Grass roots chemistry: meta-Tyrosine, an herbicidal nonprotein amino acid

Cécile Bertin1†, Leslie A. Weston1, Tengfang Huang1, Georg Jander4, Thomas Owens5, Jerrold Meinwald6†, and Frank C. Schroeder7¶

Departments of 1Horticulture, 2Plant Biology, and 3Chemistry and Chemical Biology and 4Boyce Thompson Institute, Cornell University, Ithaca, NY 14853

Contributed by Jerrold Meinwald, July 31, 2007 (sent for review May 28, 2007)

Fine fescue grasses displace neighboring plants by depositing large quantities of an aqueous phytotoxic root exudate in the soil rhizosphere. Via activity-guided fractionation, we have isolated and identified the nonprotein amino acid m-tyrosine as the major active component. m-Tyrosine is significantly more phytotoxic than its structural isomers o- and p-tyrosine. We show that m-tyrosine exposure results in growth inhibition for a wide range of plant species and propose that the release of this nonprotein amino acid interferes with root development of competing plants. Acid hydrolysis of total root protein from plant species and propose that the release of this nonprotein amino acid interferes with root development of competing plants. Acid hydrolysis of total root protein from Arabidopsis thaliana showed incorporation of m-tyrosine, suggesting this as a possible mechanism of phytotoxicity. m-Tyrosine inhibition of A. thaliana root growth is counteracted by exogenous addition of protein amino acids, with phenylalanine having the most significant effect. The discovery of m-tyrosine, as well as a further understanding of its mode(s) of action, could lead to the development of biorational approaches to weed control.

Results and Discussion

In an initial field evaluation of 80 fine fescue cultivars, 8 cultivars with strong weed suppressive potential were identified and their allelopathic potential in laboratory settings was confirmed (4). Based on both field and laboratory results, we selected “Intrigue,” a common Chewing’s fescue cultivar (Festuca rubra L. ssp. commutata), for further studies.

To identify the allelopathic compound(s) contained in Intrigue root exudates, we developed an activity-guided separation scheme based on the inhibition of lettuce (Lactuca sativa L.) radicle elongation in a filter paper-based assay. By using this assay, we compared the phytotoxicity of root surface washes (hexanes, dichloromethane, methanol, and water) prepared from 2-week-old Intrigue seedlings grown under soil-free conditions. The aqueous root wash, which showed the strongest inhibition of lettuce root growth, was selected for further fractionation (Fig. 2) by reverse-phase column chromatography on C18-coated silica gel, followed by size exclusion chromatography with Sephadex LH20 beads. At all stages, biological activity was monitored by using the same filter paper assay.

More than 80% of the resulting active fraction consisted of one major component, which was characterized without additional purification via a standard set of two-dimensional NMR spectra, including DQF-COSY, (1H,13C)-heteronuclear multiple-quantum correlation spectra, and (1H,13C)-heteronuclear multiple-bond correlation spectra (6). The NMR-spectroscopic data suggested a 3-substituted phenylalanine derivative as the structure of the major component. Additional analyses by high-resolution positive-ion electrospray mass spectrometry yielded a molecular formula of C9H11NO3. In combination with the results from UV and infrared spectroscopic analysis, these data indicated that 3-hydroxyphenylalanine, commonly known as m-tyrosine, is the major component of the active fraction isolated from the root exudates. This structural assignment was confirmed via an NMR-spectroscopic mixing experiment, whereby a small amount of synthetic m-tyrosine was added to the isolated active fraction (7). Finally, the absolute configuration of the isolated m-tyrosine was determined to be L by NMR-spectroscopic comparison of its [(S)-methoxytrifluoromethylphenyl]acetic acid [(S)-MTPA] derivative with the (R)- and (S)-MTPA derivatives of synthetic m-tyrosine (8).

Abbreviations: MTPA, methoxytrifluoromethylphenylacetic acid; MS medium, Murashige and Skoog medium.

