Running Head: Mutations in TIR1 that confer auxin hypersensitivity

Corresponding Author:

Mark Estelle
UCSD
9500 Gilman Dr.
La Jolla, CA 92030-0116
858-539-5128
mestelle@ucsd.edu

Research Areas: Signaling and Response; Genes, Development, and Evolution.
Mutations in the TIR1 auxin receptor that increase affinity for Aux/IAA proteins result in auxin hypersensitivity

Hong Yu, a Britney Moss, b Seunghee S. Jang, c Michael Prigge, a Eric Klavins, c Jennifer Nemhauser, b and Mark Estelle a

a Howard Hughes Medical Institute and Section of Cell and Developmental Biology, University of California San Diego, La Jolla, California, USA.
b Department of Biology, University of Washington, Seattle, Washington, USA.
c Department of Electrical Engineering, University of Washington, Seattle, Washington, USA.

Summary: A directed genetic screen for novel mutations in the TIR1 auxin receptor produced auxin receptors with increased activity both in vitro and in the plant.
Footnotes

Financial Information: This work was supported by grants from the NIH (GM43644 to ME), the Howard Hughes Medical Institute (to ME), the Gordon and Betty Moore Foundation (to ME) and the Paul G. Allen Family Foundation (to EK and JN).

Corresponding Author: Mark Estelle, mestelle@ucsd.edu
ABSTRACT

The phytohormone auxin regulates virtually every aspect of plant development. The hormone directly mediates the interaction between the two members of the auxin co-receptor complex, a TIR1/AFB F-box protein and an Aux/IAA transcriptional repressor. To learn more about the interaction between these proteins, a mutant screen was performed using the yeast two-hybrid system. Two tir1 mutations were identified that increased interaction with Aux/IAAs. The D170E and M473L mutations increase affinity between TIR1 and the degron motif of Aux/IAAs and enhance the activity of the SCFTIR1 complex. This results in faster degradation of Aux/IAAs and increased transcription of auxin responsive genes in the plant. Plants carrying the pTIR1:tir1 D170E/M473L-Myc transgene exhibit diverse developmental defects during plant growth and display an auxin-hypersensitive phenotype. This work demonstrates that changes in the LRR domain of the TIR1 auxin co-receptor can alter the properties of SCFTIR1.
INTRODUCTION

The plant hormone indole-3-acetic acid (IAA) is the most important natural auxin with the ability to regulate virtually every aspect of plant development (Woodward and Bartel, 2005; Moller and Weijers, 2009; Sundberg and Ostergaard, 2009; Takahashi et al., 2009; Overvoorde et al., 2010; Vernoux et al., 2010). Auxin signaling is mediated by at least three protein families; the TIR1/AFB F-box proteins, the Aux/IAA transcriptional repressors, and the ARF transcription factors. At low auxin levels, the transcriptional activity of the ARF proteins is inhibited through an interaction with an Aux/IAA protein and the co-repressor TOPLESS (TPL) (Reed, 2001; Tiwari et al., 2001; Weijers et al., 2005; Guilfoyle and Hagen, 2007; Szemenyei et al., 2008). Auxin promotes the recruitment of the Aux/IAA protein to the SCFTIR1/AFB E3 ubiquitin ligase leading to degradation of the Aux/IAA proteins by the ubiquitin-proteasome pathway, thus allowing ARF-dependent transcription (Ruegger et al., 1998; Gray et al., 2001; Mockaitis and Estelle, 2008).

In Arabidopsis, 29 Aux/IAAs have been identified, most of which share a similar protein structure (Reed, 2001). Domain I of the Aux/IAAs binds TPL and is required for transcriptional repression (Tiwari et al., 2004; Szemenyei et al., 2008). The core region of domain II is a 6-amino acid sequence (VGWPPV/I) called the degron, which is required for proteolytic degradation of the Aux/IAA proteins (Worley et al., 2000; Ramos et al., 2001; Dreher et al., 2006). Several dominant or semi-dominant gain-of-function mutants, mutated within the conserved degron sequence, have been isolated (Reed, 2001). Later studies showed that these mutations stabilize the affected Aux/IAAs leading to auxin resistance (Worley et al., 2000; Ramos et al., 2001). Domains III and IV mediate homo- and hetero-dimerization, including with the ARF proteins (Mockaitis and Estelle, 2008).

TIR1 is a member of a small family of F-box proteins that contains 5 additional AFB proteins, AFB1-5 (Dharmasiri et al., 2005). All six members function as auxin receptors...
(Dharmasiri et al., 2005; Dharmasiri et al., 2005; Greenham et al., 2011). The TIR1 structure in complex with auxin, ASK1 (Arabidopsis SKP1) and the degron motif from IAA7 revealed that the 18 Leucine-Rich-Repeat (LRRs) domain forms an auxin binding pocket and binding surface for the Aux/IAA protein (Tan et al., 2007). Auxin acts as a “molecular glue” to direct the interaction between TIR1 and the degron motif of Aux/IAAs (Tan et al., 2007). Recent studies have shown that different combinations of TIR1/AFB and the Aux/IAA proteins form co-receptor complexes with a wide range of auxin-binding affinities, which may function in different auxin-regulated processes (Navarro et al., 2006; Parry et al., 2009; Vidal et al., 2010; Calderon Villalobos et al., 2012).

To learn more about the interactions between auxin, TIR1/AFBs and Aux/IAAs, we performed a mutant screen based on the yeast two-hybrid system (Prigge et al., 2010; Calderon Villalobos et al., 2012). Two TIR1 mutations, D170E and M473L, were identified that increase the interaction between TIR1 and Aux/IAAs. We present data showing that these two mutations increase the affinity between TIR1 and the degron motif of Aux/IAAs, and further enhance the degradation of Aux/IAAs in the plant. Plants expressing the mutant genes exhibit an auxin-hypersensitive phenotype.

