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Tuning a timing device that regulates lateral root development in rice

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Abstract

Peptidyl Prolyl Isomerases (PPIases) accelerate *cis-trans* isomerization of prolyl peptide bonds. In rice, the PPIase LRT2 is essential for lateral root initiation. LRT2 displays *in vitro* isomerization of a highly conserved W-P peptide bond (¹⁰⁴W-P¹⁰⁵) in the natural substrate OsIAA11. OsIAA11 is a transcription repressor that, in response to the plant hormone auxin, is targeted to ubiquitin-mediated proteasomal degradation via specific recognition of the *cis* isomer of its ¹⁰⁴W-P¹⁰⁵ peptide bond. OsIAA11 controls transcription of specific genes, including its own, that are required for lateral root development. This auxin-responsive negative feedback circuit governs patterning and development of lateral roots along the primary root. The ability to tune LRT2 activity via mutagenesis is crucial for understanding and modeling the role of this bimodal switch in the auxin circuit and lateral root development. We present characterization of the thermal stability and isomerization rates of several LRT2 mutants acting on the OsIAA11 substrate. The thermally stable mutants display activities lower than that of wild-type (WT) LRT2. These include binding diminished but catalytically active P125K, binding incompetent W128A, and binding capable but catalytically incompetent H133Q mutations. Additionally, LRT2 homologs hCypA from human, TaCypA from *Triticum aestivum* (wheat) and PPIB from *E. coli* were shown to have 110%, 50% and 60% of WT LRT2 activity on the OsIAA11 substrate. These studies identify several thermally stable LRT2 mutants with altered activities that will be useful for establishing relationships between *cis-trans* isomerization, auxin circuit dynamics, and lateral root development in rice.

Keywords

Auxin circuit; *cis-trans* isomerization; LRT2; OsIAA11; LRT2 mutations; exchange kinetics

Introduction

Proteins are highly dynamic molecules in which motions are utilized for multiple aspects of function. NMR is uniquely suited for elucidating protein motions at atomic resolution, and has been widely applied to investigate relationships between dynamics and function (recently reviewed by Torchia¹). From the early demonstration of the role of mobility in the

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viscoelastic behavior of elastin², to the elucidation of contributions of conformational entropy to ligand binding energy^{3,4} and the importance of motions in facilitating access to enzyme active sites⁵, the development and application of protein NMR methods has enhanced our understanding of many functional aspects of protein motions.

More recently, the functional implications of protein conformational ensembles have come to light^{6–12}. A protein conformational ensemble consists of a set of chemically identical but structurally distinct conformers populated according to their relative free energies that interconvert on timescales governed by the activation barriers between them. This ensemble can include conformers that are uniquely capable of interacting with highly specific binding partners. NMR enables the detection of even very low populated conformers, as well as the measurement of exchange rates between conformers^{11,13}. A very simple example of a conformational ensemble is the distinct *cis* and *trans* isomers of a prolyl peptide bond. The *cis* and *trans* conformers are separated by a high activation barrier and undergo very slow *cis-trans* exchange (isomerization), providing a bimodal switch with an intrinsically slow flipping rate¹⁴. Peptidyl-prolyl *cis-trans* isomerase (PPIase) enzymes are required to accelerate this exchange into the shorter timescales of biological signaling and regulatory processes¹⁵. Application of NMR methods enables the full quantification of PPIase-catalyzed *cis-trans* isomerization of biological substrates^{16,17}.

A critical function of prolyl *cis-trans* isomerization is exemplified by its role in a negative feedback gene regulation circuit in plants that is responsive to the phytohormone auxin^{18–20}. The Aux/IAA family of transcription repressor proteins, which are involved in controlling many auxin-responsive developmental processes in plants²¹, contain a highly conserved sequence, the GWPPV ‘degron motif’, that is a signal for ubiquitin-mediated proteasomal degradation²². Importantly, only the *cis* W-P isomer of the Aux/IAA degron motif (Aux/IAA-WP^{*cis*}) binds along with auxin to the E3 ubiquitin ligase SCF^{TIR1}, resulting in *cis*-specific proteasomal degradation of Aux/IAA proteins in response to auxin^{23,24}. As Aux/IAA-WP^{*cis*} is degraded, a PPIase is needed to facilitate depletion of the total Aux/IAA protein level by rapidly maintaining the substrate (i.e., the *cis* conformer) at its equilibrium population. Depletion of the Aux/IAA transcription repressor activates transcription of auxin-responsive genes, which includes the gene encoding the Aux/IAA protein itself, thereby generating a classic negative feedback circuit referred to as the auxin circuit^{25,26}. The auxin circuit is a profound example of a direct link between conformer exchange kinetics (PPIase-catalyzed *cis-trans* isomerization of a W-P peptide bond in an Aux/IAA protein) and a phenotype at the whole organism level (the patterning and development of lateral roots in plants)²⁰.

