

High-Affinity Auxin Transport by the AUX1 Influx Carrier Protein

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Summary

In plants, auxin is a key regulator of development and is unique among plant hormones in that its function requires polarized transport between neighboring cells to form concentration gradients across various plant tissues [1–5]. Although putative auxin-influx [6, 7] and -efflux [8–15] transporters have been identified by using molecular genetic approaches, a detailed functional understanding for many of these transporters remains undetermined. Here we present the functional characterization of the auxin-influx carrier AUX1. Upon expression of AUX1 in *Xenopus* oocytes, saturable, pH-dependent uptake of ³H-IAA was measured. Mutations in AUX1 that abrogate physiological responses to IAA in planta resulted in loss or reduction of ³H-IAA uptake in AUX1-expressing oocytes. AUX1-mediated uptake of ³H-IAA was reduced by the IAA analogs 2,4-D and 1-NOA, but not by other auxin analogs. The measured K_m for AUX1-mediated uptake of ³H-IAA was at concentrations at which physiological responses are observed for exogenously added IAA and 2,4-D. This is the first report demonstrating detailed functional characteristics of a plant auxin-influx transporter. This biochemical characterization provides new insights and a novel tool for studying auxin entry into cells and its pivotal roles in plant growth and development.

Results and Discussion

Although molecular genetic studies have identified both putative auxin-influx and putative auxin-efflux carriers, the biochemical characterization of their activities remains largely undetermined. We therefore initiated studies to specifically determine whether AUX1 transports IAA and other auxin forms by using a *Xenopus* oocyte expression system. Because EYFP-AUX1 fusions have been shown to complement the *aux1-22* phenotypes in planta [16], we used a EYFP-AUX1 fusion to show that

AUX1 was properly targeted to the plasma membranes when expressed in *Xenopus* oocytes (Figure 1A). Oocytes expressing untagged AUX1 took up significantly higher amounts of ³H-IAA as compared to water-injected controls (Figure 1B). Transport of ³H-IAA in AUX1-expressing oocytes was saturable, with a K_m value of ~800 nM (Figure 1C). These observations provided a direct estimation for the affinity of AUX1 for IAA. A previous report calculated the K_m of auxin transport in plant suspension-culture cells by mathematically adjusting for the anionic form of IAA and found that carrier-mediated auxin uptake was between 1 and 5 μM [3].

To confirm that the saturable transport of ³H-IAA in AUX1-expressing oocytes was due to the activity of this protein, we created point mutations in the AUX1 protein. These mutations had been identified and partially characterized in planta as failing to respond to exogenously added IAA to the same degree as *aux1* null mutants [17]. To reduce the possibility that mutated forms of AUX1 would not be properly trafficked to the plasma membrane, we selected three AUX1 mutants (*aux1-7*, *aux1-102*, and *aux1-117*) that contain single amino acid substitutions in flexible loop regions of the AUX1 protein (Figure 2A). After confirming that the YFP-tagged forms of these mutants were properly sorted to the plasma membrane of injected oocytes (see Figure S1 in the Supplemental Data available online), we tested their ability to transport added ³H-IAA. ³H-IAA uptake was completely abolished in oocytes expressing either *aux1-7* or *aux1-117* mutants, but oocytes expressing *aux1-102* displayed a ~60% reduction in ³H-IAA transport activity (Figure 2B). Furthermore, the expression of the double mutant in oocytes containing both *aux1-102* and *aux1-117* substitutions abolished uptake activity (Figure 2B). Taken together, the observations that *aux1-7* and *aux1-117* abolish and that *aux1-102* modulates uptake of ³H-IAA provide additional support for the conclusion that expression of a functional AUX1 transporter protein in oocytes is required for the increased uptake of auxin that we measured in *Xenopus* oocytes.

