Genetic and chemical analyses of the action mechanisms of sirtinol in *Arabidopsis*

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The synthetic molecule sirtinol was shown previously to activate the auxin signal transduction pathway. Here we present a combination of genetic and chemical approaches to elucidate the action mechanisms of sirtinol in *Arabidopsis*. Analysis of sirtinol derivatives indicated that the ''active moiety'' of sirtinol is 2-hydroxy-1-naphthaldehyde (HNA), suggesting that sirtinol undergoes a series of transformations in *Arabidopsis* to generate HNA, which then is converted to 2-hydroxy-1-naphthoic acid (HNC), which activates auxin signaling. A key step in the activation of sirtinol is the conversion of HNA to HNC, which is likely catalyzed by an aldehyde oxidase. Mutations in any of the genes that are responsible for synthesizing the molybdopterin cofactor, an essential cofactor for aldehyde oxidases, led to resistance to sirtinol, probably caused by the compromised capacity of the mutants to convert HNA to HNC. We also showed that sirtinol and HNA could bypass the auxin polar transport system and that they were transported efficiently to aerial parts of seedlings, whereas HNC and 1-naphthoic acid were essentially not absorbed by *Arabidopsis* seedlings, suggesting that sirtinol and HNA are useful tools for auxin studies.

auxin | polar transport | molybdopterin | aldehyde oxidase

With the advancements of combinatory chemistry and availability of affordable commercial collections of chemical libraries, more and more laboratories are exploring the use of synthetic molecules as tools to solve biological problems (1–6). One of the challenges of using a synthetic compound in a biological system is to define the action mechanisms of the used compound. We previously reported the use of a small molecule, sirtinol, to modulate auxin-signal transduction (7). Sirtinol was first found as an inhibitor of the Sirtuin family of NAD-dependent deacetylases in *Saccharomyces cerevisiae* and later found to inhibit *Arabidopsis* root elongation, phenotypes similar to those caused by exogenous auxin treatment (8). Furthermore, sirtinol specifically activates the expression of auxin-inducible genes (7). Similar to auxin treatment, sirtinol treatment also caused a rapid degradation of the AXR3-NT-β-glucuronidase (GUS) fusion protein, indicating that sirtinol probably activates auxin gene expression by regulated degradation of negative regulators such as AUX/indole-3-acetic acid (IAA) proteins (7). Although sirtinol activates auxin-inducible genes and leads to auxin phenotypes, sirtinol causes additional phenotypes including cup-shaped true leaves in the aerial parts of *Arabidopsis* seedlings (7). Unlike sirtinol, exogenous IAA or other synthetic auxins mainly inhibit root elongation, suggesting that sirtinol serves as a useful tool for auxin studies. Understanding the action mechanisms of sirtinol will not only enable better use of sirtinol in auxin studies but also may serve as a model to dissect action mechanisms of other small molecules used in *Arabidopsis* research.

Here we present the elucidation of sirtinol action mechanisms by identification of the active core structure in sirtinol and genetic analysis of sirtinol-resistant mutants. An analysis of the effects of a series of sirtinol derivatives on *Arabidopsis* seedling growth demonstrated that the active core moiety of sirtinol is 2-hydroxy-1-naphthaldehyde (HNA). Our genetic studies of sirtinol-resistant mutants revealed that enzymes involved in the biosynthesis of molybdopterin cofactor (moco), a necessary cofactor for aldehyde oxidases and xanthine dehydrogenases, play an essential role in sirtinol activities. Together with the analysis of sirtinol derivatives, the genetic studies suggest that sirtinol undergoes a series of metabolic transformations to generate HNA, which then is converted by an aldehyde oxidase to generate 2-hydroxy-1-naphthoic acid (HNC), an active auxin. This work demonstrates the power of combining genetic studies and chemical analysis in determining the action mechanisms of a synthetic compound in multicellular organisms.

Materials and Methods

Materials. Sirtinol was purchased from ChemBridge (San Diego). HNC, 1-naphthoic acid, and 1-naphthaleneacetic acid (NAA) were from Sigma. Ethyl methanesulfonate-mutagenized M2 *Arabidopsis* seeds were purchased from Lehle Seeds (Round Rock, TX). Murashige and Skoog (MS) medium was from Invitrogen.

Isolation, Characterization, and Cloning of Sirtinol-Resistant Mutants. Genetic screens for mutants resistant to sirtinol were carried out according to published protocols (7, 9). Putative mutants were backcrossed to clean up background mutations and outcrossed to other ecotypes to generate mapping populations as described (7, 9). Mutants were characterized and cloned by using a map-based positional cloning approach (7, 9).

Analysis of Transfer DNA Insertion Mutants. T-DNA (portion of the tumor-inducing plasmid that is transferred to plant cells) insertion mutants were identified from the Salk collection (http://signal.salk.edu/cgi-bin/tdnaexpress) (10). T-DNA insertion in the gene of interest was confirmed by PCR with a gene-specific primer and a T-DNA-specific primer. The exact insertion site was determined by DNA sequencing.