A hallmark of developmental processes is oscillating gene expression that gives rise to spatial patterning during organism development²⁷. Roles of prolyl isomerases in regulating gene transcription are well established, including controlling the stability of transcription factors²⁸. In plants, the patterning of lateral roots along the primary root axis depends critically on the auxin circuit. This “biological clock” periodically marks cells for later differentiation into branch initiation sites as they move from the root tip upward along the primary root, thereby transforming the dynamics of a gene regulation circuit into the spatial patterning of lateral roots. In rice, by mutagenizing seedlings and screening for the absence

of lateral roots (the lateral rootless phenotype), the Aux/IAA protein OsIAA11 and the PPIase LRT2 (a PPIase classified as cyclophilin) were identified as essential proteins for lateral root development^{29–31}. The role of LRT2 in regulating OsIAA11 levels is further demonstrated by higher accumulation of OsIAA11 in LRT2 knockout rice plants, and lower proteasome-dependent degradation of purified recombinant His-OsIAA11 incubated with protein extracts from LRT2 knockout plants as compared to that of WT plants²⁰. Similarly, a rice strain that harbors the OsIAA11 P106L mutation (directly adjacent to the W₁₀₄-P₁₀₅ peptide bond) evades proteasomal degradation by preventing binding to the E3 ubiquitin ligase SCF^{TIR1}, and also shows no induction of lateral root development²⁹. Together with the demonstration of direct LRT2-catalyzed isomerization of the OsIAA11 degron motif by NMR²⁰, these studies indicate a central role of this *cis-trans* bimodal switch in an important auxin-responsive circuit. While LRT2 mutants studied to date have provided essentially “off” states of this enzyme^{20,30,31}, the central question of the quantitative relationship between isomerization rate and auxin circuit dynamics (i.e., *how fast is fast enough?*) has not yet been addressed.

Here, we have generated several LRT2 mutants with near-wild type (WT) thermal stability and have applied NMR to measure the observed OsIAA11 W₁₀₄-P₁₀₅ *cis-trans* exchange rates catalyzed by each thermally stable LRT2 mutant. Three of these stable mutants (P125K, W128A and H133Q) were also evaluated for their OsIAA11 binding affinities, and one mutant that retained significant catalytic activity (P125K) was further characterized by titration and NMR lineshape analysis to discern the impact of this mutation on the microscopic parameters of the catalytic reaction cycle. An approach is presented for guiding the lineshape analysis based on the independently measured *cis-trans* exchange rates by ROESY and Nzz-exchange spectroscopy. The OsIAA11 W₁₀₄-P₁₀₅ *cis-trans* exchange rates catalyzed by LRT2 homologs hCypA (human cyclophilin A), PPIB (petidyl-prolyl isomerase B from *E. coli*) and TaCypA (cyclophilin A from *Triticum aestivum*, wheat) were also measured for comparison, showing similar but slower isomerization rates than WT LRT2. These findings identify specific LRT2 mutations that yield stably folded and functionally altered enzyme, providing a range of LRT2 activities on the natural OsIAA11 substrate. These LRT2 mutants represent important tools for future studies in the context of live cells and *in planta* of relationships between the LRT2:OsIAA11 bimodal switch, auxin circuit dynamics, and lateral root development.

Materials and Methods

Plasmids, protein expression and purification.

The plasmids for expression of WT LRT2, hCypA (a gift from C. Kalodimos, Rutgers University, Piscataway, NJ) and OsIAA11^{72–125} in *E. coli* have been described previously^{16,32,33}. Mutations in the LRT2 coding sequence were achieved either by site directed mutagenesis Polymerase Chain Reaction (PCR) or by overlap extension PCR³⁴. For the H61Q, R62A, Q118A, W128A, and H133Q mutations, the QuickChange II Site-Directed Mutagenesis kit (Agilent) was used according to manufacturer's instructions. For the G72A, F120A, P125K, and V124A/P125K/C126T/S127E (hereafter referred to as VPCS) mutations, the overlap extension method was used. This method consists of creating

two initial PCR products followed by generation of a final PCR product. Briefly, one initial PCR product was created with a nested forward primer (LRT2-NdeI-FW) and the reverse primer with the desired mutation, while the other initial PCR product was created with a forward primer with the desired mutation and a reverse nested primer (LRT2-HindIII-Rev). Primer sequences are provided in Online Resource 1. Using both initial PCR products as DNA templates and both nested primers, a long final PCR product that contains the entire LRT2 gene with the desired mutation was obtained. This final PCR product was then digested with NdeI (New England Biolabs) and HindIII (New England Biolabs) according to manufacturer's instructions. Ligation with T4 DNA Ligase (New England Biolabs) of the final PCR product to the original pET28a vector was achieved following manufacturer's instructions.

Custom vectors for bacterial expression of PPIB (Uniprot entry P23869) and TaCypA (Uniprot entry Q93W25) were purchased from GenScript (Piscataway, NJ). Each gene was placed in the pET28a (+) kanamycin resistance vector that adds an N-terminal His tag followed by a tobacco etch virus (TEV) cleavage site to the expressed protein.

All LRT2 mutants, hCypA, PPIB, and TaCyp were expressed and purified as previously described for WT LRT2³², with the exception that for hCypA (which lacks a cleavage site) the His-tag was not cleaved. Each protein was concentrated with a 10K or 3K MWC centrifugal device (Pall Corporation, Ann Arbor, MI, or Millipore Corporation, Bedford, MA) to either 5 μ M in assay buffer (50mM KCl, 20mM KPO₄, 1mM TCEP, pH=6.67) for thermostability testing or to higher concentrations in NMR buffer (50mM KCl, 20mM KPO₄, 3mM TCEP, 5mM NaN₃, 0.1% P.I. cocktail, pH=6.67) for NMR experiments. LRT2 mutant, hCypA, PPIB and TaCypA protein concentrations were determined by UV absorption at 280nm, using the theoretical extinction coefficient of 9970 cm⁻¹M⁻¹ for all except LRT2 W128A which has a theoretical extinction coefficient of 4470 cm⁻¹M⁻¹, determined as described³⁵.

