was fitted to the log-transformed data to assess the genotype (WT and max2–1) and treatment (mock, strigolactone) main effects and the genotype.treatment interaction on protein abundance. Fig. 2 shows all proteins for which at least one of the terms (genotype, treatment, or their interaction) was significant with $p < 0.01$ (red dots). All ratio values for these proteins are given in supplemental Table S1.

In total, 33 proteins at $p$ value $< 0.01$ differed significantly in abundance after rac-GR24 treatment, whereas 117 ($p$ value $< 0.01$) were differentially abundant when the root proteomes of max2–1 and WT plants were compared (Fig. 2). Finally, the interaction between treatment and genotype had a statistically significant effect on the abundance of 9 ($p$ value $< 0.01$) proteins (Fig. 2). Ratios of all proteins as well as $p$ values (when the proteins were included in the statistical analysis), are given in supplemental Table S2.

Upon examination, four out of the nine proteins that have a significant interaction term (genotype.treatment) have been shown to be involved in different steps of flavonoid biosynthesis. For three of these proteins, phenyl ammonia-lyase (PAL1), CFI family protein and flavanone 3’-hydroxylase (F3’H), their abundance increases only in the WT upon rac-GR24, suggesting that a functional MAX2 protein is necessary for this change to occur (Fig. 3). More broadly, multiple proteins involved in the flavonoid metabolism are significant for the genotype term, including PAL2, and enzymes more specifically involved in flavonoid biosynthesis and transport, such as flavonol synthase 1 (FLS1), flavanone 3-hydroxylase (F3H), chalcone synthase (CHS), UDP-glucosyl transferase 78D2 (UGT78D2), cinnamate-4-hydroxylase (C4H), and the nonintrinsic ABC protein 9 (NAP9). These proteins were more abundant in WT than in max2–1 roots. Taken together, these results suggest that in the absence of a functional MAX2, a large set of enzymes responsible for flavonol biosynthesis are less present and that at least for some of these enzymes, their abundance increases in a MAX2-dependent manner upon rac-GR24 treatment.

**Transcript Analysis Reveals a MAX2-Dependent rac-GR24-Induced Regulation of Genes Coding for Flavonoid Biosynthesis Enzymes**—With a detected enrichment for proteins involved in phenylpropanoid and, more specifically, flavonoid synthesis, we wanted to investigate whether these changes between genotype and/or upon rac-GR24 treatment were regulated at the transcript level. WT and max2–1 roots grown in the presence or absence of rac-GR24 were used to study the gene expression of markers for phenylpropanoid and flavonol biosynthesis, such as enzymes catalyzing early steps of the phenylpropanoid pathway (PAL1 and PAL2) and proteins more specifically involved in flavonol biosynthesis (CHS, UGT78D2, and F3’H) via qRT-PCR analysis. For all genes tested, no differences in expression levels were detected when untreated WT and max2–1 samples were compared (Fig. 4). In contrast, the transcript levels of all tested genes increased statistically significantly (Student’s t test with $p < 0.05$) upon rac-GR24 treatment in WT background, a response that was completely abolished in the max2–1 mutant. These results indicate that the flavonoid biosynthesis pathway is transcriptionally activated by rac-GR24 treatment in a MAX2-dependent manner.

**Secondary Metabolite Profiling Pinpoints Specific Flavonols to Accumulate upon rac-GR24 Treatment in a MAX2-Dependent Manner**
dent Manner—As rac-GR24 treatment and MAX2 function appeared to regulate enzymes involved in flavonoid biosynthesis and, more generally, phenylpropanoid biosynthesis, at the transcript and protein levels, metabolite profiling experiments were conducted. In a first experiment, methanol extracts from the roots of WT plants grown on mock or rac-GR24-containing medium were compared and, in a second experiment, metabolite profiles of untreated root tissues of WT and max2–1 plants were evaluated (Fig. 5A). Methanol extracts were analyzed via Ultra-HPLC-MS (for details, see Experimental Procedures).

In total, 1,121 compound ions were detected in experiment 1 (Fig. 5A). Prior to univariate analysis, two filters were applied to increase the stringency. An intensity threshold of 500 spectrum counts in at least one group and an average peak width threshold of minimum 0.05 min in at least one group were applied, resulting in 474 remaining compound ions. By Student’s t test analysis and multiple testing corrections, 93 and 48 compound ions were found to be significantly more and less abundant, respectively, in WT upon rac-GR24 treatment (Fig. 5A). A principal component analysis (Fig. 5B) was carried out and showed a separation between two groups, indicating that plants grown on mock-treated medium or on rac-GR24-supplemented medium had different phenolic profiles.

In the second experiment, 1,512 compound ions were detected. With the same filters as in experiment 1, 701 compound ions remained for univariate analysis. The Student’s t test analysis indicated that 134 and 167 compound ions were significantly more and less abundant, respectively, in max2–1 mutants. The second principal component analysis (Fig. 5B) showed difference in the phenolic profiles of max2–1 and WT roots grown under mock conditions.