RESULTS
Identification of TIR1 Mutations with Increased Affinity for Aux/IAAs

Previous studies showed that the LexA yeast two-hybrid system can be used to study the interaction between auxin receptors TIR1/AFB1-3 with their substrates Aux/IAAs (Prigge et al., 2010; Calderon Villalobos et al., 2012). The TIR1/AFB1-3 proteins interact with the Aux/IAAs in an auxin-concentration-dependent manner. However, the interaction does not occur between TIR1/AFB1-3 and degron-mutated Aux/IAAs even in the presence of auxin (Prigge et al., 2010; Calderon Villalobos et al., 2012).

Using error-prone polymerase chain reaction, random mutations were introduced into the full length TIR1 to create an extensive library of TIR1 mutants. The TIR1 mutant library
was then fused to the LexA DNA binding domain (DB) and introduced into a strain expressing the IAA12 protein fused to the B43 activation domain (AD). IAA12 was chosen because it has a relatively low affinity for TIR1, making it easier to identify mutations that increase the interaction. By screening the mutant library, we aimed to find TIR1 mutations that increased interaction with AD-IAA12 upon auxin treatment. Twelve yeast colonies were recovered in the screen and the tir1 plasmids were sequenced.

Two TIR1 mutations, D170E and M473L, were identified that increase the strength of the interaction between TIR1 and IAA12 compared to control yeast colonies as measured by β-gal activity (Fig. 1A). To determine if this behavior was specific for IAA12, we tested the mutant TIR1 proteins against a number of additional Aux/IAA proteins. In every case the mutations increased the level of β-gal activity (Fig. 1A). Further, the TIR1 mutant proteins do not interact with degron-mutated IAA7 mutants in the presence or absence of auxin (Fig. 1B). The results of protein blots show that the TIR1 mutants are expressed at a similar level as the wild type, suggesting that the enhanced interaction in yeast may be caused by changes in affinity between TIR1 and Aux/IAAs (Supplemental Fig. 1A). To address this possibility, GST-tagged IAA3 was purified from E.coli and an in vitro pull-down assay was performed with mutant and wild-type TIR1-Myc synthesized by TNT® coupled wheat germ extract (TNT). For this experiment, we also generated a double mutant protein carrying both the D170E and M473L substitutions. The results show that GST-IAA3 pulls down more mutant protein than the wild type TIR1 both in the absence and presence of auxin (Fig. 2A). In addition, the effects of each mutation were additive such that the double mutant protein had even higher affinity for IAA3. To further verify this result, the tir1 D170E/M473L-Myc construct regulated by the native TIR1 promoter was created and introduced into a pHS:AXR3NT-GUS transgenic line. This line can express domain I and II of AXR3/IAA17 (AXR3NT) upon heat shock and is used as a reporter of auxin-dependent degradation of Aux/IAAs (Gray et al., 2001). After heat shock and treatment with MG132, an inhibitor of the 26S proteasome, total protein extract was...
prepared and incubated with and without auxin. TIR1-Myc was pulled down by anti-c-Myc beads and the amount of AXR3NT-GUS in the complex was determined. The results show that tir1 D170E/M473L-Myc pulls down much more AXR3NT-GUS than the control (Supplemental Fig. 1B). These experiments indicate that the mutations enhance the affinity between TIR1 and Aux/IAA proteins. However, the effect of the mutations can be abolished with anti-auxin compound, auxinole (Hayashi et al., 2012). Auxinole strongly interacts with Phe82 of TIR1, a residue crucial for Aux/IAA recognition, and blocks the formation of the TIR1-IAA-Aux/IAA complex. The result shows that GST-IAA3 cannot pull down additional tir1 D170E/M473L-Myc in the presence of auxinole, similar to the TIR1 control (Fig. 2B).

D170 and M473 Are Required for the Function of TIR1

The structure of TIR1 shows that D170 and M473 are outside of the auxin binding pocket. D170 is located on the top of the LRRs (Leucine-Rich-Repeats) domain while M473 is in a helix region within the hydrophobic core of this domain (Supplemental Fig. 2A). Interestingly, D170 is conserved among the 6 TIR1/AFB proteins. In addition, the residue corresponding to TIR1 D170 in AFB4 (D250) is mutated to an N in the abf4-2 mutant (Greenham et al., 2011). To determine the effects of the D to N substitution on TIR1, we made this mutant protein and tested it in the pull-down assay. The tir1 D170N protein has reduced interaction with GST-IAA3 (Supplemental Fig. 2B).

To further explore the function of residues D170 and M473, the tir1 mutations D170A and M473A were generated and tested in the two-hybrid system. The results show that DBD-tir1 D170A-Myc displays a reduced interaction with AD-Aux/IAAs in the presence of auxin, while DBD-tir1 M473A-Myc does not interact with AD-Aux/IAAs compared to the control (Fig. 2C). Similar results were obtained by in vitro pull-down assay. The D170A substitution reduced recovery of TIR1 while the M473A-Myc protein did not respond to auxin (Fig. 2D). These results indicate that D170 and M473 are required for TIR1 function.
The TIR1 Mutants Enhance the Interaction with the Degron Motif of Aux/IAAs

Structural studies have established the spatial relationship between TIR1 and the degron motif of IAA7. However, the structure of other domains of IAA7 and their potential interaction with TIR1 is not known. To further study the effect of D170M and M473L, the interaction between the TIR1 mutants and different fragments of IAA7 was determined by yeast two-hybrid test. The results show that the mutations increase the interaction between TIR1 and different IAA7 fragments including DI/II, DI/II/III and DII/III/IV, suggesting that the mutations may enhance the interaction with DII of IAA7 (Fig. 3A). To address this possibility, a yeast 2-hybrid experiment was performed to test the interaction between the degron motif from IAA7 and the tir1 D170E M473L. The results show that the mutant protein interacts better with domain II of IAA7 upon auxin treatment than does the control (Fig. 3B and 3C). Similar results were observed in pull-down assays. The tir1 D170E/M473L protein does not interact with GST-DI or DIII/IV of IAA7 (Supplemental Fig. 1C). However, GST-degron motif pulls down much more tir1 D170E/M473L-Myc than the TIR1-Myc control in the absence and presence of auxin (Fig. 3D). This indicates that the TIR1 mutations enhance the interaction with the degron motif of Aux/IAAs and that this effect is at least partially independent of auxin.