Natural abundance and uniformly ¹⁵N-labeled OsIAA11⁷²⁻¹²⁵ were expressed and purified as previously described¹⁶. For NMR experiments, OsIAA11⁷²⁻¹²⁵ or ¹⁵N-OsIAA11⁷²⁻¹²⁵ was concentrated in NMR buffer to the desired final concentration using a 3K MWCO centrifugal device (Pall Corporation, Ann Arbor, MI, or Millipore Corporation, Bedford, MA).

The synthetic peptide composed of OsIAA11 residues 98–109, blocked at each end with an N-terminal acetyl group and a C-terminal amine group, was custom synthesized and purified by Tufts Peptide Core Facility (Tufts University, Boston, MA). Both natural abundance (OsIAA11⁹⁸⁻¹⁰⁹) and isotope labeled forms were purchased, where the isotope labeled peptide was synthesized with ¹⁵N-Gly at G₁₀₃ and ¹³C₅-¹⁵N-Pro at P₁₀₅ (¹⁵N_{GP}/¹³C_P-OsIAA11⁹⁸⁻¹⁰⁹).

Thermal shift assay

Five repeats for each mutant and a control with no protein were prepared by mixing 45 μ l of 5 μ M protein (or buffer) and 5 μ l of 200X SYPRO Orange dye (Fischer). The thermal cycle was set up according to the Protein Thermal Shift Studies User Guide (Applied Biosystems),

and the thermal shift assay was carried out in a 96 well plate using an Applied Biosystems ViiA 7 Real-Time PCR System (ThermoFisher Scientific). The average fluorescence signal over the five repeats was determined and the fluorescence of the control sample was subtracted from each average. Additionally, signal for each sample was normalized to the maximum signal for that sample to allow comparison between all mutants.

Approach to determining overall exchange rates and microscopic reaction parameters for enzyme-catalyzed *cis-trans* isomerization of OsIAA11 W₁₀₄-P₁₀₅ peptide bond

The reaction scheme for enzyme-catalyzed *cis-trans* isomerization of a substrate involves four states, with each of the two isomers in free and enzyme-bound states (Fig. 1a). The microscopic (individual) parameters describing the isomerization and binding steps in this reaction scheme fully characterize the activity of a given isomerase enzyme on a specific substrate, providing the ability to predict the activity at any enzyme and substrate concentrations¹⁶. This can be very useful for modeling reactions at very low protein concentrations such as those found in living cells.

The application of ROESY or Nzz exchange spectroscopy to a given enzyme:substrate sample yields measurement of the overall observed catalyzed exchange rate, k_{ex}^{obs} , which reflects the combined binding and on-enzyme isomerization steps (Fig. 1b). Notably, k_{ex}^{obs} is dependent on the concentrations of enzyme and substrate, and is related to the microscopic parameters by Eq. 1, derived from the reversible Michaelis Menten equation¹⁷:

$$k_{ex}^{obs} = \left(\frac{k_{cat}^{tc}}{K_D^{trans}} + \frac{k_{cat}^{ct}}{K_D^{cis}} \right) \frac{[E]^{total}}{1 + \frac{[trans]^f}{K_D^{trans}} + \frac{[cis]^f}{K_D^{cis}}} \quad \text{Eq. 1}$$

where $[E]^{total}$ is the total enzyme concentration, $[trans]^f$ and $[cis]^f$ are the isomer concentrations in the free state, K_D^{trans} and K_D^{cis} are the dissociation constants for each isomer, and k_{cat}^{tc} and k_{cat}^{ct} are the on-enzyme *trans*-to-*cis* and *cis*-to-*trans* rates, respectively. The free *cis-trans* equilibrium constant (K_{eq}^f) and the on-enzyme exchange rate (k_{ex}^b) and equilibrium constant (K_{eq}^b) are defined as:

$$K_{eq}^f = \frac{[trans]^f}{[cis]^f} \quad \text{Eq. 2}$$

$$k_{ex}^b = k_{cat}^{tc} + k_{cat}^{ct} \quad \text{Eq. 3}$$

$$K_{eq}^b = \frac{k_{cat}^{ct}}{k_{cat}^{tc}} = \frac{[E:trans]}{[E:cis]} \quad \text{Eq. 4}$$

The isomer-specific dissociation constants K_D^{trans} and K_D^{cis} can be expressed in terms of the apparent dissociation constant K_D^{App} (the two-state approximation of the binding affinity from the enzyme perspective, Fig. 1c), and the free and on-enzyme equilibrium constants K_{eq}^f and K_{eq}^b , respectively, by the following equations:

$$K_D^{cis} = K_D^{App} \frac{(1 + K_{eq}^b)}{(1 + K_{eq}^f)} \quad \text{Eq. 5}$$

$$K_D^{trans} = K_D^{App} \frac{(K_{eq}^f)(1 + K_{eq}^b)}{(K_{eq}^b)(1 + K_{eq}^f)} \quad \text{Eq. 6}$$

Using equations 2–6 and assuming that the total substrate concentration is much greater than the total enzyme concentration, the measured k_{ex}^{obs} for a given sample can be expressed in terms of the unknowns K_{eq}^b and k_{ex}^b , the independently measured parameters K_{eq}^f and K_D^{App} , and the sample-specific total enzyme and substrate concentrations $[E]^{total}$ and $[S]^{total}$ as follows:

$$k_{ex}^{obs} = \frac{k_{ex}^b \cdot K_{eq}^b (K_{eq}^f + 1)}{(K_{eq}^b + 1)^2 \cdot K_D^{App}} \left(1 + \frac{1}{K_{eq}^f} \right) \frac{[E]^{total}}{\left(1 + \frac{[S]^{total}}{K_D^{App}} \right)} \quad \text{Eq. 7}$$