According to the chemiosmotic model of auxin transport in plants, uptake of IAA from the extracellular space to the cytoplasm can occur by two mechanisms, diffusion of the protonated form of IAA across the plasma membrane and carrier-mediated uptake of deprotonated IAA [3, 18, 19]. If the increased ³H-IAA uptake observed in oocytes expressing AUX1 represents carrier-mediated uptake of the deprotonated form, we would expect to observe changes in rates of ³H-IAA uptake in water-injected oocytes as compared to AUX1-expressing oocytes in different pH conditions. At low pH, when ³H-IAA is predominantly protonated, uptake rates of ³H-IAA were high in both AUX1-expressing and water-injected oocytes as a result of the increased diffusion of protonated IAA across the plasma membrane (Figure 3A). In water-injected oocytes, ³H-IAA uptake was rapidly reduced to levels <20% of maximal uptake (Figure 3A, open circles) at higher pH conditions in which

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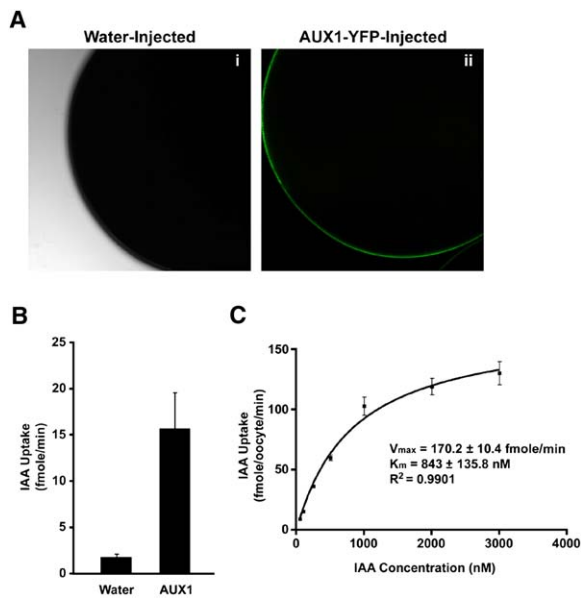


Figure 1. AUX1 Transports IAA into *Xenopus* Oocytes

(A) EYFP-AUX1 is properly targeted to the plasma membrane in *Xenopus laevis* oocytes. In panel (A_i), water-injected oocytes in both bright-field and confocal images are shown. In panel (A_{ii}), a confocal image of a *Xenopus* oocyte expressing the EYFP-AUX1 fusion protein (green) indicates localization to the plasma membrane. (B) *Xenopus* oocytes expressing the AUX1 transporter display increased IAA uptake. Uptake studies of ³H-IAA into *Xenopus* oocytes injected with water or AUX1-cRNA at pH 6.4 were performed. The oocytes injected with AUX1 cRNA displayed >10 times increase of ³H-IAA uptake in comparison to water-injected controls. Values indicated represent the mean ± standard deviation (SD); n = 8 oocytes. (C) Kinetic analysis of IAA uptake by AUX1. Uptake studies of ³H-IAA into *Xenopus* oocytes injected with AUX1-cRNA at pH 6.4 were performed. Mean IAA uptake rates at indicated concentration are shown ± SD; n = 8 for each concentration (experiments were repeated on oocytes from four different frogs and showed similar results).

the majority of IAA is deprotonated (pK_a = 4.7). In contrast, AUX1-expressing oocytes maintained higher rates of ³H-IAA uptake at >80% of maximal uptake until pH ≥ 6.5 (Figure 3A, filled circles). Although we cannot exclude that higher pH may also affect activity of the AUX1 transporter itself, at pH 6.5 the ratio of deprotonated IAA to protonated IAA is ~100:1; therefore, the majority of ³H-IAA uptake at this pH should occur via a carrier-mediated transport of the ³H-IAA anion. Measurements of extracellular pH in planta under specific conditions range from 5.3 to 5.7 [20, 21]. Even under these conditions, the predicted ratios of deprotonated IAA to protonated IAA would be ~4–10:1. Carrier-mediated uptake of ³H-IAA, defined as the difference between ³H-IAA uptake in AUX1-expressing oocytes minus ³H-IAA uptake in water-injected oocytes, displayed maximal activity between pH 6.0 and 6.5 (Figure 3A, triangles). In an earlier study, maximal carrier-mediated IAA uptake activity in suspension cells was estimated to occur at pH 5.9 [3]. Importantly, in this study the use of a heterologous oocyte expression system provided the ability to measure the specific stimulation of IAA uptake induced by AUX1 expression under different pH conditions without additional auxin carriers being present in the membrane. The observation that increased external pH up to 7.0