To determine whether the effect of the mutations is specific to IAA, different auxins were tested, including 4-Cl-IAA, NAA, 2,4-D and picloram. The results show that each of these auxins except picloram dramatically increased the interaction. The behavior of picloram is consistent with earlier studies showing that TIR1 does not recognize picloram (Calderon Villalobos et al., 2012) (Supplemental Fig. 1D).

The TIR1 Mutations D170E and M473L Enhance the Degradation of Aux/IAAs

Although neither D170 nor M473 are in the F-box domain, it is possible that the mutations affect the interaction with the ASK1 adaptor protein. We tested this possibility in
yeast. The results show that the TIR1 mutants exhibited a similar interaction with ASK1 as the control, suggesting that the mutations do not affect the formation of the TIR1-ASK complex (Supplemental Fig. 3).

To explore the effects of the mutations on degradation of Aux/IAAs, we used the $pTIR1::tir1\ D170E/M473L-Myc\ pHS::AXR3NT-GUS$ transgenic line. GUS activity was measured after heat shock treatment. Compared to the control, the $tir1\ D170E/M473L-Myc$ transgenic seedlings display lower GUS activities after heat shock treatment in the absence or presence of auxin, suggesting that the $tir1\ D170E/M473L-Myc$ transgenic plants degrade AXR3NT faster than the control (Fig. 4A and 4B). A protein blot showed that the transgenic lines expressed TIR1 at a similar level (Fig. 4C).

As a complement to this experiment we also assessed the effects of the mutation on Aux/IAA degradation in Saccharomyces cerevisiae (Havens et al., 2012). Since eukaryotes share conserved components in the SCF-dependent degradation pathway, YFP-Aux/IAA fusion proteins are rapidly degraded by SCFTIR1 complex upon auxin treatment in yeast (Nishimura et al., 2009; Havens et al., 2012). The amount of YFP-Aux/IAA fusion proteins was determined by flow cytometry and used to calculate the Aux/IAA degradation rate [$k_5$ according to the nomenclature in (Havens et al., 2012)]. Faster degrading Aux/IAAs have larger $k_5$ values, while more stable Aux/IAAs have lower $k_5$ values. Initially, we tested degradation of IAA7 and IAA28. Our results show that both the D170E and M473L dramatically enhance the degradation of both Aux/IAA proteins upon auxin treatment compared to the control (Fig 5A, Table 1). The mutations appear to act independently on degradation since the effects are additive or synergistic in the double mutant. The D170E M473L double mutant also increased the rate of degradation of IAA1, IAA6, and IAA17 indicating that the mutations do not act selectively with respect to Aux/IAA protein (See Supplemental Fig. 4).

We also examined degradation of two truncated IAA28 proteins containing DII; t2, centered on the DII domain, and t2v, shifted N-terminal to include the upstream KR
sequence (Fig 5B and Table 1). Both proteins were rapidly degraded by D170E M473L, consistent with our two-hybrid and pulldown assays, indicating that the mutations affect the interaction with the DII domain. Finally, we examined degradation of IAA20, which lacks the DII domain. This protein is stable confirming that the DII region is required for degradation by either wild-type or mutant proteins (Fig. 5B).

The TIR1 Mutant Transgenic Lines Display Diverse Developmental Defects and Exhibit an Auxin-hypersensitive Phenotype

To explore the effect of the tir1 mutations on plant development, the phenotype of two pTIR1::tir1 D170E/M473L-Myc transgenic lines, 6-10 and 10-10, was characterized. Levels of tir1 D170E/M473L-Myc are similar to the control (Fig. 4C). Compared with the control, the tir1 D170E/M473L-Myc plants initiate many more lateral roots and have smaller rosette leaves (Fig. 6A-C). In addition, the 6-10 plants develop fewer axillary branches after bolting compared to the control (Fig. 6D and 6E).

To characterize the auxin response in these lines, the effect of auxin on root growth was determined. 6-day old seedlings were transferred onto fresh ATS medium with different concentrations of the synthetic auxin 2,4-D. After another two days of growth, the length of newly grown primary root was measured and expressed relative to growth on control plates. The result shows that the primary root elongation is strongly inhibited in pTIR1::tir1 D170E/M473L-Myc transgenic seedlings at low concentrations of 2,4-D compared with the control (Fig. 7A). This suggests that tir1 D170E/M473L causes an auxin-hypersensitive phenotype in the plant.

Next we measured the expression of auxin responsive genes in the tir1 D170E/M473L-Myc transgenic lines using real time Q-PCR. Our results show that expression of the tir1 D170E/M473L proteins increase transcription of auxin responsive genes in the plant. For example, the tir1 D170E/M473L-Myc transgenic seedlings display a significantly increased IAA3 RNA level compared with the wild type TIR1 control with or
without 2,4-D treatment (Fig. 7B). Similar effects can be observed for other auxin responsive genes as well (Fig. 7B).