GUS Activity Measurement. For the histochemical GUS assay, the seedlings were washed with buffer A [100 mM sodium phosphate, pH 7.0/10 mM EDTA/0.5 mM $K_3Fe(CN)_6$/0.1% Triton X-100] and then incubated in a staining buffer (buffer A with 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide) at 37°C until sufficient staining developed (11). For quantitative fluorometric GUS assays, whole seedlings (n = 10–20) were homogenized in an extraction buffer as described in ref. 11. After centrifugation to remove cell debris, GUS activity was measured with 1 mM 4-methyl umbelliferyl β-D-glucuronide as a substrate at 37°C (11).

Abbreviations: GUS, β-glucuronidase; IAA, indole-3-acetic acid; HNA, 2-hydroxy-1-naphthaldehyde; moco, molybdopterin cofactor; HNC, 2-hydroxy-1-naphthoic acid; NAA, 1-naphthaleneacetic acid; T-DNA, portion of the tumor-inducing plasmid that is transferred to plant cells; BAC, bacteria artificial chromosome.

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PLANT BIOLOGY

Table 1. Isolation of sirtinol-resistant mutants

Mutants	Alleles	Recessive/dominant	Chromosome location
sir1	3	Recessive	Chr. V between BACs MUP24 and K6M13
sir3	12	Recessive	Chr. I between markers nga 63 and nga 248
sir4	1	Recessive	Chr. V between markers nga 139 and nga 151
sir5	1	Recessive	Chr. IV between markers nga 8 and ciw6
sir6	1	Recessive	Chr. III between markers ciw4 and AtGAPab
sir7	2	Recessive	Chr. V between BACs MUP24 and MCO15

Sir1 has been described (7). SIR2 was not used to name any of our *sir* mutants, because SIR2 has been widely used in the literature for a family of NAD^+-dependent histone/protein deactylases.

Synthesis of Sirtinol Derivatives. The sirtinol derivatives 2-[(2-hydroxy-naphthalen-1-ylmethylene)-amino]-benzoic acid (**1**) and 2-[(2-hydroxy-naphthalen-1-ylmethylene)-amino]-benzamide (**2**) were synthesized by the condensation of HNA and corresponding amines. HNA (400 mg, 2.33 mmol) and amine (320 mg, 2.34 mmol, 2-aminobenzoic acid for **1**; 320 mg, 2.35 mmol, 2-aminobenzamide for **2**) were dissolved in 10 ml of benzene, and 2 g of molecular sieves 3A was added into this solution. The reaction mixture was heated under reflux for 8 h, diluted with 50 ml of hot methanol, and then filtered. The filtrate was concentrated *in vacuo* to give a yellow (**1**) or orange (**2**) solid. The crude products were recrystallized from MeOH to give compound **1** (366 mg, 54%); mp 204 C°; UV λnm(logϵ) 227 (5.1), 261 (4.6), 316 (4.5), 441 (4.6), 460 (4.6); IR 3,428, 1,640, 1,591 cm^{-1}: high-resolution fast-atom-bombardment mass spectrometry *m/z* 314.0772 $[M + Na]^+$, (Δmmu 2.1, Calcd. for $C_{18}H_{13}NO_3Na$); ^1H NMR (DMSO-d_6) δ 6.78 (d, *J* = 9.3, 1H), 7.31 (t, *J* = 7.6, 1H), 7.32 (t, *J* = 7.6, 1H), 7.48 (t, *J* = 8.1, 1H), 7.67 (t, *J* = 8.0, 1H), 7.68 (t, *J* = 7.8, 1H), 7.82 (d, *J* = 9.3, 1H), 7.97 (d, *J* = 7.6, 2H), 8.36 (d, *J* = 8.4, 1H), 9.32 (s, 1H), and compound **2** (412 mg, 61% yield); mp 148 C°; UV λnm(logϵ) 229 (5.1), 258 (4.7), 316 (4.6), 438 (4.5), 461 (4.4); IR 3,390, 3,178, 1,664, 1,622 cm^{-1}; high-resolution fast-atom-bombardment mass spectrometry *m/z* 313.0963 $[M + Na]^+$, (Δmmu 1.1, Calcd. for $C_{18}H_{14}N_2O_2Na$); ^1H NMR (DMSO-d_6) 6.89 (d, *J* = 9.2, 1H), 7.32 (t, *J* = 7.8, 2H), 7.51 (t, *J* = 7.8, 1H), 7.56 (s, 1H, NH), 7.59 (d, *J* = 8.0, 1H), 7.60 (t, *J* = 7.8, 1H), 7.74 (d, J = 7.8, 1H), 7.86 (d, *J* = 7.8, 1H), 7.87 (d, *J* = 9.2, 1H), 7.98 (s, 1H, NH), 8.41 (d, *J* = 8.4, 1H), 9.42 (s, 1H).

Results

Isolation of Sirtinol-Resistant Mutants. Sirtinol was shown to activate the expression of auxin-inducible genes and to cause auxin-related developmental phenotypes (7). As a first step in elucidating the mechanisms by which sirtinol activates auxin signaling, we screened for sirtinol-resistant mutants. As expected, alleles of the previously known auxin-resistant mutants (*axr1*, *axr2*, *axr3*, *axr6*, *tir1*, *ibr5*, *rce1*, *ecr1*, *ask1*, and *msg1*) emerged from the screen (data not shown) (For auxin mutants, see review in ref. 12.) In addition to the known auxin-resistant loci, we also isolated several previously uncharacterized auxin-resistant mutants including *Atcand1* (9). Finally, six sirtinol-resistant (*sir*) mutants, but auxin-sensitive loci, were also identified and are described further here (Table 1). The mutant *sir1* has been described (7).