Equation 7 can be written in quadratic form in terms of the unknown K_{eq}^b :

$$0 = K_{eq}^{b2} + (2 - k_{ex}^b \cdot C) \cdot K_{eq}^b + 1 \quad \text{Eq. 8}$$

where C contains all known or measured values:

$$C = \frac{(K_{eq}^f + 1)}{K_D^{App} \cdot k_{ex}^{obs}} \left(1 + \frac{1}{K_{eq}^f} \right) \frac{[E]^{total}}{1 + \frac{[S]^{total}}{K_D^{App}}} \quad \text{Eq. 9}$$

Solving Equation 9 yields the two solutions

$$K_{eq}^b = \frac{-(2 - k_{ex}^b \cdot c) \pm \sqrt{(2 - k_{ex}^b \cdot C)^2 - 4}}{2} \quad \text{Eq. 10}$$

These two solutions provide insight on the possible K_{eq}^b and k_{ex}^b values that are consistent with measured k_{ex}^{obs} values at specific enzyme and substrate concentrations. It is interesting to note that the intersection of the + and – solutions given by Eq. 10 occurs at $K_{eq}^b = 1$ (i.e., when the bound *trans* and bound *cis* isomer populations are equal). This intersection point corresponds to $k_{ex}^b = 4/C$ (i.e., when $\sqrt{(2 - k_{ex}^b \cdot C)^2 - 4} = 0$). Since the square root term is

imaginary when $k_{ex}^b < 4/C$, the *minimum* physically possible on-enzyme exchange rate is defined as $k_{ex}^b = 4/C$. Notably, the + and – solutions correspond to $K_{eq}^b \geq 1$ and $0 < K_{eq}^b \leq 1$, respectively, where K_{eq}^b simply reflects the relative populations of bound *trans* and bound *cis* isomers (i.e., either solution is physically possible). In addition to providing a lower bound on k_{ex}^b , Eq. 10 evaluated at specific k_{ex}^{obs} , $[E]^{total}$, $[S]^{total}$, K_{eq}^f and K_D^{App} values provides pairs of possible K_{eq}^b and k_{ex}^b values that can be used to guide and/or evaluate the results of lineshape analysis.

Measurement of overall observed *cis-trans* exchange rate (k_{ex}^{obs}) by ROESY or Nzz-exchange spectroscopy

To determine the activities of LRT2 mutants and homologs, the overall catalyzed *cis-trans* isomerization of the W₁₀₄-P₁₀₅ peptide bond in the OsIAA11 substrate, k_{ex}^{obs} , was measured by ROESY and/or Nzz-exchange spectroscopy as previously described¹⁶. For ROESY experiments, samples with 1.84mM of OsIAA11^{98–109} and 120μM concentration of enzyme (R62A, P125K, W128A, H133Q, VPCS, or TaCypA) were prepared. For each sample, a series of ROESY spectra were acquired at mixing times of 30, 50, 70, 90, 110, and 130 ms. For Nzz experiments, samples with 800μM of ¹⁵N-OsIAA11^{72–125} and 16μM of enzyme (P125K, W128A, VPCS, or PPIB) in NMR buffer were prepared. For each sample, a series of Nzz spectra were acquired at mixing times of 0.011, 0.11, 0.22, 0.44, 0.55, 0.67, 0.89 and 1.00 s. The acquired ROESY and Nzz data were processed and analyzed to obtain the corresponding observed *cis-trans* exchange rates, k_{ex}^{obs} , and their uncertainties as previously described^{17,36}.

Measurement or estimation of K_D^{App} for binding of selected LRT2 mutants to OsIAA11

The apparent dissociation constant, K_D^{App} (Fig. 1c), for the LRT2-P125K mutant was determined as previously described for LRT2¹⁶. Briefly, the concentration of ¹⁵N-P125K was held constant at 0.120 mM, and an ¹⁵N-¹H HSQC spectrum was acquired at each step of a reverse titration with natural abundance OsIAA11^{98–109} (0, 0.13, 0.26, 0.53, 1.1, 2.1, 3.2 and 4.7 mM). Peaks that displayed chemical shift perturbations and could be tracked throughout the titration were fit individually to a two-state binding model to obtain K_D^{App} values from multiple ‘observers’ of the same reaction, and the average and standard deviation of these values was calculated.

To obtain a rough estimate of the very weak interaction of LRT2-W128A with OsIAA11, the concentration of ¹⁵N-LRT2-W128A was held constant at 0.26 mM, and a ¹⁵N-¹H HSQC spectrum was acquired at each step of a reverse titration with natural abundance OsIAA11^{98–109} (0, 0.24, 0.48, 0.96, 1.4, 2.2 mM). Chemical shift perturbation was used to obtain an approximate K_D^{App} value as described in the Results section.

To detect binding of LRT2-H133Q to OsIAA11, the site-specifically labeled peptide ¹⁵N_{GP}/¹³C_P-OsIAA11^{98–109} was held at a concentration of 0.7 mM, and a ¹H-¹⁵N HSQC spectrum

was acquired at each step of a reverse titration with unlabeled LRT2-H133Q (0, 0.95, 1.4, and 2.1 mM). The resulting chemical shift perturbations of the G₁₀₃ ¹⁵N-¹H group provide evidence for binding as described in the Results section.