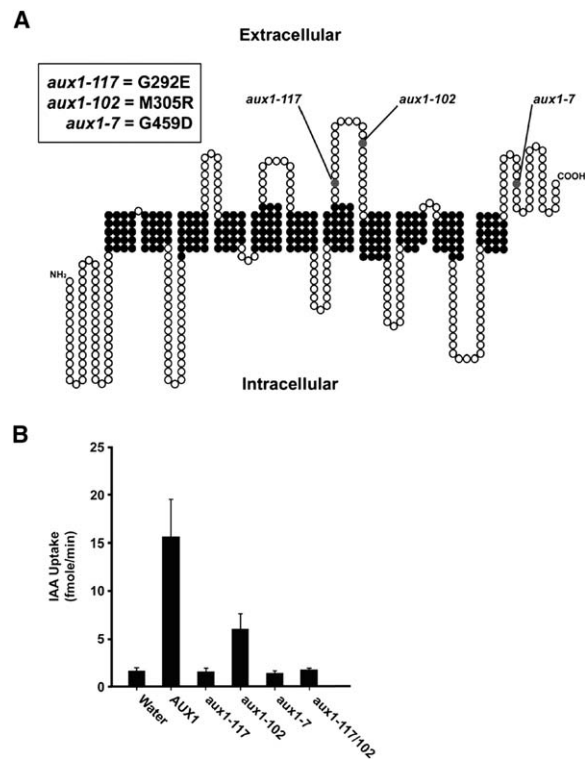


Figure 2. Mutations in AUX1 Inhibit IAA Uptake into Oocytes

(A) A schematic diagram illustrating the membrane topology of AUX1. Black circles represent AUX1 amino acid residues predicted to represent transmembrane α helices. Open circles represent amino acid residues predicted to be intra- α -helical flexible loops that are located either intracellularly or extracellularly. Point mutations analyzed in this study are indicated by gray circles, and details of these mutations are presented in the inset. (B) AUX1 mutants reduce or abolish IAA uptake. Uptake studies of ³H-IAA into *Xenopus* oocytes expressing AUX1 cRNA or AUX1 cRNA containing the indicated point mutations alter or abolish auxin uptake. Mean IAA uptake rates for the different point mutations were calculated ± SD; n = 8.

did not reduce rates of IAA uptake into AUX1-expressing oocytes in the same manner that was observed in the water-injected oocytes strongly suggests that AUX1 is transporting IAA in its anionic form.

In whole-plant tissues and in plant cells, it is difficult to assess the specific effects of auxin analogs on auxin transporters because there are multiple mechanisms for auxin uptake and efflux operating in the same membrane. The ability to measure specific uptake of ³H-IAA in AUX1-expressing oocytes provided a powerful tool for detailed examination of how specific auxin analogs modulate AUX1-mediated IAA transport across the plasma membrane. First, uptake of ³H-IAA in AUX1-expressing oocytes was effectively competed by addition of unlabeled IAA (Figure 3B). The addition of the auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D) competitively inhibited uptake of ³H-IAA in AUX1-expressing oocytes (Figure 3B). In contrast, the addition of the lipophilic auxin analog 1-naphthaleneacetic acid (NAA) did not decrease ³H-IAA uptake in AUX1-expressing oocytes (Figure 3B). These results are consistent with whole-plant studies because, whereas aux1 mutant plants are less sensitive to both IAA and 2,4-D, aux1