See Commentary on page 16729.

†To whom correspondence may be addressed. E-mail: circe@cornell.edu or fs31@cornell.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0707198104DC1.

© 2007 by The National Academy of Sciences of the USA
tyrosine accounted for the majority of observed toxicity (Fig. 3A). In assays using enantiomerically pure samples of m-tyrosine, the D and L enantiomers proved equally effective in inhibiting lettuce root growth, with the concentrations required to achieve 50% reduction of lettuce root growth (IC50) being 17 and 21 µM, respectively. Whereas lettuce roots exposed to m-tyrosine showed stunting and brownish, discolored roots, shoot growth of 5-day-old lettuce seedlings was only marginally affected (Fig. 3A Inset). This could indicate that roots are uniquely sensitive, or that m-tyrosine is not transported to the shoots. After 7 days of m-tyrosine treatment, lettuce seedlings showed a significant reduction in shoot growth, possibly as a result of insufficient root development. In contrast to the root growth inhibition by m-tyrosine, p-tyrosine and o-tyrosine actually stimulated lettuce root growth at concentrations as low as 50 µM (Fig. 3B).

Because our previous studies suggested that fine leaf fescue may impact growth of a wide range of plant species (12), we explored the m-tyrosine susceptibility of a selection of monocot and dicot plants by measuring root growth in a filter paper assay [Fig. 4 and supporting information (SI) Table 1]. The IC50 of m-tyrosine ranged from 10 to 260 µM for the tested species. Root elongation of fescue species that produce m-tyrosine, the D and L enantiomers proved equally effective in inhibiting root elongation (9).

- Analysis of aqueous extracts of root exudates from multiple fine fescue cultivars and related species showed that all Arizona fescue (F. arizonica), creeping red fescue (F. rubra ssp. rubra), and Chewing’s fescue (F. rubra ssp. commutata) cultivars produced large amounts of m-tyrosine, whereas hard (Festuca longifolia), sheep (Festuca ovina), and Idaho (Festuca idahoensis) fescues did not produce detectable amounts of m-tyrosine (9).
- m-Tyrosine has been detected in only one other plant species, Euphorbia myrsinitis (10), and we are not aware of any reports of this nonprotein amino acid in natural soils.

HPLC analyses showed that m-tyrosine constitutes 33–43% of the dry weight of Intrigue aqueous root exudate extract and is also an abundant metabolite in intact plant tissue. In 1-week-old seedlings, m-tyrosine was 10-fold more abundant in roots (6,500 pmol/mg wet weight; mean ± SD of n = 6) than in leaves (590 ± 160 pmol/mg wet weight; mean ± SD of n = 5). Free m-tyrosine was present at much lower concentrations in seeds (24 ± 8 pmol/mg; mean ± SD of n = 6), suggesting that the biosynthesis of this metabolite is initiated after germination. Because metabolite production is often highly dependent on plant age and environmental factors (11), the absolute amounts of m-tyrosine produced by the various fescue species and cultivars will vary with plant growth stage and in response to changes in growth conditions.

To determine whether the phytotoxicity of aqueous extracts of fine fescue root exudate can be attributed to m-tyrosine, the activity was compared with that of authentic DL-m-tyrosine solutions. By using an array of equivalent concentrations of aqueous root exudates extracts and DL-m-tyrosine, nearly identical dose–response curves were obtained, indicating that m-tyrosine accounted for the majority of observed toxicity (Fig. 3A). In assays using enantiomerically pure samples of m-tyrosine, the D and L enantiomers proved equally effective in inhibiting lettuce root growth, with the concentrations required to achieve 50% reduction of lettuce root growth (IC50) being 17 and 21 µM, respectively. Whereas lettuce roots exposed to m-tyrosine showed stunting and brownish, discolored roots, shoot growth of 5-day-old lettuce seedlings was only marginally affected (Fig. 3A Inset). This could indicate that roots are uniquely sensitive, or that m-tyrosine is not transported to the shoots. After 7 days of m-tyrosine treatment, lettuce seedlings showed a significant reduction in shoot growth, possibly as a result of insufficient root development. In contrast to the root growth inhibition by m-tyrosine, p-tyrosine and o-tyrosine actually stimulated lettuce root growth at concentrations as low as 50 µM (Fig. 3B).