Determination of LRT2-P125K microscopic reaction parameters by lineshape analysis

Titration and NMR lineshape analysis was applied to determine the microscopic reaction parameters (Fig. 1a) for LRT2-P125K catalysis of the W₁₀₄-P₁₀₅ peptide bond in OsIaa11. An ¹⁵N-¹H HSQC spectrum was acquired at each of the following combinations of ¹⁵N-OsIAA11^{72–125}/unlabeled LRT2-P125K: 0.260/0, 0.254/0.125, 0.248/0.250, 0.235/0.500, 0.210/1.0 and 0.160/2.172 (all concentrations in mM). Analysis of lineshapes utilized the TITration ANalysis (TITAN) software³⁷ and its built-in 4-state model as previously described¹⁶. The resulting fits were evaluated with regard to the possible K_{eq}^b and k_{ex}^b values obtained from Eq. 10 using the independently measured k_{ex}^{obs} (at known $[E]^{total}$, $[S]^{total}$), K_{eq}^f and K_D^{App} values as described in the Results section.

Results

Rational design and thermostability testing yield a set of stably folded LRT2 mutants.

In order to alter the activity of LRT2, specific amino acid residues were chosen for mutation based on prior mutagenesis studies on human cyclophilin A (hCypA, Fig. 2a), which shares 68.6% sequence identity with LRT2^{38–40}. Our previously reported LRT2 homology model^{16,40} was built using the crystal structure of the wheat cyclophilin TaCypA-1 (4hy7.1)⁴¹, which is the closest LRT2 sequence homolog (87.7% identity, Fig. 2a) for which a structure is determined. Structural alignment of this LRT2 model with hCypA (1AWR.1.pdb) shows a highly conserved fold (C^α rmsd = 0.55 Å), with six of the conserved active site residues (H61, R62, Q118, F120, W128 and H133 relative to LRT2 sequence numbering) displaying an all-atom rmsd of 0.38 Å (Fig. 2b). In hCypA studies, when these residues were individually mutated to Gln (both His) or Ala (all others), the resulting activities ranged from 15% to 0.1% of WT hCypA activity, as measured by the chymotrypsin assay on the substrate N-succinyl-AAPI-p-nitroaniline⁴⁰. We hypothesized that making these same mutations in LRT2 (H61Q, R62A, Q118A, F120A, W128A, and H133Q) would create a library of mutants with lower isomerization activity on the OsIAA11 W-P peptide bond. Moreover, the importance of the four residues preceding the highly conserved W128 in LRT2 that are part of a loop that differs between hCypA (₁₁₇AKTE₁₂₀) and LRT2 (₁₂₄VPCS₁₂₇) (Fig. 2c,d) was investigated by the P125K and quadruple VPCS (V124A/P125K/C126T/S127E) mutants. An additional LRT2 mutant of interest is G72A, since this mutation in rice causes a significant reduction in lateral root development³⁰.

Expression plasmids for the nine LRT2 mutants rationalized above were constructed, each mutant was expressed and purified, and the thermal stability of each was tested using the ThermoFluor assay to ensure that any differences in measured isomerization rates would not be due to instability of the protein. This assay detects protein unfolding by utilizing the emission of fluorescence by SYPRO Orange dye when it binds to hydrophobic protein surfaces⁴². As temperature rises, the protein unfolds, hydrophobic regions are exposed to

solvent, SYPRO Orange binds to exposed hydrophobic surfaces, and fluorescence is emitted. The resulting sigmoidal fluorescence curve reflects cooperative protein unfolding, with the melting temperature (T_m) defined as the inflection point in the curve⁴³. Using this thermal shift assay, T_m values for WT and LRT2 mutants were determined, revealing a broad range of stabilities (Fig. 3, Table 1). Notably, the G72A mutant is already unfolded at 25°C in the buffer conditions used, suggesting that this LRT2 mutant is also predominantly unfolded in the rice plant, potentially explaining its lateral rootless phenotype³⁰. Additionally, the T_m of H61Q is significantly reduced (28% lower than WT), while T_m values for the rest of the mutants are within 18% of WT. Four mutants (R62A, P125K, W128A and H133Q) are within 10% of WT stability. For further analysis by NMR, we selected these four most stable mutants and the VPCS mutant (T_m value 16% lower than WT) that generates the corresponding hCypA loop in LRT2.

Stable LRT2 mutants exhibit a range of activities on the OsIAA11 substrate.

NMR provides direct observation of *cis* and *trans* populations due to the intrinsically slow exchange timescale which gives rise to distinct peaks, and enables determination of PPIase-catalyzed isomerization rates^{17,44}. Using the same protocol as previously described¹⁶, we measured LRT2 mutant catalysis of the degron peptide OsIAA11^{98–109} by Rotating frame Overhauser Effect Spectroscopy (ROESY) for samples containing 120 μM LRT2 mutant and 1.84 mM OsIAA11^{98–109}. The presence of exchange crosspeaks between the indole proton autopeaks of $^{104}\text{WHe}^{cis}$ and $^{104}\text{WHe}^{trans}$ in the ^1H - ^1H ROESY spectrum, which provides clear evidence of isomerization, was observed for only the P125K and VPCS mutants (Fig. 4a). ^1H - ^1H ROESY spectra were acquired in the absence and presence of LRT2 mutants as function of mixing time, which allows determination of k_{ex}^{obs} (Fig. 4b, Table 2). The P125K and VPCS mutants have slightly lower k_{ex}^{obs} values compared to WT values at the same conditions¹⁶. However, since ROESY crosspeaks were not detected at any mixing times for the R62A, W128A, and H133Q LRT2 mutants, k_{ex}^{obs} values could not be determined for these mutants (Table 2).