When grown on 25 μM sirtinol in light, the mutant *sir3* displayed long roots and normal cotyledons, whereas wild-type (WT) *Arabidopsis* seedlings had short hypocotyls, no roots, and epinastic cotyledons (Fig. 1 *A* and *B*). In the dark, *sir3* had a long hypocotyl, an elongated root, and an apical hook when grown on sirtinol-containing medium, whereas WT did not have a primary root and an apical hook (Fig. 1 *C* and *D*). Similar to *sir3*, both *sir4* and *sir5* were resistant to 25 μM sirtinol in the light (data not shown). Moreover, both *sir4* and *sir5* had long hypocotyls and roots but lacked an apical hook when grown in the dark in the presence of sirtinol (Fig. 1 *E* and *F*).

Molecular Cloning of Sirtinol-Resistant Mutants *sir3*, *sir4*, and *sir5*. The *sir3* mutant was mapped to a 125-kb interval between molecular markers F3O9B and F19K19A on the chromosome I (Fig. 1*G*). DNA sequencing of selected ORFs in the mapping interval revealed a G-to-A transversion in the gene At1g16540 in *sir3-1*. The mutation was at the splice junction of exon 11 and intron 11 (Fig. 1*G*), which probably leads to aberrant RNA processing, as is the case for many splice-junction mutants.

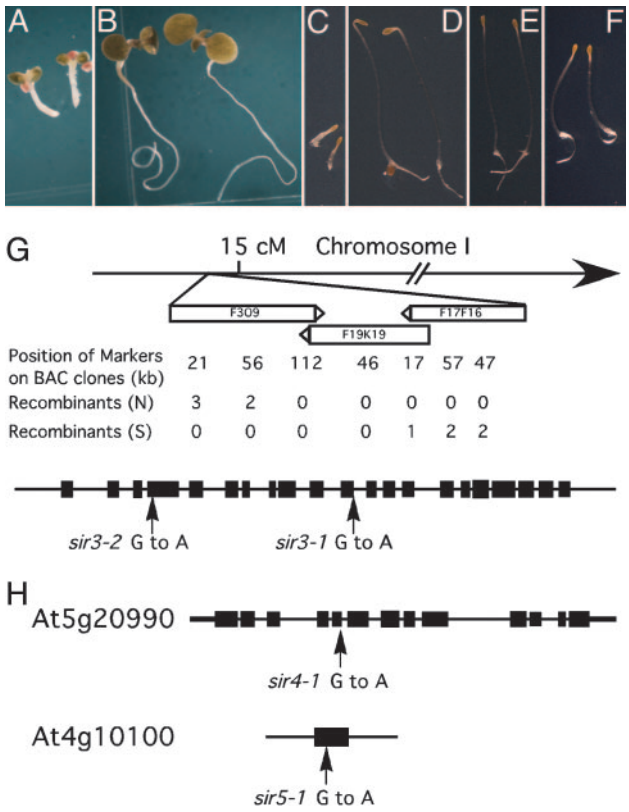

Fig. 1. Characterization and cloning of *sir3*, *sir4*, and *sir5*. (*A*) Seven-day-old *Arabidopsis* WT seedlings grown on medium containing 25 μM sirtinol in light. (*B*) *sir3-1* grown on 25 μM sirtinol for 7 days in the light. (*C*) Three-day-old WT *Arabidopsis* seedlings grown on 5 μM sirtinol in total darkness. (*D*) Dark-grown *sir3-1* seedlings on 5 μM sirtinol for 3 days. (*E*) Mutant *sir4* grown on 5 μM sirtinol for 3 days in the dark. (*F*) *sir5* grown on 5 μM sirtinol for 3 days in the dark. (*G*) Cloning of *sir3*. cM, centimorgan; BAC, bacteria artificial chromosome; N, north; and S, south. F19K19, F3O9, and F17F16 are three BACs. The arrows on the BACs indicate the directions of the BACs. (*G Lower*) The intron and exon structures are shown. Mutations of *sir3-1* and *sir3-2* are indicated. (*H*) Cloning of *sir4* and *sir5*. The intron and exon structures of At5g20990 (*SIR4*) and At4g10100 (*SIR5*) and mutations of the *sir4* and *sir5* mutants are shown.

Sequencing other alleles of *sir3* demonstrated that *sir3-2* had a G-to-A change in the fourth exon (G317 from the ATG in the mRNA sequence) of At1g16540, which led to a glycine-to-glutamate change. The mutants *sir3-3* and *sir3-4* contained mutations at the C terminus of At1g16540. *sir3-3* had a G-to-A transversion at nucleotide 2168 in mRNA that caused an arginine-to-lysine change, whereas *sir3-4* had an alanine-to-valine change, which was caused by a C-to-T change at 2156 from ATG in the mRNA sequence (Fig. 1*G*). Finding mutations in At1g16540 among several *sir3* alleles indicated that At1g16540 was the gene responsible for the *sir3* phenotypes.