Because our previous studies suggested that fine leaf fescue may impact growth of a wide range of plant species (12), we explored the m-tyrosine susceptibility of a selection of monocot and dicot plants by measuring root growth in a filter paper assay [Fig. 4 and supporting information (SI) Table 1]. The IC50 of m-tyrosine ranged from 10 to 260 µM for the tested species. Root elongation of fescue species that produce m-tyrosine was not affected by 20 to 160 µM synthetic m-tyrosine, whereas nonproducing fescue species were strongly sensitive (Fig. 4C).

Further experiments to characterize m-tyrosine toxicity were conducted with the model plant Arabidopsis thaliana (Arabidopsis). Whereas D- and L-m-tyrosine were equally toxic...
higher than that of m-tyrosine, but the overall phenotypic effects were similar. p-Hydroxyphenylpyruvate, the deaminated form of p-tyrosine, was nontoxic at concentrations up to 40 μM.

We have considered several mechanisms by which m-tyrosine could inhibit root growth, including direct interference with amino acid metabolism, inhibition of cell wall formation, and alteration of plant hormone signaling. In addition, we considered the possibility of m-tyrosine interfering with soil microbial ecology. However, growth of Escherichia coli, Bacillus cereus, Bacillus subtilis, and the soil fungus Metarhizium anisopliae was not inhibited at concentrations as high as 25 mM. Given the >1,000-fold higher susceptibility of Arabidopsis (and most other monocots and dicots), it seemed likely that m-tyrosine toxicity results from direct interference with plant metabolism.

Other root-deposited allelochemicals such as juglone and sorgoleone appear to interfere directly with photosynthesis and other redox processes in the plant cell or cell membrane. However, m-tyrosine affected neither photosynthetic efficiency nor chlorophyll production in lettuce seedlings, suggesting a different mode of action. The structures of both juglone and sorgoleone contain a quinonoid system, and their mode of action appears to be directly related to the chemical properties of the m-tyrosine structural feature (15). Although m-tyrosine could not serve as a biosynthetic precursor of a quinonoid amino acid, this is unlikely to play a significant role for its toxicity because o-tyrosine, which also is a potential precursor for the same para-quinone, is devoid of herbicidal activity (Fig. 3B and SI Fig. 6). Furthermore, L-DOPA, which could represent a downstream metabolite and intermediate in the conversion of m-tyrosine to a corresponding ortho-quinone, is at least 15-fold less toxic to Arabidopsis than m-tyrosine (data not shown).

Our observations that low concentrations of m-tyrosine inhibit the primary root and promote lateral root elongation in Arabidopsis and some lettuce isolates suggested that interference with plant growth hormones, in particular auxin (indole-3-acetic acid), could be a mechanism of m-tyrosine toxicity. Generally, high concentrations of auxin inhibit root growth, whereas very low concentrations stimulate root development (16). Furthermore, the chemical structures of m-tyrosine and especially its metabolite 3-hydroxyphenylpyruvic acid suggest that they could interfere with auxin-dependent growth regulation. However, expression of an auxin-responsive DR5-GUS fusion (17) was unaffected by m-tyrosine treatment, and six Arabidopsis auxin-response mutants (axr1-1, aux1-7, tgirl-1, axr1-3, axr2-1, and axr3-1) did not show altered sensitivity to m-tyrosine, suggesting that m-tyrosine does not interfere directly with auxin metabolism or activity.