To probe a different timescale window and to provide a different condition of enzyme and substrate concentrations, Nzz-exchange spectroscopy was applied for samples containing 16 μM LRT2 mutant and 800 μM OsIAA11^{72–125}. As we previously demonstrated¹⁶, LRT2 catalysis of the W-P peptide bond is the same in the context of the shorter peptide OsIAA11^{98–109} and in the longer recombinant substrate OsIAA11^{72–125}. Use of the longer recombinant substrate OsIAA11^{72–125} enables ^{15}N -labeling as needed for this experiment, and provides well resolved ^{15}N - ^1H peaks for $^{104}\text{WHe}^{cis}$ and $^{104}\text{WHe}^{trans}$ in the 2D Nzz-exchange spectra (Fig. 5a). Just as the exchange timescale detectable by the ROESY experiment is limited by the transverse relaxation time constant $T_{1\rho}$ (on the order of 0.1 s for OsIAA11^{98–109}, as displayed by the exponential decay of autopeaks in Fig. 4), the Nzz-exchange experiment is limited by the longitudinal relaxation time constant T_1 . For the recombinant ^{15}N -labeled OsIAA11^{72–125} construct, T_1 is on the order of 1 s (as displayed by autopeak decays in Fig. 5). Hence, the Nzz-exchange experiment allows measurement of slower k_{ex}^{obs} values as is the case for lower LRT2 concentrations, enabling a significantly different sample condition to be characterized.

The Nzz-exchange experiment was applied to measure k_{ex}^{obs} for catalysis of the $^{104}\text{W-P}^{105}$ peptide bond in $^{15}\text{N-OsIAA11}^{72-125}$ by the LRT2 mutants P125K, W128A, H133Q and VPCS. The build up of exchange crosspeaks was not observed in the absence of LRT2¹⁶, was observed for LRT2 mutants P125K and VPCS (Fig. 5a,b), and was not observed in presence of LRT2 mutants W128A or H133Q (Fig. 5c,d). The full set of $^1\text{H-}^{15}\text{N}$ ZZ exchange spectra at different mixing times was collected for each of these mutants, and for the P125K and VPCS mutants the resulting curves were fit to determine the corresponding k_{ex}^{obs} values (Fig. 5e,f, Table 2). For mutants that lack exchange crosspeaks at all mixing times, k_{ex}^{obs} values were obtained from just the two autosequences which are each dependent on the chemical exchange and longitudinal ^{15}N relaxation rates. The relatively slow longitudinal ^{15}N relaxation rates (e.g., significantly slower than two-spin order relaxation⁴⁵) enable more robust detection of chemical exchange from the Nzz autosequences.

Affinities of LRT2 mutants P125K, W128A and H133Q for OsIAA11 substrate.

The above characterization of LRT2 mutants has identified the P125K mutant as having intermediate (~75% of WT) activity on the OsIAA11 substrate, which would be useful *in planta* as a single-site mutation that ‘tunes’ the LRT2 activity on this natural substrate to a lower level. The W128A and H133Q mutants, which show no activity in the assays performed here, are of particular interest because in hCypA they have been implicated as binding defective (W128A) and catalytically deficient (H133Q)⁴⁰, which would be useful for probing these separable roles of LRT2 binding and catalysis *in planta*. As a first step toward characterizing the origins of the functional deficiencies of these mutations in LRT2, the binding of these three mutants to the OsIAA11 degron was evaluated.

To determine the apparent dissociation constant (K_D^{App}) from the perspective of the enzyme for the P125K mutant, $^{15}\text{N-LRT2-P125K}$ was titrated with unlabeled OsIAA11⁹⁸⁻¹⁰⁹, and a series of 2D HSQC spectra were acquired. The 20 residues whose peaks displayed the largest movements and remained resolved throughout the titration were selected and used to determine the apparent affinity, K_D^{App} . The chemical shift trajectory of each of the selected residues was fit individually to the simple two-state binding model (Fig. 6a), and the average and standard deviation of the resulting K_D^{App} values was determined to be 5.2 ± 0.9 mM. Due to the weakness of this affinity that prevents measurement of the full binding curve, this K_D^{App} value serves primarily as a lower limit of the actual value.

Similar studies were performed by titrating $^{15}\text{N-LRT2-W128A}$ with unlabeled OsIAA11⁹⁸⁻¹⁰⁹. Very small shifts in peaks were observed for this LRT2 mutant, with the A108 peak displaying the largest displacement. Notably, the trajectory of the A108 peak is in the same direction as the WT A108 peak (Fig. 6b). To approximate the K_D^{App} for LRT2-W128A, we assumed the same A108 chemical shift in the bound state as determined for WT LRT2¹⁶. The A108 chemical shift values observed in the LRT2-W128A titration were then fit to the simple 2-state binding model to yield the approximation $K_D^{App} = 31$ mM. We note that this extremely weak affinity value represents only a rough estimate, since observed peak

shifts are limited to the initial linear region of the binding curve, and the bound chemical shift could differ from that determined for WT LRT2.

Although the same ^{15}N -LRT2 titration has not yet been performed for the LRT2-H133Q mutant, the ability of the LRT2-H133Q mutant to bind to OsIAA11 is supported by titration of $^{15}\text{N}_{\text{GP}}/^{13}\text{C}_{\text{P}}$ -OsIAA11^{98–109} with unlabeled LRT2-H133Q (i.e., the peptide perspective of the interaction). Small peak shifts were observed (Fig. 6c), similar to those induced by titration with WT LRT2¹⁶. The Gly *cis* peak tracks in the same direction as WT LRT2 during titration (Fig. 6c). If we assume that LRT2-H133Q has WT affinity and the WT $K_{\text{d}}^{\text{cis}}$ value of 1.5 mM¹⁶ is applied, the highest H133Q concentration corresponds to ~50% bound. Indeed, the peak position for the highest H133Q concentration point is at the predicted position (Fig. 6c), suggesting that LRT2-H133Q has near-WT binding affinity. This is in agreement with mutation of the corresponding His residue in hCypA to either Q or A, which shows 0.5% of WT PPIase activity and retains the ability to bind inhibitor and substrate^{38,40}.