**The Function of the Mutations May be Unique for the TIR1 Protein**

TIR1 belongs to a small family of F-box proteins that contains 5 additional AFB proteins, AFB1-5 (Dharmasiri et al., 2005). As previously noted, D170 is conserved in all 6 proteins, and the *afb4-2* mutation affects this residue (Greenham et al., 2011). However we find that the corresponding D to E substitution in AFB1 and AFB2 does not increase binding to the Aux/IAAs (See Supplementary Fig. 5B and 5C). In the case of AFB2, the mutation decreased the interaction. Either M or L can be found in other family members at positions homologous to TIR1 M473. The M468L mutation in AFB2 does not affect interaction with the Aux/IAAs. Thus, the effect of TIR1 D170E and M473L are dependent on the TIR1 context.

**DISCUSSION**

The ubiquitin-proteasome pathway is one of the most important proteolytic pathways in eukaryotes. In this pathway, the small protein ubiquitin (Ub) is attached to protein substrates and the Ub-protein conjugates are recognized and degraded by the 26S proteasome (Fang and Weissman, 2004). The SCF complexes are a major class of ubiquitin ligase enzyme (Gagne et al., 2002; Petroski and Deshaies, 2005). In this complex, the F-box protein plays a critical role in the determination of substrate specificity (Petroski and Deshaies, 2005). The plant hormone auxin directly induces rapid degradation of the Aux/IAA family of transcriptional repressors by SCF^{TIR1/AFB} E3 ubiquitin ligase (Gray et al., 2001; Dharmasiri et al., 2005; Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). In *Arabidopsis*, TIR1 is a member of a small group of F-box proteins that also includes AFB1 through AFB5 and the jasmonic acid receptor, COI1 (Dharmasiri et al., 2005). The TIR1 protein plays a critical role in regulating most aspects
of auxin response.

In this study, two TIR1 mutations, D170E and M473L, were isolated that increase the interaction between TIR1 and the Aux/IAA proteins (Fig. 1A and 1C). Further studies show that the TIR1 mutations increase the affinity for the degron motif of the Aux/IAAs (Fig. 2A and Supplemental Fig. 1B). Both D170 and M473 are located outside of the TIR1 auxin-binding pocket and do not directly contact auxin or the degron motif of Aux/IAAs (Tan et al., 2007). However, when they are mutated to A, they either reduce the interaction with IAA7 for D170A, or abolish the interaction in the case of M473A, indicating that these two positions are essential for TIR1 function (Fig. 2C and 2D).

At this point it is not clear how the two mutations impact Aux/IAA binding. It is possible that they induce structural changes in the auxin-binding pocket that result in more efficient contact between TIR1 and the degron motif. For example, the two oxygens in the carboxyl group of D170 form hydrogen bonds with two backbone amide groups (A195 and S196) in an adjacent loop. The D170E substitution may strengthen these interactions while D170A would eliminate them. Alternatively because D170 is on the surface of the LRR domain, the longer side chain of glutamic acid may promote an interaction with either the Aux/IAA or another unknown interacting protein. Because M473 is within the cores of the LRR domain, mutation to alanine is likely to affect folding of the domain. M473 is buried within the LRR domain and hence cannot contact other interacting proteins. Substitution of L for M may result in a subtle change in the LRR domain, somehow facilitating interaction with the degron.

Experiments with the pHS:AXR3NT-GUS transgenic line show that the pTIR1:tir1 D170E/M473L-Myc plants degrade AXR3 faster than the wild type, consistent with the observation that the mutations increase the affinity with Aux/IAAs and further enhance the function of the SCFtir1 complex in yeast (Fig. 4 and 5). The root elongation of the pTIR1:tir1 D170E/M473L-Myc line is more sensitive to low concentrations of auxin treatment; meanwhile, an increased transcription of auxin responsive genes has been observed.
even without auxin treatment, indicating that tir1 D170E/M473L enhances auxin signal transduction in the plant (Fig. 8).

Previous studies demonstrated that the TIR1-IAA-degron module is a powerful tool to study protein function. The auxin-inducible degron (AID) system can be used to control the degradation of target proteins upon auxin treatment in a reversible manner in nonplant cells such as yeast, chicken, mouse, hamster, monkey and human cells (Nishimura et al., 2009; Kanke et al., 2011; Holland et al., 2012). The system is particularly useful for investigating the function of proteins in specific developmental processes (Holland et al., 2012). In our yeast analysis, the tir1 D170E/M473L protein increases the rate of degradation of YFP-Aux/IAA7/28 approximately 15 to 25 fold compared to wild-type TIR1 (Fig. 6). Our results suggest that it may be possible to modify the TIR1/AFB proteins from Arabidopsis or other plant species to increase the flexibility of the AID system.

MATERIAL AND METHODS

Generation of mutant library and mutant screen

Full length TIR1 cDNA was mutagenized by error–prone PCR with a mutation ratio of approximately 2 mutations per tir1 molecule. The PCR products were ligated into the pGILDA vector and transformed into E. coli competent cells to generate a library of approximately 1.3×10⁴ colonies. The library was transformed into the pB42AD:IAA12 yeast strain (EGY48) and yeast colonies were screened on SD-U-H-W-L medium supplemented with 10μM IAA. The number of total yeast colonies screened was about 2.5×10⁵. Yeast colonies that grew fastest were isolated and tested on X-gal plates. The pGILDA:tir1-Myc plasmids from positive colonies were extracted and sequenced.