We also mapped the mutant *sir4-1* to the middle of chromosome V and identified a G-to-A transversion in the gene At5g20990 (Fig. 1*H*). The mutant *sir5* was mapped to the top of chromosome IV. We identified a G-to-A mutation 128 bp downstream from the ATG of the mRNA sequence in the gene At4g10100, which led to a replacement of serine 43 with an asparagine (Fig. 1*H*). Because both At5g20990 and At4g10100 are predicted to participate in the pathway involved in SIR1 and SIR3 (defined below), it is likely that the identified mutations in At5g20990 and At4g10100 are responsible for the *sir4* and *sir5* phenotypes, respectively.

Sirtinol-Resistant Mutants Define a Moco Biosynthesis Pathway. At1g16540/SIR3 was annotated as a moco sulfurase, which also was called ABA3 for its role in abscisic acid biosynthesis (13–15). The moco sulfurase was proposed to catalyze the replacement of an oxygen atom with an inorganic sulfur to the molybdenum center of moco, a step necessary for activation of certain molybdenum enzymes including aldehyde oxidases and xanthine dehydrogenases (16) (Fig. 2*A*).

At5g20990/SIR4 is the putative *Arabidopsis* CNX1, an enzyme responsible for insertion of the molybdenum metal ion into molybdopterin to make moco (17, 18) (Fig. 2*A*). At4g10100/SIR5 is the putative small subunit (CNX7) of molybdopterin synthase (16) (Fig. 2*A*). We previously reported that SIR1 is the *Arabidopsis* homolog of Uba4 from *S. cerevisiae*, which is also homologous to molybdopterin synthase sulfurylase (CNX5) (7) (Fig. 2*A*). Identification of sirtinol-resistant mutants *sir3*, *sir4*, and *sir5* as moco biosynthesis enzymes indicates that SIR1 likely plays a role in molybdopterin biosynthesis rather than in protein degradation as we had suggested previously (7). Taken together, our genetic analysis of sirtinol-resistant mutants clearly indicated that moco is critical for sirtinol functions in *Arabidopsis*.

There are at least seven genes (CNX1–CNX7) known to participate in moco biosynthesis (16) (Fig. 2*A*), although it is unclear what the role of CNX4 plays in moco biosynthesis. The first stage of moco biosynthesis is to convert GTP to an intermediate called precursor Z (Fig. 2*A*). The biosynthesis of precursor Z from GTP is catalyzed by two proteins, CNX2 and CNX3, but little is known about the mechanisms of these two proteins (19–21). The precursor Z already has all of the carbon skeleton of a molybdopterin structure and is converted to molybdopterin by the moco synthase, a heterotetramer of two subunits of CNX6 and two subunits of CNX7 (19, 22) (Fig. 2*A*). The insertion of two sulfur atoms in precursor Z is achieved by transferring sulfur atoms from the small subunit CNX7, where the sulfur atom is bound to the C terminus of CNX7 as a thiocarboxylate. The CNX7 subunit then is recharged with a thiocarboxylate at its C terminus by CNX5, a moco sulfurylase (Fig. 2*A*). The next step in moco biosynthesis is the insertion of molybdenum metal ion into molybdopterin to form the holomoco, a step catalyzed by CNX1 (17) (Fig. 2*A*). Moco is an essential cofactor for important enzymes such as nitrate reductase and sulfite oxidase. Moco is modified further by a sulfurase (At1g16540/ABA3/SIR3) to generate thiolated moco that functions as an essential cofactor for aldehyde oxidases and xanthine dehydrogenases (13, 14) (Fig. 2*A*).