After manual fragment ion clean-up and assessment of numbers and types of compound ions that displayed a MAX2-dependent and rac-GR24-induced profile, 28 compounds were retained from the two combined experiments (supplemental Table S3) that could be structurally characterized based on MS/MS fragmentation (Fig. 5A). Nine compounds could be classified as phenylpropanoids, such as several glycosyl derivates of p-coumaric acid, caffeic acid, and ferulic acid, two as guaiacylglycerol-β-ferulic acid ethers, 11 as flavonols, and one as flavanone naringenin (supplemental Table S3; Fig. 5A). Regarding the flavonols, derivates from each of the three main flavonol families, kaempferol, quercetin, and isorhamnetin, accumulated in WT roots upon rac-GR24 treatment and were less abundant in max2–1 mutants than in WT plants (supplemental Table S3). These phenolic profiling results indicate that rac-GR24 treatment gives rise to a MAX2-mediated flavonol accumulation in Arabidopsis roots, in line with the results from the transcriptional analysis (Fig. 4).
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A New Flavonol Readout to Dissect Strigolactone Signaling—To confirm the MAX2-dependent rac-GR24 metabolic response in roots, we separated methanol extracts on HPTLC, followed by flavonol-specific DPBA staining and UV/VIS spectrophotometry. Firstly, to independently confirm the large-scale metabolome analysis, new methanol extracts were prepared from roots of WT and max2–1 plants grown with or without rac-GR24. Rac-GR24 treatment of WT roots resulted in the accumulation of compounds stained mainly orange and blue, corresponding to quercetin and kaempferol derivates, respectively (Fig. 6A). Furthermore, this rac-GR24-triggered flavonol accumulation was abolished in the max2–1 mutant background (Fig. 6A), confirming the UHPLC-MS data that revealed an increase in flavonol production upon rac-GR24 treatment in roots.

The applied rac-GR24 consisted of two enantiomers, GR24DS (GR24+) and GR24ent-SDS (GR24–), thought to mimic naturally occurring strigolactones and potentially karrikins or other unknown compounds, respectively (18). Next, the specificity of the observed flavonoid response to one of the two enantiomers was evaluated. Flavonols accumulated after treatment with both 1 μM GR24+ or 1 μM GR24– in roots of 5-day-old plants (Fig. 6B). Additionally, the roles were examined of the two receptor proteins D14 and HTL/KAI2 that can mediate the response to rac-GR24 (17) in the observed strigolactone response. The d14 mutant still accumulated...
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![Visualization of flavonol induction on HPTLC plates.](image)

**Fig. 6. Visualization of flavonol induction on HPTLC plates.** A, HPTLC plate with DPBA-stained methanol extracts from WT and max2–1 roots treated with rac-GR24 or not. B, HPTLC plate with DPBA-stained methanol extracts from a mock-treated WT root and grown either with 1 μM GR24+ or 1 μM GR24-. C, HPTLC plate with DPBA-stained methanol extracts from a mock-treated WT root and d14 mutant roots grown either with 1 μM GR24+ or 1 μM GR24-. D, HPTLC plate with DPBA-stained methanol extracts from a mock-treated WT (accession Wassilewskija (Ws)) root and a htl-1 mutant root grown either with 1 μM GR24+ or 1 μM GR24-.

flavonols in response to rac-GR24 (Fig. 6C) as did the htl-1/ kai2 mutant, available in the Arabidopsis Wassilewskija (Ws) accession and responding similarly as the Ws control (Fig. 6D). Taken together, these results show that the uncovered flavonol response is common to both rac-GR24-containing enantiomers and can be induced both through D14 and/or KAI2.

**DISCUSSION**

With the present study, we shed more light on the processes that are at play downstream of the rac-GR24 perception and underline the multiplicity of roles played by MAX2 in the roots of Arabidopsis. A protein profiling approach led to the identification of 4,260 proteins in the root proteome in four biological replicates. By means of a linear mixed model analysis of variance, a total of 147 proteins displayed a statistically significant difference in abundance (p value < 0.01), either when max2–1 and WT root proteomes were compared, upon rac-GR24 treatment, or because of the interaction of both genotype and treatment.