Given the chemical structure of m-tyrosine, it seemed possible that this compound would interfere with plant amino acid metabolism. The toxicity of 3 μM DL-m-tyrosine for Arabidopsis root growth was counteracted to some extent by the addition of 14 of the 20 protein amino acids at 40 μM concentrations (Fig. 5B). Addition of charged amino acids caused little or no improvement in root growth, which may indicate that aromatic and neutral amino acids compete with m-tyrosine for uptake or transport within the roots. In control experiments, the protein amino acids by themselves did not significantly improve root growth at these concentrations (data not shown).

Although the pattern of amino acid rescue (Fig. 5B) is similar to the profile of amino acids that are substrates for the AAP1 transporter, two Arabidopsis land race Wassilewskija aap1 mutants (18) did not show elevated resistance to m-tyrosine (data not shown). However, the Arabidopsis genome encodes a large number of predicted and proven amino acid transporters, and it is possible that other such proteins play a nonredundant role in the uptake or within-plant movement of m-tyrosine. For instance, the recently identified LHT1 transporter, which contrib-
However, because significant growth inhibition is observed at >100-fold lower concentrations in Arabidopsis than in B. subtilis (23) or Chinese hamster ovary cells (24), plant proteins would have to be uniquely sensitive to m-tyrosine incorporation.

In summary, our results show that several fine fescue species release large amounts of m-tyrosine into the rhizosphere, and that this nonprotein amino acid functions as a broad-spectrum phytotoxin. Although several hundred nonprotein amino acids, including several with potential allelopathic properties, have been identified in plants (29), m-tyrosine is unique in being both root-exuded and inhibitory to root growth of other plants at low (micromolar) concentrations. Given the increasing public concern about the use of synthetic herbicides, there is great need for new approaches to weed management. Therefore, the identification of m-tyrosine as a naturally produced phytotoxin may contribute to the development of effective and more environmentally friendly weed management systems.

Materials and Methods

Plant Material and Chemicals. Seeds of fine leaf fescue cultivar Intrigue (F. rubra spp. commutata) were donated by Preferred Seed Company (Buffalo, NY). Other fescue seeds were obtained from Scott’s (Maryville, OH), Turf Merchants (Tangent, OR), Lebanon Seaboard (Lebanon, PA), and Seed Superstore (Buffalo, NY). Lettuce (L. sativa L.) seeds were purchased from Johnny’s Selected Seed (Winslow, ME). Seeds of A. thaliana land race Columbia-0 (Col-0) and mutant lines were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). The following weed seeds were purchased from Herbi-seed (Twyford, U.K.): dandelion (Taraxacum officinale Weber in Wiggers), large crabgrass (Digitaria sanguinalis L.), black medic (Medicago lupulina L.), cress (Lepidium sativum L.), barnyardgrass [Echinochloa crus-galli (L.) Beauv.], annual bluegrass (Poa annua L.), birdfoot trefoil (Lotus corniculatus L.), broadleaf plantain (Plantago major L.), mouse-ear chickweed (Cerastium vulgatum L.), common chickweed (Stellaria media L.), velvetleaf (Abutilon theophrasti Medicus), purslane (Portulaca oleracea L.), and white clover (Trifolium repens L.).

Seeds used for the capillary mat system or for Petri dishes bioassays were surface sterilized by suspension for 1 min in 5% ethanol, followed by three rinses with distilled water. Approximately 50 g of sterilized fine leaf fescue seeds were placed between two layers of wet cheesecloth (40 × 50 cm) arranged on the capillary mat system and grown for 14 days.

Method A. Roots were harvested by separation from the adjacent screen with a razor blade. The fresh roots were carefully submerged for 15 min in 200 ml of hexanes, 200 ml of methanol, 200 ml of dichloromethane, or 200 ml of water.