Lineshape analysis of P125K mutant reveals efficient on-enzyme catalysis

Of these three mutants, only P125K exhibits sufficient activity for full characterization of the reaction parameters by lineshape analysis. Lineshape analysis of NMR titration spectra is a powerful method for determining the thermodynamic and kinetic parameters associated with the reversible PPIase reaction cycle^{16,17}. NMR titration data sets comprised of ^{15}N - ^1H HSQC spectra at each titration point were acquired using ^{15}N -labeled OsIAA11^{72–125} titrated with unlabeled LRT2-P125K. The resolved *cis* and *trans* NH peaks corresponding to the $^{104}\text{W-P}^{105}$ indole NH and the G103 backbone NH groups provide two observers of the isomerization reaction.

For analysis of the titration data using TITAN³⁷, the $K_{\text{D}}^{\text{App}}$ value was fixed at the independently determined value of 5.2 ± 0.9 mM. The ^{15}N - ^1H peaks for $^{104}\text{W}_{\text{e}}^{\text{cis}}$ and $^{104}\text{W}_{\text{e}}^{\text{trans}}$ were fit separately from the ^{15}N - ^1H peaks for $^{103}\text{G}^{\text{cis}}$ and $^{103}\text{G}^{\text{trans}}$ because TITAN requires a single $^3\text{J}_{\text{HNH}\alpha}$ value for all peaks in the analysis, and Trp indole NH $^3\text{J}_{\text{HNH}\alpha}$ values are significantly smaller than backbone $^3\text{J}_{\text{HNH}\alpha}$ values. Optimization of the fitting showed $^3\text{J}_{\text{HNH}\alpha} = 3.5$ Hz for the $^{104}\text{W}_{\text{e}}^{\text{cis}}$ and $^{104}\text{W}_{\text{e}}^{\text{trans}}$ peaks, and $^3\text{J}_{\text{HNH}\alpha} = 8.5$ Hz for the $^{103}\text{G}^{\text{cis}}$ and $^{103}\text{G}^{\text{trans}}$ peaks. Additionally, the bound R_2 values were constrained to those expected for the bound complex as approximated from R_2 values for ^{15}N -labeled LRT2-P125K (42.1 ± 9.6 s⁻¹ and 8.7 ± 2.6 s⁻¹ for ^1H and ^{15}N , respectively). As was found for WT LRT2, the dissociation rates for the *cis* and *trans* binding reactions, $k_{\text{off}}^{\text{cis}}$ and $k_{\text{off}}^{\text{trans}}$, are too fast to influence the analysis, and were set to 17,000 s⁻¹ and 15,000 s⁻¹ consistent with a diffusion-limited on-rate as described in more detail elsewhere¹⁶. Fitted parameters included the bound chemical shifts for each N-H group, and the microscopic reaction parameters K_{eq}^{b} and k_{ex}^{b} that characterize the on-enzyme catalysis step. Based on Eq. 10 and the independently measured $k_{\text{ex}}^{\text{obs}}$ values, the K_{eq}^{b} and k_{ex}^{b} values must fall along a distinct curve formed by the plus and minus solutions (Fig. 7a), which provides guidance for the fitting of lineshapes. The resulting optimized values, $K_{\text{eq}}^{\text{b}} = 2.9 \pm 0.2$ and $k_{\text{ex}}^{\text{b}} = 211 \pm 5$ s⁻¹ yield good fits (Fig. 7b–g, Online Resource 2), and predict $k_{\text{ex}}^{\text{obs}}$ values using Eq. 7 (2.8 s⁻¹

and 0.43 s^{-1}) that match closely to those measured by ROESY and Nzz ($2.7 \pm 0.3 \text{ s}^{-1}$ and $0.47 \pm 0.04 \text{ s}^{-1}$). These K_{eq}^b and k_{ex}^b values indicate that the P125K mutation favors binding of the *trans* isomer and accelerates the on-enzyme reaction relative to WT ($K_{eq}^b = 1.12 \pm 0.11$ and $k_{ex}^b = 95 \pm 8 \text{ s}^{-1}$)¹⁶.

LRT2 homologs display similar activities as LRT2 on the W-P peptide bond in OsIAA11.

The activities of several LRT2 homologs have been reported in the literature, with significantly higher k_{ex}^{obs} values reported for different (not W-P) substrates^{17,44}. We previously showed that LRT2 displays typical cyclophilin activity on a standard cyclophilin substrate (containing a G-P peptide bond), suggesting that the relatively slow catalyzed isomerization rates observed for OsIAA11¹⁰⁴W-P¹⁰⁵ are due to the W-P substrate¹⁶. However, the question still remains whether homologs that display activities higher than hCypA on other substrates might also exhibit higher k_{ex}^{obs} values than LRT2 WT on the OsIAA11 substrate. We selected hCypA since it and its mutants are widely studied on other substrates^{38,40,46}, and the LRT2 homolog Peptidyl-Prolyl Isomerase B (PPIB) from *E. coli* because it was reported to have more than an order of magnitude higher k_{cat}/K_m than hCypA on the Succinyl-Ala-Ala-Pro-Phe-NH-Mec peptide substrate^{47,48}. Additionally, we selected TaCypA from wheat to provide a close homolog from an organism with a lateral root system⁴⁹. Application of Nzz-exchange spectroscopy to hCypA and PPIB (Fig. 8a,b) and ROESY spectroscopy to TaCypA (Fig. 8c) resulted in clear exchange crosspeaks, and curve fitting (Fig. 8d–f) yielded k_{ex}^{obs} values that are within 50–110% of rates determined for LRT2 WT at the same sample conditions (Table 2). These results further support the conclusion that the slow isomerization rate of the OsIAA11 degron sequence is an intrinsic property of the W-P substrate, and show that the higher activity of PPIB on a different substrate does not translate to faster isomerization of the OsIAA11 substrate studied here. Notably, the closest homolog tested, TaCypA from an organism with lateral roots, displays the largest difference in catalyzed rate.