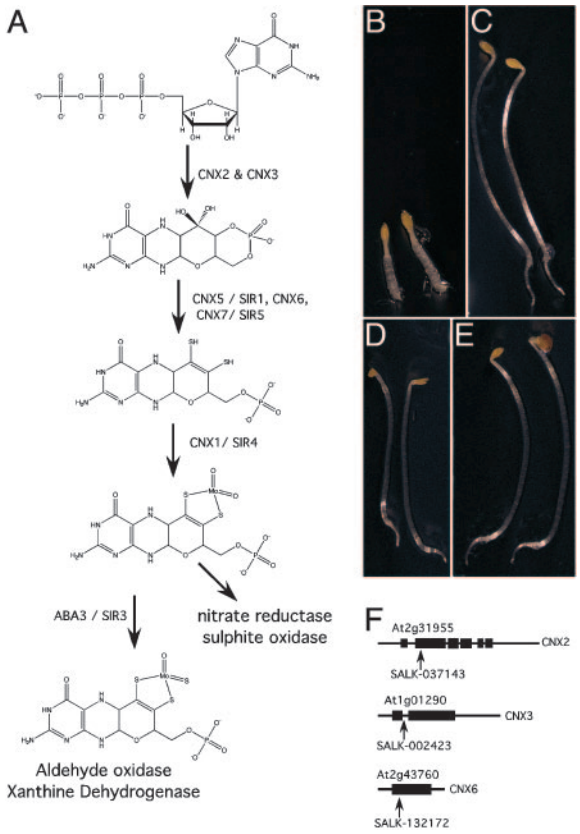

Fig. 2. Moco biosynthesis pathway and analysis of T-DNA insertion mutants of the putative moco biosynthesis genes. (*A*) The proposed moco biosynthesis pathway. Moco is synthesized from GTP by at least seven proteins named CNX1–CNX7. The biosynthesis starts from the conversion of GTP to the precursor Z catalyzed by CNX2 and CNX3. The precursor Z is then converted to molybdopterin by moco synthase, composed of CNX6 and CNX7 heterotetramer. CNX5 is proposed to activate CNX7 by generating a thiocarboxylate at the C terminus of CNX7. The synthesized molybdopterin is then inserted with a molybdenum atom catalyzed by the CNX1 gene to form the complete moco. Moco is an essential cofactor for nitrate reductase and sulfite oxidase. Moco can be modified further by a sulfurase to generate thiolated moco, which is essential for aldehyde oxidases and xanthine dehydrogenases. (*B*) Dark-grown WT seedlings on 5 μM sirtinol. (*C*) A T-DNA insertion mutant (Salk_037143) of CNX2 grown on 5 μM sirtinol for 3 days in the dark. (*D*) Dark-grown seedlings of a T-DNA line (Salk_002423) of CNX3 on 5 μM sirtinol for 3 days. (*E*) Seedlings of the T-DNA line (Salk_132172) that has an insertion in the CNX6 gene grown on 5 μM sirtinol in the dark. (*F*) The T-DNA insertion mutants of cnx2, cnx3, and cnx6. The intron and exon structures and the location of the T-DNA insertions are indicated schematically.

Because sirtinol-resistant mutants *sir1/cnx5*, *sir3/aba3*, *sir4/cnx1*, and *sir5/cnx7* all participate in moco biosynthesis, we investigated whether other molybdopterin biosynthesis enzymes are also important for sirtinol activities. As shown in Fig. 2 *B–D*, T-DNA insertion mutants in either *CNX2* or *CNX3* led to sirtinol resistance, i.e., both long hypocotyls and long roots were grown on sirtinol in the dark. Loss of function of *CNX6* by T-DNA insertion also conferred sirtinol resistance (Fig. 2*E*). T-DNA line Salk_037143 was inserted in the second exon of the CNX2 gene, and the line Salk_132172 had an insertion in the exon of CNX6 gene (Fig. 2*F*). We isolated two alleles of CNX3, T-DNA insertion mutant Salk_002423, which has a T-DNA insertion in the first intron of CNX3, and Salk_106237, the T-DNA insertion of which was in the first exon (Fig. 2*F*). In summary, loss-of-function mutations in any of the presumed molybdopterin biosynthesis enzymes led to sirtinol resistance.

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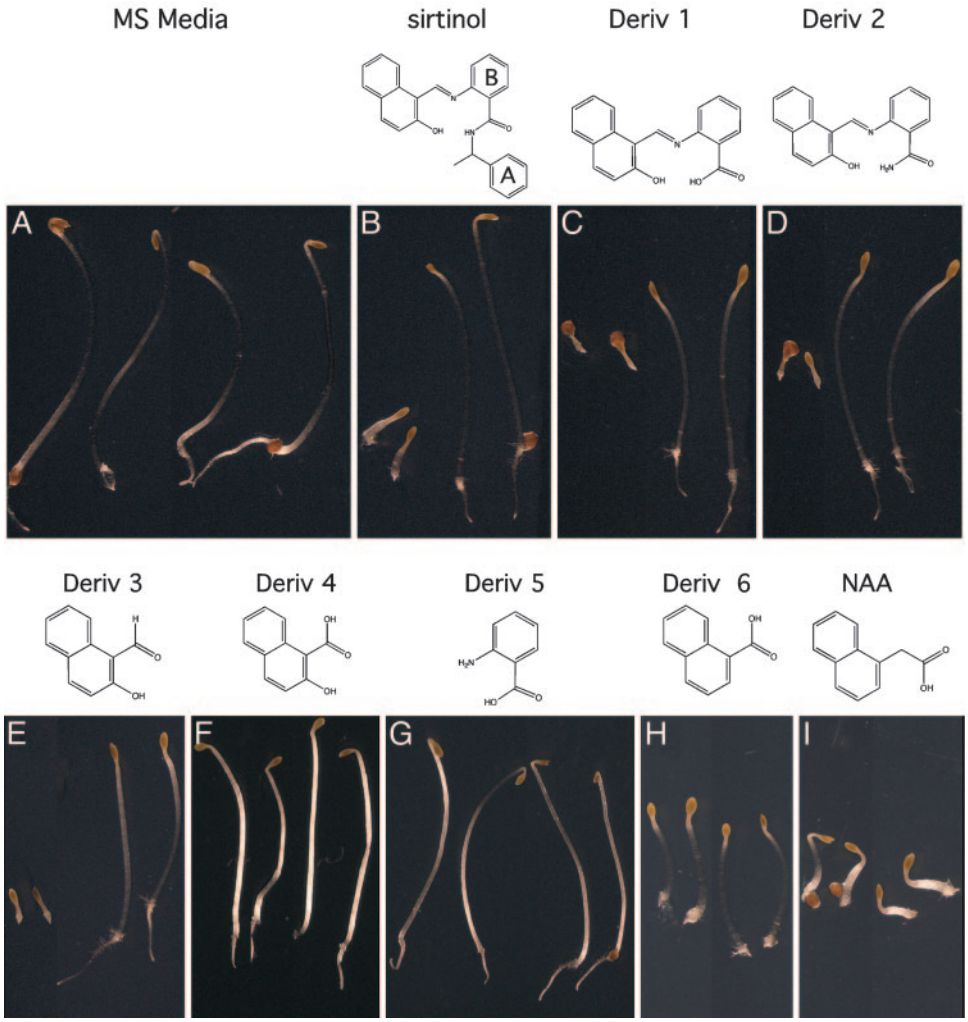