Interestingly, our data set of significantly regulated proteins presented a clear enrichment for phenylpropanoid/flavonoid metabolism-related proteins, which we further explored via transcriptional and metabolome analyses. For several of the genes encoding these enzymes, qRT-PCR data revealed a MAX2-dependent increase in transcript levels upon rac-GR24 treatment. Accordingly, metabolome analysis confirmed the rac-GR24-induced accumulation of flavonols requiring a functional MAX2. As flavonol compounds are known to be stress responsive in some cases (44), it is imperative to underline the MAX2-dependent character of this response, hinting at a specific response to rac-GR24 and ruling out the possibility that merely a general stress response is observed. On the metabolite level, 11 flavonols, one direct flavonol precursor, narigenin and, more generally, nine phenylpropanoids displayed a MAX2-dependent increase in response to the rac-GR24 treatment, supporting a clear link between strigolactones and flavonols in the root. Previously, a rac-GR24-triggered induction of CHS expression, comparable to the one described here, had been observed in whole seedlings (45), implying that flavonol might accumulate in different plant tissues. Accordingly, a transcriptome analysis has revealed that flavonol biosynthesis genes are induced at lower levels in max2–1 than in WT upon drought stress in leaves (31). Moreover, flavonol production has been shown to be misregulated in the strigolactone biosynthesis mutant max1 in the shoot (46). However, because mutants affected in flavonol biosynthesis have no enhanced branching phenotype, flavonols probably do not play a main role in strigolactone-controlled shoot branching (47). As flavonol accumulation and aspects of the root architecture have been linked (48–50), the next challenge will be to examine the role of flavonols in rac-GR24-affected processes in the root.

We have translated the connection between strigolactones and flavonols in the root into a cost-effective and user-friendly HPTLC tool that allowed us to acquire more insight into the rac-GR24 signaling pathways. Recently, the use of rac-GR24 as a generic strigolactone analog has been questioned, because rac-GR24 is actually a mixture of two enantiomers. Whereas GR24+ mimics natural strigolactones and is perceived via D14, GR24- is active via the KAI2 receptor and represents a noncanonical strigolactone analog. Importantly, both enantiomers have been shown to signal via MAX2. In this context, some strigolactone-related phenotypes have been linked to specific stereo-isomers of rac-GR24 or specific receptors, although these observations were not absolute (18). On the one hand, shoot branching is elicited by GR24+ via D14 signaling, whereas on the other hand, GR24- and KAI2 affect hypocotyl elongation and aberrant cotyledon morphology (18, 25). Therefore, we tested whether the flavonol
response was specific to an enantiomer receptor pair. The application of the specific enantiomers revealed that both GR24+ and GR24- could increase the flavonol production. In addition, both the d14 and kai2 signaling mutants were examined for their capacity to transduce the rac-GR24 and give rise to the flavonol read out. In agreement with the enantiomer experiment, the flavonol induction was maintained in both mutants. Together, these results imply that the rac-GR24-induced and MAX2-controlled flavonol production is not stereo-selective and, hence, can occur upon activation of either D14 or KAI2. This observation suggests that, at least in the roots, a crosstalk exists between D14 and KAI2 pathways and raises the question whether other known root phenotypes can also be instigated by both receptors.

Besides flavonols, our data indicate that also other secondary metabolites could contribute to strigolactone-mediated effects in Arabidopsis roots. Several antioxidant phenylpropanoids, sharing p-coumaric acid as a precursor, accumulate with the same strigolactone-related abundance profiles as flavonols. From the proteomics results, we can infer that this effect might be caused by a change in production of CINNAMATE 4-HYDROXYLASE (C4H), the enzyme producing this effect might be caused by a change in production of this compound. Moreover, two hexosylated G(8-O-4)ferulic acid compounds were found to accumulate similarly as the flavonols. The in planta function of these neo lignan-like compounds is unknown, but we can postulate that their accumulation is the consequence of an increase in (hexosylated) ferulic acid.

Previously, a comparable proteome analysis in the context of strigolactone signaling had been conducted (36). Only a limited overlap could be observed with our data (10% at the protein level), potentially arising from technical differences. We used a 5-fold lower concentration of rac-GR24 and sampled roots in contrast to whole plants. Nevertheless, a more attractive explanation is also plausible: we used the signaling mutant max2–1 instead of the biosynthesis mutant max3. Thus, the previous approach focused on proteome changes upon signaling of natural strigolactones (36), whereas our work spans an enlarged signaling network, uncovering all downstream effects of MAX2. In this context, it is important to note that the role of MAX2 is broader than strigolactone signaling alone and also to encompass signal transduction of unknown molecules (18, 51). Although not yet biochemically characterized, additional MAX2 activity elicitors are expected to exist based on genetic studies, as illustrated by the increase in LRD in the max2–1 mutant, which is not phenocopied in the max3 and max4 mutants, despite their inability to synthesize strigolactones (1, 2).

Additionally, we detected a set of proteins that responded to rac-GR24, both in the max2–1 background and in the WT control, possibly pointing toward the existence of a MAX2-independent response to strigolactones. In agreement, MAX2-independent responses in root growth and development to rac-GR24 were reported (2, 35).

In conclusion, the large set of proteins shown to be regulated by the MAX2 function provides a comprehensive resource that can serve as a foundation for studies aiming to elucidate the roles of MAX2 in roots. Finally, the link between strigolactones and flavonols will allow the dissection of the molecular networks that act between strigolactone signaling and the induction of transcriptional changes.

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