Method B. Uninjured roots were carefully rinsed with water by placing the entire mat system into a shallow water bath. After filtration, the exudate extracts obtained through these methods A or B were evaporated to dryness in vacuo by using a rotary evaporator at ambient temperature. The dried root exudate extracts were weighed and then stored at −20°C until further use.

Filter Paper Bioassays with Lettuce. Hexanes, dichloromethane, methanol, and aqueous fescue root exudate extracts were used in Petri dish bioassays to assess their effect on lettuce growth. Whatman no. 1 filter paper (Whatman, Middlesex, U.K.) in

Fig. 5. m-Tyrosine inhibition and amino acid rescue of Arabidopsis root growth. (A) Photograph of 5-day-old Arabidopsis seedlings exposed to a series of m-tyrosine concentrations. (B) Rescue of 3 μM m-tyrosine toxicity by 40 μM or individual amino acids or NH₄NO₃. Root length after 1 week of growth on MS agar with 3 μM m-tyrosine and other amino acids at 40 μM. Shown is mean ± SD of n = 16–23. * P < 0.01; t-test, relative to the 3 μM m-tyrosine-only treatment.
10-cm-diameter Petri dishes was treated with 1.0 ml of a solution consisting of 0.125, 0.25, 0.5, and 1.0 mg of root exudate extracts per milliliter of the solvent. To avoid toxic effects of the solvents, filter paper treated with hexanes, methanol, and methylene chloride extract solutions were placed in a fume hood for 1 h to allow complete solvent evaporation. Subsequently, 1.0 ml of water was added to each filter paper disk. Filter paper from which 1.0 ml of solvent had been evaporated before addition of 1.0 ml water, as well as filter paper with 1.0 ml of water alone, was used for control experiments. In additional experiments, the filter paper was treated with 1 ml of aqueous solutions of L-p-tyrosine, L-o-tyrosine, D-m-tyrosine, L-m-tyrosine, and DL-m-tyrosine at 10, 20, 40, 80, 160, and 320 μM concentrations. Ten lettuce seeds were placed on the moist paper filter in each Petri dish. After 5 days in a controlled environment (22°C, 45 μmol m^-2 s^-1 photosynthetic photon flux density), radicle and shoot length of experimental and control plants were measured.

Filter Paper Bioassays with Weed and Crop Species. The weed and crop species listed in the plant material section in SI Table 1, as well as the fescue species F. rubra ssp. commutata (Chewing’s fescue cv. Intrigue and Sandpiper), F. rubra ssp. rubra (creeping red fescue cv. Jasper) seeds, F. longifolia (hard fescue cv. Oxford), and F. ovina (sheep fescue), were tested for their sensitivity to m-tyrosine by using the filter paper assay described above. Seeds were sterilized with 50% ethanol as described above and were subsequently placed on filter paper treated with 0, 10, 20, 40, 80, or 160 μM DL-m-tyrosine (SI Table 1).

Agar Plate Bioassays with Arabidopsis. To assess effects of m-tyrosine and other compounds on Arabidopsis root growth, formulated solutions of each were added to half-strength Murashige and Skoog medium (0.5× MS) (31), 1% Phytagar (Invitrogen, Carlsbad, CA), and 1% sucrose in Petri dishes. Arabidopsis seeds were sterilized by shaking in 30% bleach, 0.3% Triton X-100 for 10 min, followed by three rinses with sterile distilled water. Petri dishes with seeds on agar medium were cold-stratified for 24 h at 4°C, and were subsequently placed vertically in Conviron (Winnipeg, MB, Canada) growth chambers at 23°C, 180 μmol m^-2 s^-1 photosynthetic photon flux density, and a 16:8 h light/dark cycle. After 5 days of growth, the root lengths of 10 seedlings per plate were measured. Experiments were repeated three times, and each replicate consisted of three agar plates. By using this assay, the effects of DL-m-tyrosine on Arabidopsis root growth were assessed at concentrations ranging from 0 to 320 μM (Fig. 3B). Rescue of 3 μM m-tyrosine toxicity was assessed by adding the 20 protein amino acids individually at 40 μM concentration to the assay. Phytotoxicity of L-, D-, and DL-m-tyrosine was compared at 0.25, 0.5, 1, 2, 5, and 10 μM concentrations. Toxicity of L-p-tyrosine, DL-o-tyrosine, and m-hydroxyinnamate and l-dopa (L-DOPA) was assessed at concentrations ranging from 1.25 to 40 μM.