Fig. 3. Effects of sirtinol derivatives on *Arabidopsis* seedling growth and development. In *A–I*, the left two seedlings represent WT Columbia seedlings, and the two seedlings on the right side represent mutant *sir1*. (*A–I Upper*) The structure of the sirtinol derivatives. (*A–I Lower*) Seedlings grown on 5 μM compound in the dark for 3 days. (*A*) Three-day-old WT and *sir1* seedlings grown on MS medium in the dark. (*B*) Effects of sirtinol. The two benzene rings are labeled A and B to help in discussing the structures of the derivatives. (*C*) Effects of the sirtinol derivative **1**. (*D*) Effects of derivative **2**. (*E*) Effects of derivative **3**. (*F*) Effects of HNC, derivative **4**. (*G*) Effects of derivative **5**. (*H*) Effects of derivative **6** (1-naphthoic acid). (*I*) Inhibitory effects of NAA.

The Active Moiety of Sirtinol Is HNA. We synthesized a series of sirtinol derivatives to determine the core structure responsible for sirtinol activities. We first examined whether the phenylethyl amino moiety in sirtinol is necessary for sirtinol activity by removing the entire moiety from sirtinol to generate the sirtinol derivative **1** {2-[(2-hydroxy-naphthalen-1-ylmethylene)-amino]-benzoic acid}, which does not have the benzene ring A (Fig. 3 *B* and *C*). Similar to sirtinol, derivative **1** inhibits the growth of primary root and hypocotyl of WT *Arabidopsis* seedlings (Fig. 3 *A–C*). Sirtinol-resistant mutant *sir1* was also resistant to derivative **1** (Fig. 3 *A–C*), indicating that the phenylethyl amino moiety is not necessary for the sirtinol activities. We then investigated whether the carboxyl group in derivative **1** is important for sirtinol activity. Converting the carboxyl group in derivative **1** to an amide to generate derivative **2** {2-[(2-hydroxy-naphthalen-1-ylmethylene)-amino]-benzamide} did not abolish the sirtinol activity, and *sir1* was also resistant to this compound (Fig. 3*D*). We next removed both benzene rings A and B in sirtinol to generate derivative **3**: HNA (Fig. 3*E*). To our surprise, WT seedlings grown on derivative **3** had identical phenotypes to those of seedlings grown on sirtinol and derivatives **1** and **2**, suggesting that both the rings A and B were not necessary for sirtinol activities. Interestingly, derivative **3** was also found to inhibit NAD-dependent deacetylase in yeast but not as potently as sirtinol (8). Furthermore, sirtinol-resistant mutant *sir1* was also resistant to derivative **3** (Fig. 3*E*).

Because the oxidation product of HNA is HNC (derivative **4**), a compound that is structurally related to known synthetic auxin NAA, we suspect that the active molecule from sirtinol is HNC (derivative **4**). When WT *Arabidopsis* seedlings were grown on 5 μM HNC, however, no inhibition of root and hypocotyl elongation was observed, indicating that HNC either did not have activities similar to those of sirtinol under the testing conditions or may not be absorbed by the seedlings (Fig. 4*F*). We also tested derivative **5** and observed that it did not display sirtinol activity (Fig. 4*G*). We further tested the effects of 1-naphthoic acid (derivative **6**) on *Arabidopsis* growth and found that derivative **6** had weak sirtinol activities (Fig. 3*H*). Seedlings of WT *Arabidopsis* grown on 5 μM derivative **6** did not have primary roots and lacked an apical hook, but the hypocotyl is apparently longer than that of seedlings grown on sirtinol or derivatives **1**, **2**, or **3** (Fig. 3*H*). Interestingly, sirtinol-resistant mutant *sir1* showed the same responses to derivative **6** as the WT seedlings, indicating that SIR1 is not important for the auxin activity of derivative **6**.

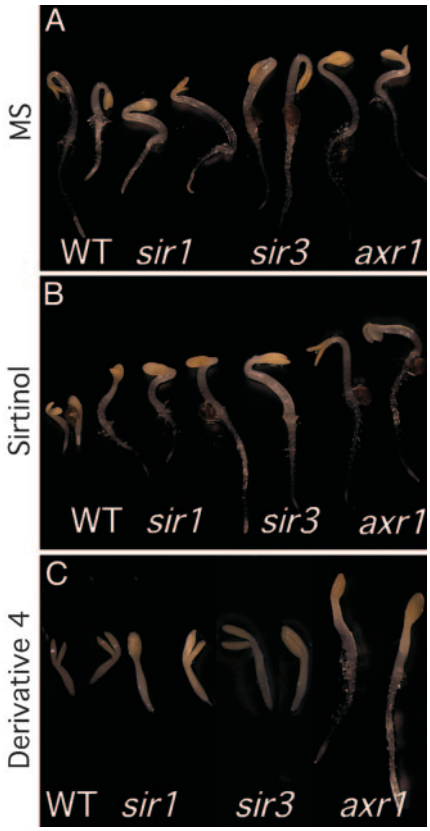

Fig. 4. Effects of sirtinol and HNC on seedling growth in liquid culture. (*A*) Seedlings of WT, *sir1*, *sir3*, and *axr1-12* grown in MS liquid medium for 3 days in total darkness. (*B*) Effects of sirtinol on seedling growth in liquid culture. Seedlings were grown in liquid culture containing 5 μM sirtinol in the dark for 3 days. (*C*) Seedlings grown in liquid culture containing 5 μM derivative **4** (HNC) in the dark for 3 days.