Plant materials and conditions

All Arabidopsis thaliana mutants used in this study were generated in the Columbia-0 (Col-0) ecotype. Seeds were surface sterilized for 20min in 30% commercial bleach,
plated on ATS medium (Arabidopsis thaliana solution) supplemented with 0.8% agar, and stratified for 2 - 4 days at 4°C. ATS medium consists of 1% sucrose, 5mM KNO₃, 2.5mM KPO₄, 2mM MgSO₄, 2mM Ca(NO₃)₂, 50μM Fe-EDTA, and 1mL/L of micronutrients. All seedling experiments were performed under long day conditions (16h light and 8h dark) a growth chamber (80μmol/m²/s, 22°C), unless otherwise stated. Plants in soil were grown in long day conditions at 22°C.

**Plasmid constructs and generation of transgenic lines**

The pTIR1:TIR1/tir1-Myc constructs were made by introducing a 2kb 5’ upstream region of the TIR1 gene adjacent to the TIR1/tir1 cDNA into pGWB16 vector. In all cases, plasmids were introduced into Col-0 plants as described in (Clough and Bent, 1998)

**RNA extraction and real time RT-PCR**

Total RNA was extracted from 7-day-old seedlings by the RNeasy plant mini kit (Qiagen) and RNA yield was quantified using a Thermo Scientific NanoDrop 2000. 1μg of RNA was used for cDNA synthesis using the Superscript III First-Strand Synthesis kit (Invitrogen). Quantitative RT-PCR was performed as previously described (Greenham et al., 2011). Data was normalized to the reference PP2AA3 according to the △Ct method.

**Protein extraction and pull-down assays**

To perform pull-down assays from plant extracts, tissue was harvested, ground to a powder in liquid nitrogen, and vortexed vigorously in extraction buffer (50mM Tris pH7.5, 150mM NaCl, 10% glycerol, 0.1% NP-40, complete protease inhibitor (Roche), 20μM MG132). Cellular debris was removed by centrifugation and total protein concentration was determined using the Bradford assay. Total protein extract (1mg) was incubated with or without 50μM IAA at 4°C for 4hr. TIR1-Myc was recovered with 20μl of anti-c-Myc agarose beads (clontech) and washed by extraction buffer for 5 times. The protein
sample was eluted by SDS-PAGE sample buffer, heated for 8 min at 90°C, cooled on ice for 2 min and fractionated by SDS-PAGE. The AXR3NT-GUS protein was detected by immunoblotting with anti-GUS (Invitrogen) and visualized using the ECL Plus Western Blotting Detection System (Amersham).

The in vitro pull-down assay from TNT® coupled wheat germ extracts was described previously (Parry et al., 2009). Briefly, GST-Aux/IAA was expressed in E.coli and purified with glutathione agarose beads (Sigma). TIR1/tir1-Myc proteins were synthesized in the TNT® coupled wheat germ extract system (Promega). 20 μl of extract was incubated with > 10 μg of GST-Aux/IAA beads in 200 μl of lysis buffer (50 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, 0.1% Tween-20, protease inhibitors) in the presence or absence of IAA at 4°C for 1 hr. The pull-down reaction was then transferred to a Micro Bio-Spin Chromatography Column (Bio-Rad). After washing with lysis buffer, samples were eluted using reduced glutathione (Sigma) and separated by SDS/PAGE. Proteins were detected by anti-c-Myc-peroxidase antibody (Roche) and visualized.

**Measurement of β-glucuronidase and β-galactosidase activity**

GUS staining was performed on 7-day old seedling. The seedlings were collected in GUS staining solution (100 mM Na₂PO₄ pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1.0 mM K₃Fe(CN)₆ and 2 mM X-Gluc), vacuum-infiltrated for 20 min and stained overnight at 37°C. The seedlings were cleared in 70% (v/v) ethanol and imaged with a Nikon SMZ1500 dissecting microscope. MUG assays were performed to quantify β-glucuronidase activity (Ge et al., 2010). To measure β-galactosidase activity, proteins were extracted from yeast cells using Y-PER Reagent (Thermo) and the yield was determined by Bradford assay. The assay was performed using 100 μl protein extract, 200 μl 4 mg/ml ONPG with 700 μl Z-buffer (16.1 g Na₂HPO₄·7H₂O, 5.5 g NaH₂PO₄·H₂O, 0.75 g KCl, 0.246 g MgSO₄·7H₂O in 1 L water, pH 7.0) at 37°C. The reaction was stopped by adding 400 μl of 1 M Na₂CO₃ and OD₄₂₀ of the supernatant was measured.
Root inhibition assay

For root growth assays, 6-day old seedlings were transferred onto fresh ATS medium with different concentrations of 2,4-D for 2 days and the length of new primary root was measured using ImageJ software.

Aux/IAA Degradation Assays in Yeast

Yeast degradation assays were carried out as in (Havens et al., 2012). Briefly, yeast strains co-expressing stably-integrated TIR1 variants and YFP-tagged IAA proteins were prepared by transferring a freshly grown colony from YPD plates into Synthetic Complete (SC) media. Flow cytometry was used to estimate the cell density (in events $\mu L^{-1}$) and dilute cells to such that cultures were in log phase 16 h later and for the duration of the experiment. All cultures were grown at 30°C with shaking. Pre-auxin measurements were taken to ascertain baseline expression, followed by addition of auxin (10$\mu M$ indole-3-acetic acid) or mock treatment (95% [v/v] ethanol). Measurements were acquired over the course of 120-150 min following auxin treatment, with intervals ranging from 3 min early in the YFP-IAA degradation phase to 20 min later in the degradation phase. Controls were measured every hour for the duration of the experiment.