Activity-Guided Fractionation of Aqueous Root Exudate Extracts and Chemical Analysis. Crude aqueous root exudate extracts (method A) were subjected to reverse-phase column chromatography on C18-coated silica gel, using a methanol–water solvent gradient for elution, increasing the methanol content from 0 to 100%. Three fractions were collected for the filter paper bioassay described above (SI Fig. 7). The aqueous fraction, which showed by far the strongest toxicity, was subjected to size-exclusion column chromatography on Sephadex LH20, using a 1:1 mixture of methanol and water as solvent. Ten fractions were collected, which were evaporated separately and then submitted to 1H NMR spectroscopic analysis using a 600 MHz Varian (Palo Alto, CA) INOVA spectrometer. Fractions with similar 1H NMR spectroscopic profiles were combined, which resulted in four fractions that were tested in the filter paper bioassay. One single fraction showed strong phytotoxic activity, whereas the other three fractions were inactive. The active fraction was subjected to a series of two-dimensional NMR-spectroscopic experiments, including phase-sensitive DQF-COSY, heteronuclear multiple-quantum correlation spectroscopy, and heteronuclear multiple-bond correlation (SI Table 2).

After NMR-spectroscopic analysis, the sample was subjected to mass spectrometry, using a Micromass (Manchester, U.K.) Quattro I tandem mass spectrometer operated in positive-ion electrospray mode with direct infusion of the sample dissolved in a 50:50 (vol/vol) solution of methanol and water containing 1% formic acid. Molecular mass calculated for C30H21NO3 (M+H)+ was m/z 182.07 and found was m/z 182.07.

Determination of the Absolute Configuration of Fescue-Produced m-Tyrosine. Reference samples of the (S)- and (R)-2-methoxy-2-trifluoromethyl-2-phenylacetic acid ([S]- and (R)-MTPA) derivatives of L-3′-hydroxyphenylalanine were prepared as follows. To a well stirred solution of 0.5 mg of L-3′-hydroxyphenylalanine in 0.5 ml of water at 0°C were added 0.5 ml of aqueous NaHCO3 solution, 1 ml of acetone, and 4 μl of either (R)- or (S)-2-methoxy-2-trifluoromethyl-2-phenylacetic acid chloride [(R)- and (S)-MTPA-Cl]. The resulting mixture was stirred for 1 h at 20°C. Subsequently, the acetone was evaporated in vacuo by using a rotary evaporator, and the aqueous residue was acidified by addition of 1 M aqueous HCl and extracted with 1 ml of ether. The organic extract was filtered over a pad of anhydrous Na2SO4 and evaporated to dryness in vacuo. The residue was dissolved in 0.6 ml of acetone-d6, and the resulting solution was analyzed by 1H-NMR spectroscopy. The diastereomeric (S)- and (R)-MTPA derivatives of L-3′-hydroxyphenylalanine showed significant differences in their 1H-NMR spectra. Characteristic signals include protons 3-Hα and 3-Hβ ([(R)-MTPA derivative [from (S)-MTPA-Cl] δ 3.09 ppm and 3.24 ppm; (S)-MTPA derivative [from (R)-MTPA-Cl] δ 3.02 ppm and 3.19 ppm). Subsequently, a portion of fescue-produced m-tyrosine (isolated from the aqueous fescue root exudate extracts) was reacted with (R)-MTPA-Cl in the same manner as described above. NMR-spectroscopic analysis of the resulting (S)-MTPA derivative produced an 1H-NMR spectrum showing signals for protons 3-Hα and 3-Hβ at 3.02 and 3.19 ppm, indicating that the root exudate fraction contained the L-isomer of 3′-hydroxyphenylalanine (m-tyrosine).