As a control, we also tested NAA on seedling growth and, as we expected, NAA inhibited both root and hypocotyl elongation (Fig. 3*I*), but the NAA-treated seedlings still retained an obvious apical hook (Fig. 3*I*). It seemed that sirtinol or derivatives **1-3** are even more potent than the widely used NAA, as evidenced by the observation that dark-grown seedlings from sirtinol plates had even shorter hypocotyl and no apical hook, whereas seedlings from NAA plates still had an apical hook despite the very short hypocotyls and roots (Fig. 3).

Although derivatives **3**, **4**, and **6** and NAA are structurally very similar, the effects of these compounds on plant growth and development differed dramatically (Fig. 3). The differences could be attributed to the uptake and transport of the compounds. Therefore, we tested the effects of derivative **4** (HNC) on *Arabidopsis* growth in liquid culture. WT *Arabidopsis* seedlings and mutants *sir1*, *sir3*, and *axr1-12* all developed normal roots and apical hooks when grown in the dark in liquid MS medium (Fig. 4*A*). Although WT seedlings grown on sirtinol in liquid culture displayed typical sirtinol-related phenotypes, mutants *sir1*, *sir3*, and *axr1* all showed resistance to sirtinol (Fig. 4*B*). Sirtinol derivative **4** inhibited primary root, hypocotyl, and apical hook development of WT seedlings, which are characteristic phenotypes of sirtinol effects (Fig. 4*C*). It is interesting to note that both *sir1* and *sir3* were not resistant to derivative **4**, but *axr1* was (Fig. 4*C*). These findings were in contrast to our observations of seedlings grown on plates in which there was essentially no auxin activity of HNC (Fig. 3*E*). We conclude that HNC is an active auxin but could not be transported as efficiently as sirtinol and other sirtinol derivatives.

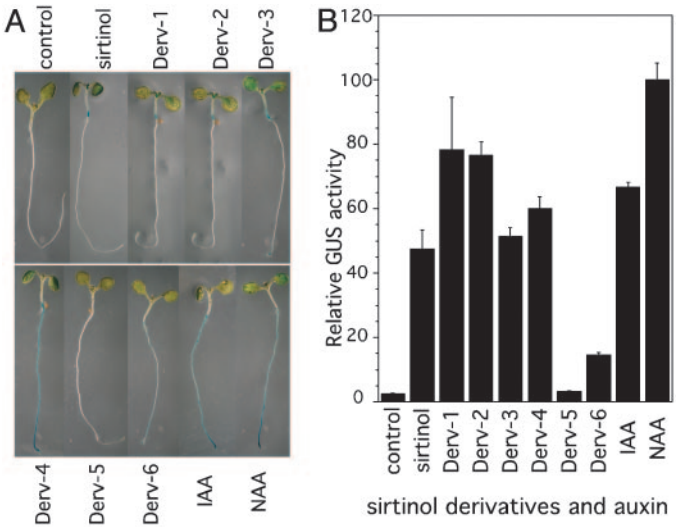

Fig. 5. Effects of sirtinol and sirtinol derivatives on the expression of auxin reporter DR5-GUS. (*A*) Seven-day-old seedlings were treated with sirtinol or the indicated compounds for 24 h in liquid culture. The concentrations of sirtinol and its derivatives were 20 μM and the concentrations of IAA and NAA were 5 μM. The expression of DR5-GUS was visualized by staining with 5-bromo-4-chloro-3-indolyl β-D-glucuronide for GUS activities. (*B*) Quantitative measurements of GUS activities. Derv, derivative.

We also tested the effects of all the sirtinol derivatives on the induction of auxin-inducible genes. Sirtinol and derivatives **1-4** all induced the expression of an auxin reporter DR5-GUS (23), and so did derivative **6**, IAA, and NAA (Fig. 5). The overall induction levels of DR5-GUS expression by sirtinol and its derivatives **1–4** are comparable to those induced by auxin IAA and NAA (Fig. 5*B*), although there was an apparent difference of DR5-GUS expression pattern induced by auxin and sirtinol/sirtinol derivatives. The GUS expression was mainly in the aerial part of the seedling treated with sirtinol or derivatives **1-3**, particularly at the root and hypocotyl junction, whereas the GUS expression was mainly in the root of IAA- or NAA-treated seedlings (Fig. 5*A*).

Discussion

In this work, we present evidence that, in *Arabidopsis*, sirtinol is converted to HNC, a molecule that activates auxin-signaling pathways and is responsible for the observed auxin phenotypes caused by sirtinol. This work, which combines both genetic and chemical approaches, provides a general strategy for elucidating the action mechanisms of synthetic molecules used in *Arabidopsis* and insights into auxin polar transport.