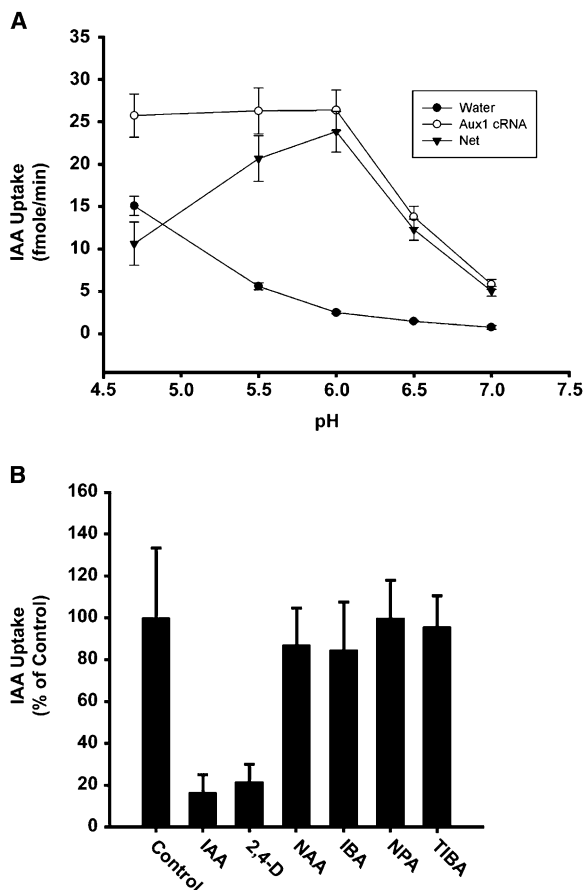


Figure 3. AUX1 Uptake in Oocytes is pH Dependent and Can Be Inhibited by IAA Analogs

(A) AUX1-mediated IAA uptake is pH dependent. Uptake studies of ^3H -IAA into *Xenopus* oocytes injected with water or AUX1 cRNA were performed at the indicated pH conditions. IAA uptake for water-injected oocytes (filled circles) decreased with the increase of pH, whereas AUX1-injected oocytes (open circles) maintained high levels of IAA uptake. Net uptake (triangles) is the AUX1-mediated uptake calculated by subtracting IAA uptake observed in water-injected oocytes from AUX1-stimulated uptake.

(B). Auxin analogs affect AUX1-mediated IAA uptake. Uptake of ^3H -IAA into *Xenopus* oocytes injected with AUX1-cRNA was examined in the presence of excess unlabeled IAA (20 μM), the auxin analogs 2,4-D (20 μM) and NAA (20 μM), and the naturally occurring auxin IBA (20 μM). In addition, uptake was reduced upon incubation with the specific auxin-uptake inhibitors 1-NOA (20 μM) and 2-NOA (20 μM), but no effect was observed upon incubation with specific auxin-efflux inhibitors NPA (20 μM) and TIBA (20 μM). Mean uptake rates for each compound were calculated \pm SD; $n = 8$.

mutant plants respond to NAA in a similar fashion as wild-type [19]. The sensitivity of *aux1* knockout plants to NAA may be due to the lipophilic nature of NAA, as has previously been proposed [22, 23], or may simply be due to the fact that NAA is not a substrate of the AUX1 influx carrier.

We also tested the ability of AUX1 to recognize other natural plant auxins. Addition of indole-butyric acid (IBA), which often accounts for 25%–30% of total auxin concentrations in some plants [24], did not competitively inhibit ^3H -IAA uptake (Figure 3B). These results support recent findings showing that whereas IAA transport is reduced, IBA polar transport is unchanged in the

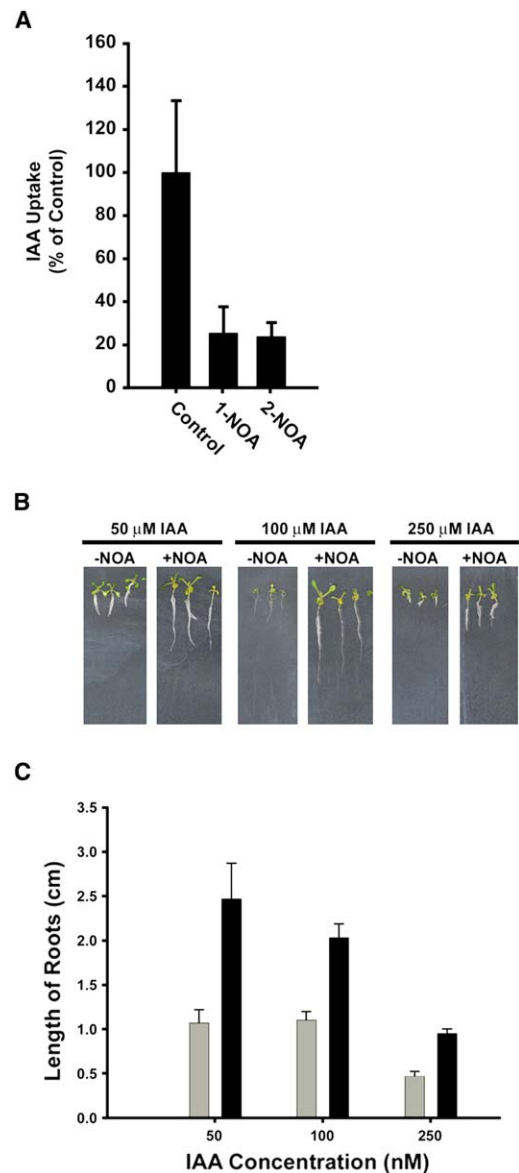


Figure 4. NOA Inhibits AUX1-Mediated IAA Uptake in Oocytes and Reduces IAA Effects on Root Growth

(A) The specific IAA uptake inhibitors 1-NOA and 2-NOA interfere with AUX1-mediated IAA uptake. Uptake of ^3H -IAA into *Xenopus* oocytes injected with AUX1-cRNA was reduced in the presence of excess unlabeled 1-NOA (20 μM) and 2-NOA (20 μM).