Quantitative Analysis of IAA Degradation Rate

Quantitative analysis of IAA degradation profiles obtained in yeast was conducted as in (Havens et al., 2012). Briefly, the auxin-induced degradation dynamics of YFP-Aux/IAA fusion proteins was characterized by a second-order differential equation model,

$$\begin{align*}
  x' &= k_1 u - k_2 x \\
  y' &= k_3 - k_4 y - k_5 x y.
\end{align*}$$

YFP-IAA degradation time courses were grouped according to TIR1 protein identity (wild type, D170E, M473L, or D170E/M473L) to compose smaller subsets of data. Within each
group, two parameters, k3 and k5, approximate the expression and degradation rates of
an Aux/IAA protein respectively and were allowed to vary in the estimation process while
holding the rest of the parameters constant. This global fitting approach ensured that the
degradation variability among the unique TIR1 and YFP-Aux/IAA pairs are represented in
the estimated k5 values. The model fit residual was computed using 2-norm of the
difference between the measured and model predicted YFP intensity at the measurement
times 0 <= t <= 150 min.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or
GenBank/EMBL databases under the following accession numbers: TIR1 (At3G62980),
AFB1 (At4G03190), AFB2 (At3G26810), IAA3 (At1G04240), IAA5 (At1G15580), IAA7
(At3G23050), IAA19 (At3G15540), IAA20 (At2G46990), IAA28 (At5G25890), GH3.1
(At2G14960), and GH3.3 (At2G23170).

ACKNOWLEDGEMENTS
We thank Professor Ning Zheng and members of the Estelle and Nemhauser groups for
helpful discussions.
References

Calderon Villalobos LI, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, Sheard LB, Tan X, Parry G, Mao H, Zheng N, Napier R, Kepinski S, Estelle M (2012) A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of auxin. Nature chemical biology 8: 477-485

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature 435: 441-445

Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jurgens G, Estelle M (2005) Plant development is regulated by a family of auxin receptor F-box proteins. Dev Cell 9: 109-119

Dreher KA, Brown J, Saw RE, Callis J (2006) The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. Plant Cell 18: 699-714

Fang S, Weissman AM (2004) A field guide to ubiquitylation. Cell Mol Life Sci 61: 1546-1561

Hatfield K, Nishimura K, Kanemaki M, Kakimoto T, Takahashi TS, Nakagawa T, Masukata H (2011) Auxin-inducible protein depletion system in fission yeast. BMC Cell Biol 12: 8

Kepinski S, Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435: 446-451

Mockaitis K, Estelle M (2008) Auxin receptors and plant development: a new signaling paradigm. Annu Rev Cell Dev Biol 24: 55-80

Moller B, Weijers D (2009) Auxin control of embryo patterning. Cold Spring Harb Perspect Biol 1: a001545

Navarro I, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312: 436-439
Nishimura K, Fukagawa T, Takisawa H, Kažimoto T, Kanemaki M (2009) An auxin-based degron system for the rapid depletion of proteins in nonplant cells. Nat Methods 6: 917-922

Overvoorde P, Fukaki H, Beeckman T (2010) Auxin control of root development. Cold Spring Harb Perspect Biol 2: a001537

Parry G, Calderon-Villalobos LI, Prigge M, Peret B, Dharmasiri S, Itoh H, Lechner E, Gray WM, Bennett M, Estelle M (2009) Complex regulation of the TIR1/AFB family of auxin receptors. Proc Natl Acad Sci U S A 106: 22540-22545

Petroski MD, Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol 6: 9-20

Prigge MJ, Lavy M, Ashton NW, Estelle M (2010) Physcomitrella patens Auxin-Resistant Mutants Affect Conserved Elements of an Auxin-Signaling Pathway. Curr Biol 20: 1907-1912

Ramos JA, Zenser N, Leyser HM, Callis J (2001) Rapid degradation of Aux/IAA proteins requires conserved amino acids of domain II and is proteasome-dependent. Plant Cell 13: 2349-2360

Reed JW (2001) Roles and activities of Aux/IAA proteins in Arabidopsis. Trends Plant Sci 6: 420-425.

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grr1p. Genes Dev 12: 198-207

Sundberg E, Ostergaard L (2009) Distinct and dynamic auxin activities during reproductive development. Cold Spring Harb Perspect Biol 1: a001628

Szemenyei H, Hannon M, Long JA (2008) TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science 319: 1384-1386

Takahashi H, Miyazawa Y, Fuji N (2009) Hormonal interactions during root tropic growth: hydrotropism versus gravitropism. Plant Mol Biol 69: 489-502

Tan X, Calderon-Villalobos LI, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446: 640-645

Tiwari SB, Hagen G, Guilfoyle TJ (2004) Aux/IAA proteins contain a potent transcriptional repression domain. Plant Cell 16: 533-543

Tiwari SB, Wang XJ, Hagen G, Guilfoyle TJ (2001) AUX/IAA proteins are active repressors, and their stability and activity are modulated by auxin. Plant Cell 13: 2809-2822.

Vernoux T, Besnard F, Traas J (2010) Auxin at the shoot apical meristem. Cold Spring Harb Perspect Biol 2: a001487

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutierrez RA (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis thaliana. Proc Natl Acad Sci U S A 107: 4477-4482

Weijers D, Benkova E, Jager KE, Schiereth A, Hamann T, Kientz M, Wilmoth JC, Reed JW, Jurgens G (2005) Developmental specificity of auxin response by pairs of ARF and Aux/IAA transcriptional regulators. Embo J 24: 1874-1885

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot (Lond) 95: 707-735

Worley CK, Zenser N, Ramos J, Rouse D, Leyser O, Theologis A, Callis J (2000) Degradation of Aux/IAA proteins is essential for normal auxin signalling. Plant J 21: 553-562
Figure Legends

Figure 1. Identification of TIR1 mutants with binding to the Aux/IAA proteins.
(A) The TIR1 mutations D170E and M473L increase the interaction between TIR1 and different Aux/IAAs upon auxin treatment. (B) The mutants proteins do not interact with degron-mutated IAA7 proteins. The degron motif of IAA7 is VGWPPV, while it is mutated to VGWSPV in iaa7-1; VGWPSV in iaa7-2 and VGWSSV in iaa7-3. (C) Measurement of β-galactosidase activity in yeast strains with and without auxin treatment. The asterisks indicate statistically significant differences between the mutants and the wild type control (t-test, P < 0.01). Error bars are SEM.