Loss-of-function mutations in any of the presumed moco biosynthesis pathway components led to sirtinol resistance (Figs. 1 and 2), suggesting that a protein using moco as an essential cofactor plays a key role in sirtinol functions. There are only four known enzymes in plants that require moco for their activities: nitrate reductase, sulfite oxidase, aldehyde oxidase, and xanthine dehydrogenase (16). The former two enzymes use moco as a cofactor, whereas the latter two enzymes use a modified moco in which an oxygen atom is replaced by a sulfur atom (Fig. 2*A*). Because the moco sulfurase, the enzyme responsible for the oxygen/sulfur replacement, is required for sirtinol activity (Fig. 2*A*), we suspected that either an aldehyde oxidase or a xanthine dehydrogenase may be involved in converting sirtinol to an active compound that activates auxin-signaling pathways. However, sirtinol is not an apparent substrate for aldehyde oxidase, because there is no aldehyde group on sirtinol itself. Sirtinol also is not an obvious xanthine dehydrogenase substrate, and we did

not observe any xanthine dehydrogenase activity when we used sirtinol as a substrate for *in vitro* xanthine dehydrogenase assays (data not shown).

We realized why moco is essential for sirtinol activities when we discovered that the active core structure of sirtinol is HNA. Removing ring A or B from sirtinol did not lead to loss of sirtinol activities, suggesting that the active moiety is derivative **3** HNA (Fig. 3). Derivative **3** is as potent as sirtinol in terms of inhibition of root/hypocotyl growth and induction of auxin-inducible genes (Figs. 3 *B* and *E* and 5). Given that a moco-containing enzyme is required for the sirtinol activities, we suspected that the true active compound was HNC and that a moco-containing aldehyde oxidase is responsible for the oxidation of HNA to HNC. If our hypothesis is true, mutants of the moco biosynthesis enzymes should all be resistant to both sirtinol and the sirtinol derivatives that are upstream of the conversion of the aldehyde to the acid but sensitive to HNC and other auxins. Indeed, mutants *sir1*, *sir3*, *sir4*, *sir5*, and T-DNA insertion mutants in *CNX2*, *CNX3*, and *CNX6* are all resistant to sirtinol and derivatives **1**-**3** (Figs. 1–3), but all of these mutants were sensitive to HNC (Fig. 4) and other auxins (data not shown). Therefore, we conclude that sirtinol likely undergoes hydrolysis of the imine bond enzymatically or nonenzymatically to generate HNA *in vivo*. HNA is then converted to HNC, catalyzed by a moco-containing aldehyde oxidase, thereby eliciting auxin responses.

We demonstrated the effectiveness of a genetic approach in elucidating the action mechanisms of the synthetic compound sirtinol. It is expected that genes involved in compound uptake, transport, and metabolism as well as targets and downstream signaling components can be isolated from genetic screens for mutants insensitive to the compound. From the sirtinol-resistant mutant screen, we identified many previously known auxin-response mutants (data not shown). Interestingly, no mutants involved in auxin polar transport such as *aux1* (24) and *pin1* (25) have been identified from the screen, consistent with the hypothesis that sirtinol is auxin polar transport-independent (7). We also identified several genes responsible for the conversion of sirtinol to an active auxin. We expect that some of the remaining uncharacterized sirtinol-resistant mutants such as *sir6* and *sir7* may be involved in either sirtinol transport or regulating the moco biosynthesis pathway. It is also expected that aldehyde oxidase genes should be isolated from the sirtinol-resistant mutant screens if indeed an aldehyde oxidase is involved in converting sirtinol to HNC. There are four annotated aldehyde oxidase genes in the *Arabidopsis* genome and two genes of xanthine dehydrogenase that sometimes can carry out aldehyde oxidation as well, suggesting that genetic redundancy may account for our failure to isolate the aldehyde oxidase genes from the sirtinol screens thus far.

One of the motivations for using sirtinol to study auxin-signaling mechanisms was that sirtinol can modulate auxin signaling exclusively without affecting auxin polar transport, a process important for maintaining an auxin gradient among neighboring cells (7). Both sirtinol and HNA activate auxin signaling effectively, but HNC and 1-naphthoic acid were much less active in plant-growth assays despite the fact that sirtinol and HNA have to be converted to HNC to be active *in vivo* (Fig. 3). The activity difference between HNA and HNC was probably caused by differences in transport efficiency for the two compounds, because HNC was as active as sirtinol when seedlings were immersed in liquid culture (Fig. 4). It seems that hydrophobicity of the compounds determines relative transport/uptake efficiency for sirtinol derivatives and NAA. HNA is not charged and is the most efficiently transported, whereas HNC has the most polar groups and probably is the least transported among all the derivatives and NAA, which is consistent with our observation that HNC essentially did not inhibit seedling growth on agar plates (Fig. 3). It is interesting to note that expression of auxin reporter gene DR5 induced by active auxins including NAA, IAA, HNC, and 1-naphthoic acid mainly occurred in roots, whereas sirtinol and derivatives **1–3** (the noncharged compounds) all induced the expression of the reporter gene in the aerial parts of the plants. These results indicated that sirtinol and its derivatives **1**-**3** can be transported efficiently to the aerial part of the plants, whereas active auxins such as IAA and NAA mainly activate auxin reporter genes in roots, which is also consistent with our observations that sirtinol treatment caused cup-shaped leaves, whereas IAA, NAA, or other synthetic auxins did not. Therefore, the difference between sirtinol/derivatives and auxin in transport/uptake provides an opportunity for the isolation of genes that may have been missed from previous genetic screens for auxin-resistant mutants.

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