Determination of m-Tyrosine Concentrations. The m-tyrosine concentration in aqueous Intrigue root exudate extracts prepared according to methods A and B (see above) and in active fractions collected during purification was determined by HPLC analysis, using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) equipped with a diode-array detector and a Supelco (Bellefonte, PA) RP-18 Discovery column (length, 250 mm; diameter, 10 mm), which was eluted with methanol–water mixtures (starting with a mixture of 3% methanol, 67% water, and 30% of 0.05% aqueous trifluoroacetic acid for an initial period of 3 min, followed by a linear gradient reaching 50% methanol, 20% water, and 30% of 0.05% aqueous trifluoroacetic acid at 30 min, at a constant flow of 3.4 ml/min). In preparation for HPLC analysis, aqueous root exudate extracts were evaporated to dryness, and the residue was reconstituted at a concentration of 13 mg/ml water. Monitoring absorption at 280 nm, peaks at 12.7 min were integrated and compared against a calibration curve obtained from serial dilutions of commercial m-tyrosine in water. Aqueous root exudate extracts prepared according to methods A or B contained similar amounts of m-tyrosine.
Comparison of the Phytotoxic Activities of m-Tyrosine and Root Exudate Extracts. Aqueous solution of commercial m-tyrosine (10, 20, 40, 80, and 160 μM concentrations) or 1 ml of an aqueous solution of dried Intrigue root exudate extract adjusted to contain m-tyrosine at identical concentrations, as determined by HPLC, were spotted onto filter paper. Root growth of L. sativa and D. sanguinalis (10 seedlings per Petri dish) were measured after 5 days (L. sativa) or 7 days (D. sanguinalis) and compared with the lengths of root and shoot of control plants grown on plates treated only with 1 ml water. Each assay was performed in triplicate and repeated at three different times. Root growth inhibition was visually observed and measured. Analysis of variance using the general linear model procedure (release 9.1; SAS Institute, Cary, NC) was carried out on the data (lettuce and crabgrass root and shoot length), and the means were separated by least significant difference at the 0.05 level. Dose–response curves were fitted to the following four-parameter logistic function as follows: \[ f = \frac{b}{1 + e^{(c-d)/a}} \]
where \( b \) is the smallest value of the dependent variable (i.e., root or shoot length, respectively), \( a \) is concentration of inhibitor, and \( \delta \) is the concentration for 50% inhibition of the test species.

Measurement of Amino Acid Concentrations in Arabidopsis and F. rubra. Eight days after germination on 0.5× MS agar plates, Arabidopsis seedlings were transferred to 0.5× MS agar plates with or without 10 μM m-tyrosine. Roots were harvested 2 days after transfer. To prepare Arabidopsis root samples for amino acid analysis, \( \approx \) 15 mg of roots was frozen in liquid nitrogen and ground to a fine powder. An extraction buffer containing 20 mM HCl and norleucine as a standard was added to each sample at the concentration of 10 μM of fresh tissue. The homogenized samples were centrifuged for 10 min at 4°C and filtered by using a Millipore (Billerica, MA) Filtrate Plate. The samples were analyzed by using the AccQ tag HPLC detection system (Waters, Milford, MA). The amino acid separations were carried out by using a 3.9 × 150 mm AccQ Tag reversed-phase column (Waters; Millipore) at 37°C, with a flow rate of 1.0 ml/min and detection at 254 nm. The two eluent systems used were as follows: eluent A, AccQ Tag eluent A (concentrate A: 190 g of sodium acetate, 17.2 g of triethylamine, 10 mg of EDTA in 1 liter of Milli-Q water (vol/vol) (for gradients used, see SI Table 3). The samples were centrifuged for 10 min at 13,000 × g for 30 min at room temperature, washed twice with 500 μl of extraction buffer, and centrifuged at 14,000 rpm. Samples were adjusted to a final volume of 100 μl using extraction buffer. Free amino acid profiles of these samples were analyzed by using the AccQ tag HPLC detection system (Waters) to confirm that they did not contain free m-tyrosine. Eighty microliters of each sample were adjusted to a final volume of 400 μl with 1% phenol and a final HCl concentration of 6 M. Samples were then transferred to Kontes valved NMR tubes (Kontes, Vineland, NJ), and the tubes were flushed with argon gas. Sealed tubes were then incubated by using a 110°C oil bath for 24 h. After hydrolysis, samples were dried by evaporation, redissolved in 20 mM HCl, and analyzed by using the AccQ tag HPLC detection system as described above, except that a modified gradient and a column temperature of 30°C were used to improve separation of the m-tyrosine derivative from the methionine derivative.