Figure 2. The function of TIR1D170 and M473 in Aux/IAA binding.
(A) In vitro pull-down assay shows that GST-IAA3 pulls down more D170E and M473L protein than wild-type TIR1 protein. (B) The anti-auxin compound auxinole abolishes the interaction between tir1 D170E/M473L-Myc and GST-IAA3. (C) Interaction between tir1D170A and tir1M473A with Aux/IAAs assessed by Y2H. The D170A mutation reduces the interaction with IAA3 and IAA7, while tir1 M473A does not interact even after auxin treatment. (D) Interaction between TIR1 mutants and GST-IAA3 assessed by pull-down assay. Numbers indicate the fold changes relative to the control sample.

Figure 3. The tir1 mutations increase binding to the IAA7 degron motif.
(A) Y2H interaction between TIR1 single and double mutant proteins with IAA7 fragments. (B) Interaction between tir1 D170E/M473L and the IAA7 degron. (C) β-galactosidase activity in yeast cells expressing the mutants and the IAA7 degron. Error bars are SEM. The asterisks denote statistically significant differences between the mutants and the wild-type control (t-test P < 0.01). (D) In vitro pull-down assay shows that GST-degron motif pulls down more tir1 D170E/M473L-Myc protein than wild-type TIR1-Myc. The
degron motif is from position 83 to 92 of IAA7. Numbers indicate the fold changes relative to the control sample.

**Figure 4.** The *ProTIR1:tir1D170E/M473L-Myc* line exhibits increased degradation of AXR3NT. (A-B) GUS activity of indicated lines after heat shock treatment for 2hr. GUS staining and measurement of GUS activity were performed in 7-day old seedlings. Bar is 1mm. (C) Plants carrying the *ProTIR1:tir1 D170E/M473L-Myc* transgene express similar TIR1 protein levels as the control.

**Figure 5.** Degradation of YFP-IAA fusion proteins in *Saccharomyces cerevisiae* in the presence of wild-type and mutant TIR1 proteins after addition of auxin. Yeast cells were imaged using time-lapse flow cytometry. Degradation curves were normalized to starting fluorescence.

**Figure 6.** *ProTIR1:tir1 D170E/M473L* plants exhibit growth defects. (A) After 10 days of growth, *ProTIR1:tir1 D170E/M473L-Myc 6-10* seedlings have more lateral roots than the wild type. (B) 4-day-old seedlings were transferred onto medium with and without 2,4-D for an additional 6 days. The number of emerged lateral roots/mm primary root length was determined. (C) *ProTIR1:tir1 D170E/M473L-Myc* plants have smaller rosettes compared to the control line. Plants are 3 weeks old (D) *ProTIR1:tir1 D170E/M473L 6-10* plants have fewer axillary branches compared to the control. Bar is 1cM. Plants are 7 weeks old. For both (B) and (E) error bars are SEM. The asterisks denote statistically significant differences between the *tir1 D170E/M473L-Myc* and the *TIR1-Myc* control plants (t-test, P < 0.05).

**Figure 7.** The *ProTIR:tir1D170E/M473L* lines are auxin hypersensitive. (A) Root growth
on increasing concentrations of 2, 4-D relative to untreated controls. (B) Expression of auxin responsive genes in *ProTIR1:tir1D170E/M473L-Myc* plants. 7-day-old seedlings were analyzed by Q-PCR. Data shown are from three biological replicates. Error bars are SEM. The asterisks denote statistically significant differences between the *tir1 D170E/M473L-Myc* and the *TIR1-Myc* control plants (*t*-test, P < 0.05).

**Supplemental Figure 1.** Characterization of the TIR1 mutations D170E and M473L.

(A) The TIR1 mutants are expressed at a similar level as the wild type in yeast. Expression of TIR1 and IAA3 is regulated by the GAL1 promoter. Galactose (GAL) induces TIR1 and IAA3 expression while glucose does not. (B) The double mutant protein tir1D170E/M473L-Myc pulls down more AXR3NT-GUS from plant extracts in the absence or presence of auxin. Numbers indicate the fold change relative to the control sample. (C) In vitro pull-down assays show that tir1D170E/M473L does not interact with DI or DIII/IV of IAA7. DI of IAA7 is from position 1 to position 82 and DIII/IV fragment is from position 93 to the C-terminus. (D) Picloram does not enhance the tir1-Aux/IAA interaction.

**Supplemental Figure 2.** (A) The position of D170 and M473 in the TIR1 structure. Both amino acids are located outside of the auxin-binding pocket. ASK1 is blue, TIR1 is green, and IAA is red. The degron motif from DII of IAA7 is cyan. D170 and M473 are purple. The afb4-2 mutation affects residue D215 corresponding to TIR1D170. (B) In vitro pull-down assay shows that GST-IAA3 pulls down less tir1D170N-Myc protein compared to the wild-type TIR1-Myc.

**Supplemental Figure 3.** D170E and M473L do not affect the interaction with ASK1. (A) Y2H interaction between the TIR1 mutant proteins and ASK1. (B) β-galactosidase activity in extracts prepared from yeast colonies from (A). Error bars are SEM.
Supplemental Figure 4. D170E and M473L enhance the function of the SCF^{TIR1} complex in yeast. Degradation of YFP-IAA fusion proteins in Saccharomyces cerevisiae in the presence of wild-type and mutant TIR1 proteins after addition of auxin. Yeast cells were imaged using time-lapse flow cytometry. Degradation curves were normalized to starting fluorescence.