(B) NOA blocks inhibition of root elongation by IAA. Shown are representative images indicating the effect of various IAA concentrations upon root elongation in wild-type *A. thaliana* (Col-0) when seedlings were grown on 1/4MS media either in the absence or presence of 20 μM NOA.

(C). Quantitation of 1-NOA effect upon IAA inhibition of *A. thaliana* root elongation. Mean lengths \pm SD of roots of wild-type plants in the presence of IAA alone (gray bars) or IAA plus 20 μM NOA (black bars) are shown \pm SD; $n = 5$ seedlings for each IAA concentration.

aux1-7 mutant background [25]. These results also further support the possibility that IAA and IBA influx carriers are distinct [26, 27]. Finally, we tested whether the auxin-efflux inhibitors 1-N-naphthylphthalamic acid (NPA) or 2,3,5-triodobenzoic acid (TIBA) effected ^3H -IAA uptake in AUX1-expressing oocytes. Neither NPA nor TIBA (Figure 3B) inhibited AUX1-mediated

³H-IAA uptake, confirming previous results indicating that these inhibitors are specific for auxin-efflux transporters [18, 28, 29] and do not affect *AUX1*-mediated uptake of IAA.

To determine whether observations made in oocytes could be correlated to *AUX1* function in whole-plant tissues, we tested the IAA influx inhibitor 1-naphthoxyacetic acid (1-NOA) on *AUX1*-expressing oocytes and *Arabidopsis*. This inhibitor competes with 2,4-D, but not NAA, as determined with root elongation assays [30]. Because IAA is the main naturally occurring auxin and is transported by *AUX1*, we tested whether 1-NOA competes for IAA uptake in the *AUX1*-expressing oocytes. We showed that the specific auxin-influx inhibitor 1-naphthoxyacetic acid (1-NOA) [30] and the isomeric 2-naphthoxyacetic acid (2-NOA) [30], reduced uptake of IAA in oocytes expressing *AUX1* (Figure 4A). Addition of exogenous IAA to wild-type *Arabidopsis* seedlings reduced root elongation up to 80% at concentrations of 50 nM (Figures 4B and 4C). However, upon addition of the auxin-influx inhibitor 1-NOA the effect of exogenous addition of IAA was effectively competed. In wild-type plants, addition of 20 μ M 1-NOA resulted in 3-fold-longer roots in the presence of 50 nM IAA as compared to 50 nM IAA alone (Figures 4B and 4C). We did not use 2-NOA to compete with IAA in the root elongation assay because this auxin analog is biologically active and 2-NOA treatment alone inhibits root elongation ([30] and data not shown). These results indicate that 1-NOA is a competitive inhibitor for IAA during carrier-mediated uptake in *AUX1*-expressing oocytes, and this inhibitory activity could be indirectly reproduced, in whole plants.

Conclusions

This report provides the first glimpse into the functional characteristics of an auxin-influx carrier protein, *AUX1*, which was previously reported to be a putative auxin-influx carrier on the basis of genetic data and the analysis of mutants in *Arabidopsis*. Here we have shown that *AUX1* is capable of transporting IAA by expressing *AUX1* in *Xenopus* oocytes. With a measured K_m of \sim 800 nM for IAA, the transport activity of *AUX1* would be significant at IAA levels previously measured in plants (\sim 1–100 nM) [31] and extracellular pHs of 5.3–5.7. We demonstrated that three independent point mutations of *AUX1* that abrogate its function in planta were successfully targeted to the oocyte plasma membrane but either eliminate or reduce the transport activity of *AUX1* in oocytes. Finally, transport of IAA was inhibited by the auxin analogs 2,4-D, 1-NOA and 2-NOA but not by NAA or by another natural plant auxin, IBA. This functional characterization of an auxin-influx carrier protein provides strong evidence to support the hypothesis that IAA can be selectively taken up by specialized plasma-membrane transporter proteins. Knowledge of the functional characteristics of this auxin transporter will facilitate the understanding of mechanisms of cellular uptake of IAA and provides new insights into how this important plant hormone is transported in plant cells.