For analysis of F. rubra seed m-tyrosine content, seeds were ground to a fine powder in liquid nitrogen, an aqueous extract was prepared, and amino acids were measured as described above.

Measurement of Protein-Incorporated m-Tyrosine. Arabidopsis land race Col-0 seeds were sterilized and sown on 0.5× MS agar plates that were placed vertically in the growth chamber. After 8 days, seedlings were transferred to new plates with control agar or agar containing 10 μM m-tyrosine. Two days after transfer, plant roots were harvested into 1.5 ml tubes (\( \approx \) 60 mg for each sample) and immediately frozen with liquid nitrogen. One 3-mlm steel ball was placed into each tube, and plant tissue was crushed by shaking on a HarBil 5G-HD paint shaker (Fluid Management, Wheeling, IL). Five hundred microliters of extraction buffer (1× PBS, pH 7.4, with 2 mM phenylmethylsulfonylfluoride) were then added to the crushed sample and mixed by using the same shaker. Samples were centrifuged for 10 min at 13,000 × g at 4°C. Supernatant was transferred to Millipore YM-10 spin columns, centrifuged at 13,000 × g for 30 min at room temperature, washed twice with 500 μl of extraction buffer, and centrifuged at 14,000 rpm. Samples were adjusted to a final volume of 100 μl using extraction buffer. Free amino acid profiles of these samples were analyzed by using the AccQ tag HPLC detection system (Waters) to confirm that they did not contain free m-tyrosine. Eighty microliters of each sample were adjusted to a final volume of 400 μl with 1% phenol and a final HCl concentration of 6 M. Samples were then transferred to Kontes valved NMR tubes (Kontes, Vineland, NJ), and the tubes were flushed with argon gas. Sealed tubes were then incubated by using a 110°C oil bath for 24 h. After hydrolysis, samples were dried by evaporation, redissolved in 20 mM HCl, and analyzed by using the AccQ tag HPLC detection system as described above, except that a modified gradient and a column temperature of 30°C were used to improve separation of the m-tyrosine derivative from the methionine derivative.

We thank Dr. Donna Gibson for assistance with bacterial and fungal assays, Drs. Kevin Vaughn and Dominick Paolillo for helpful discussions, Rose Harmon and Mia Akoagi for assistance with seedling bioassays, Ladmila Rehak for measurement of F. rubra m-tyrosine content, Dr. Andreas Weber (Michigan State University, East Lansing, MI) for providing Arabidopsis apa1 mutant seeds, and Dr. Silvina Garcia (Albany Molecular Research, North Syracuse, NY) for providing a sample of L-m-tyrosine. This work was supported in part by National Institutes of Health Grant GM55830, the New York State Turfgrass Association, National Science Foundation Grant DBI-0453331, and the Triad Foundation.

3',4'-dihydroxyphenylalanine (L-DOPA)

2',5'-dihydroxyphenylalanine