Supplementary Figure 5. The effects of D170E and M473L may be unique to TIR1. (A) Alignment at the TIR1D170 and M473 position among the auxin receptors. (B) The interaction between the AFB1/AFB2 mutant proteins and different Aux/IAAs. DBD-afb1 D166E-Myc, DBD-afb2 D165E-Myc and DBD-afb2 M468L-Myc do not display an increased interaction with AD-Aux/IAAs after auxin treatment compared to the control. (C) β-galactosidase activity of yeast colonies from (B). Error bars are SEM.
| K5   | IAA7 | IAA28 |
|------|------|-------|
| TIR1-WT | 0.023 | 0.016 |
| tir1 D170E | 0.100 | 0.081 |
| tir1 M473L | 0.150 | 0.105 |
| tir1 D170E/M473L | 0.418 | 0.232 |

| K5   | IAA28,FL  | IAA28,t2 | IAA28,t2V | IAA20 |
|------|-----------|----------|----------|-------|
| TIR1-WT | 0.024  | 0.043   | 0.009   | 0.001 |
| tir1 D170E/M473L | 0.290  | 0.730 | 0.210 | 0.001 |

**Table 1.** Degradation rates ($k_5$ value) for various IAAs in the presence of wild-type and mutant TIR1 proteins. $k_5$ values were calculated as described in the Materials and Methods.
Figure 1. Identification of TIR1 mutants with increased binding to the Aux/IAA proteins.

(A) The TIR1 mutations D170E and M473L increase the interaction between TIR1 and different Aux/IAAs upon auxin treatment. (B) The mutant proteins do not interact with degron-mutated IAA7 proteins. The degron motif of IAA7 is VGWPPV, while it is mutated to VGWSPV in iaa7-1; VGWPSV in iaa7-2 and VGWSSV in iaa7-3. (C) Measurement of β-galactosidase activity in yeast strains with and without auxin treatment. The asterisks indicate statistically significant differences between the mutants and the wild type control (t-test, P < 0.01). Error bars are SEM.
Figure 2. The function of TIR1 D170 and M473 in Aux/IAA binding.

(A) In vitro pull-down assay shows that GST-IAA3 pulls down more D170E and M473L protein than wild-type TIR1 protein. (B) The anti-auxin compound auxinole abolishes the interaction between tir1 D170E/M473L-Myc and GST-IAA3. (C) Interaction between tir1D170A and tir1M473A with Aux/IAAs assessed by Y2H. The D170A mutation reduces the interaction with IAA3 and IAA7, while tir1 M473A does not interact even after auxin treatment. (D) Interaction between TIR1 mutants and GST-IAA3 assessed by pull-down assay. Numbers indicate the fold changes relative to the control sample.
Figure 3. *tir1* mutations increase binding to the IAA7 degron motif.

(A) Y2H interaction between TIR1 single and double mutant proteins with IAA7 fragments. (B) Interaction between TIR1 D170E/M473L and the IAA7 degron. (C) β-galactosidase activity in yeast cells expressing the mutants and the IAA7 degron. Error bars are SEM. The asterisks denote statistically significant differences between the mutants and the wild-type control (t-test P < 0.01). (D) *In vitro* pull-down assay shows that GST-degron motif pulls down more tir1 D170E/M473L-Myc protein than wild-type TIR1-Myc. The degron motif is from position 83 to 92 of IAA7. Numbers indicate the fold changes relative to the control sample.
**Figure 4.** The ProTIR1:tir1 D170E/M473L-Myc line exhibits increased degradation of AXR3NT.

(A-B) GUS activity of indicated lines after heat shock treatment for 2hr. GUS staining and measurement of GUS activity were performed in 7-day old seedlings. Bar is 1mm. (C) Plants carrying the ProTIR1:tir1 D170E/M473L-Myc transgene express similar TIR1 protein levels as the control.
Figure 5. Degradation of YFP-IAA fusion proteins in *Saccharomyces cerevisiae* in the presence of wild-type and mutant TIR1 proteins after addition of auxin. Yeast cells were imaged using time-lapse flow cytometry. Degradation curves were normalized to starting fluorescence.
Figure 6. *ProTIR1:tir1 D170E/M473L* plants exhibit growth defects. (A) After 10 days of growth, *ProTIR1:tir1 D170E/M473L-Myc 6-10* seedlings have more lateral roots than the wild type. (B) 4-day-old seedlings were transferred onto medium with and without 2,4-D for an additional 6 days. The number of emerged lateral roots/mm primary root length was determined. (C) *ProTIR1:tir1 D170E/M473L-Myc* plants have smaller rosettes compared to the control line. (D) *ProTIR1:tir1 D170E/M473L-Myc 6-10* plants have fewer axillary branches compared to the control. Bar is 1cm. Plants are 7 weeks old. For both (B) and (E) error bars are SEM. The asterisks denote statistically significant differences between the *tir1 D170E/M473L-Myc* and the *TIR1-Myc* control plants (t-test, P < 0.05).
Figure 7. The ProTIR:tir1 D170E/M473L lines are auxin hypersensitive.

(A) Root growth on increasing concentrations of 2, 4-D relative to untreated controls.

(B) Expression of auxin responsive genes in ProTIR1:tir1D170E/M473L-Myc plants. 7-day-old seedlings were analyzed by Q-PCR. Data shown are from three biological replicates. Error bars are SEM. The asterisks denote statistically significant differences between the tir1 D170E/M473L-Myc and the TIR1-Myc control plants (t-test, P < 0.05).