Experimental Procedures

RNA Transcription

The cDNA of *AUX1* was cloned by RT-PCR of mRNA from wild-type *Arabidopsis* and then inserted into pOO2 vector [32]. The construct

was fully sequenced to ensure that no errors were introduced by amplification. The resulting construct was linearized with PmlI. Capped RNA (cRNA) of *AUX1* was transcribed in vitro with SP6 RNA polymerase by using the mMESSAGE mMACHINE kit (Ambion, Austin, Texas).

Generation of aux1 Mutations

Site-directed mutagenesis was used to create *aux1* mutants. Primers (*aux1-117*, forward: GCTTCGAAGACGCACTTCTCGAC CACTCC and reverse: CCAGTAAACGGCGGCAGCTGACG; *aux1-102*, forward: TCTCTTAGGCCCAAGAACGCGTGGCGTG and reverse: GAAAGCGTTGGAGTGGTCGAGAAGTG; and *aux1-7*, forward: ACTTTTGATCTCTTTGCCAAGTGTACCAATG and reverse: GTCGACTTGACGAACAAAGTTGGTTAC) were used to perform whole-plasmid PCR with pENTR/D-TOPO (Invitrogen, Carlsbad, California) containing *AUX1*. Amplified plasmids containing mutant *aux1* sequences were separated from wild-type sequences by digestion with Dpml [33] and transformed into *E.coli*. Isolated plasmids containing *aux1* mutants were confirmed by sequencing. Untagged *aux1* mutant coding sequences were then amplified from these resulting pENTR/D-TOPO plasmids with the primers BamHI-*AUX1* forward: CGGGATCCATGTCGGAAGGAGTAGAAGC and XbaI-*AUX1* reverse: GCTCTAGATCAAAGACGGTGGTGTAAAGC and were cloned into BamHI and XbaI sites of the pOO2 vector and sequenced to ensure no additional mutations had been introduced. For generation of YFP-tagged *AUX1* constructs, the pENTR/D-TOPO *aux1* mutant coding sequences were transferred into pEarlyGate 104 acceptor plasmids [34] by using the Gateway LR Clonase recombination system (Invitrogen). These YFP-*aux1* mutant fusions were then PCR amplified with the primers BamHI-YFP forward: CGGGATCCATGGCAAGGGCGAGGAGC and XbaI-*AUX1* reverse and were cloned into BamHI and XbaI sites of the pOO2 vector and sequenced.

Oocyte Preparation

Oocytes were isolated from *Xenopus laevis* frogs with the standard techniques [35]. Oocytes were incubated in Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES-NaOH, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄] with 50 μ g/ml gentamycin overnight. Stage V and VI oocytes were chosen for injection with 50 nl cRNA (50 ng/oocyte). The oocytes were incubated at 16°C for 6 days before performing ³H-IAA uptake assays. The incubation solution was changed each day.

IAA Uptake Assay

After preincubation in Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 1 mM NaHCO₃, 10 mM HEPES-NaOH, 1 mM MgCl₂) pH 6.4 for 20 min, the oocytes were transferred to Ringer solution containing IAA or other auxin analogs at specified concentrations and incubated for 30 min at room temperature, and the oocytes were then washed five times with Ringer solution containing 5 μ M unlabeled IAA. Oocytes were subsequently lysed in 2% SDS for 30 min, the scintillation mixture was added, and the sample was counted. Preliminary time-course experiments were conducted and showed that under all conditions, ³H-IAA uptake rates were linear for more than 30 min. All oocyte uptake experiments reported were repeated a minimum of three times with oocytes isolated from multiple frogs.

Growth Media and Condition

Arabidopsis plants were grown on 1/4 MS media with or without IAA and/or NOA. The plates were incubated in the growth chamber with 22°C and 16 hr day and 8 hr night.

Supplemental Data

Supplemental Data include one figure and are available with this article online at: <http://www.current-biology.com/cgi/content/full/16/11/1111-1117/DC1/>.

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