Discussion and conclusions

The cyclophilin LRT2 and the Aux/IAA protein OsIAA11, each independently linked to the lateral rootless phenotype in *Oryza sativa*, are an enzyme/substrate pair that appears to regulate lateral root development through LRT2 facilitation of OsIAA11 proteolysis²⁰. While mutant LRT2 rice strains have provided clear evidence for the essential role of LRT2 in lateral root development and the auxin circuit^{20,30,31}, the central question of the quantitative relationship between isomerization rate and auxin circuit dynamics has not yet been addressed. Moreover, the effects on lateral root development of properly folded but binding incompetent and/or catalytically inactive forms of LRT2 have not been investigated. Here, we have generated and characterized a set of LRT2 mutants that include the binding impaired but catalytically active P125K, the binding incompetent W128A, and the binding capable but catalytically impaired H133Q. These LRT2 mutants provide tools for establishing the sensitivity of the auxin response to isomerization rate, for testing the roles of

the LRT2:OsIAA11 isomerization reaction in auxin circuit dynamics, and for correlating isomerization rate to the lateral rootless phenotype.

Previously, only destabilized LRT2 mutants had been described. By mutagenizing rice seedlings and screening for the lateral rootless phenotype, three specific LRT2 mutants (*lrt2*, *cyp2-1* and *cyp2-2*) were identified^{29–31}. Specifically, *lrt2* has a 50-base pair deletion in the LRT2 gene³¹, *cyp2-1* has a premature stop codon that truncates LRT2 by 21 residues³⁰, and *cyp2-2* has the single LRT2 amino acid substitution G72A³⁰. For both *lrt2* and *cyp2-1* mutant plants, no lateral roots are formed^{30,31} and the corresponding LRT2 mutant proteins are expected to be unfolded, since each lacks key structured regions of the highly conserved cyclophilin fold. The *cyp2-2* mutant plant displays a less severe lateral rootless phenotype (a few lateral roots develop)³⁰, and the corresponding LRT2-G72A protein was previously described as unstable because it could not be detected by Western blot either in the *cyp2-2* plant or when transiently expressed in tobacco leaves²⁰. However, thermodynamic studies of the LRT2-G72A mutant had not been reported. Our thermal stability measurements demonstrate that the LRT2-G72A protein is unfolded in the observed temperature range (25–55 °C). We note that in our LRT2 homology model (as well as in hCypA and TaCypA structures), G72 adopts dihedral angles only favorable for glycine residues ($\phi \cong 98^\circ$, $\psi \cong -3^\circ$), which could explain why substitution with Ala results in unfolded protein. Hence, the only LRT2 mutants yet linked to lateral rootless phenotypes lack a stable cyclophilin fold.

The thermal stability studies reported here have established that the W128A, P125K, H133Q and R62A LRT2 mutants have near WT stability (within 10% of the T_m of WT LRT2). Moreover, these mutants display a range of activities on the OsIAA11 degron substrate, providing a set of single-residue LRT2 mutants for use in future studies of relationships between binding, isomerization rate, auxin circuit dynamics, and the lateral rootless phenotype. Inspired by the demonstration that hCypA binding and catalytic functions are separable⁴⁰, we further characterized the P125K, W128A and H133Q mutants in terms of binding and (when possible) on-enzyme catalysis. We found that the P125K mutation weakens binding to the OsIAA11 degron substrate without slowing the on-enzyme catalytic exchange rate, the H133Q mutant retains binding ability while abolishing catalysis, and the W128A mutant is incompetent for substrate binding or catalysis. While the P125K mutation has not been previously studied (in CypA the corresponding residue is K), our findings for H133Q and W128A are consistent with the impacts of the corresponding substitutions in hCypA^{38–40,46}. Notably, one previous study included measurements on a W-P substrate, showing that the hCypA W121A mutant displayed negligible catalytic activity on this substrate as measured using a chymotrypsin-based two-step assay³⁹, in agreement with our findings for the corresponding LRT2-W128A mutant acting on the W-P peptide bond in the OsIAA11 degron substrate.

Our studies of LRT2 homologs hCypA, PPIB and TaCypA show isomerization rates on the OsIAA11 substrate that are ~50 – 110% of the WT LRT2 rate at the same sample conditions. This raises the question of whether LRT2 catalysis of OsIAA11 might be finely tuned to a specific rate, or just simply needs to be above a specific threshold for proper control of the auxin circuit and lateral root development. The P125K LRT2 mutation (~75% of WT activity) will be useful for investigating whether precise LRT2 tuning is essential. If

LRT2 activity only needs to be above a certain threshold, this poses intriguing questions regarding how LRT2 is essential for lateral root development, if any cyclophilin can similarly catalyze the OsIAA11 degron motif. Certainly, differences in tissue expression and subcellular localization of LRT2 versus the other 27 rice cyclophilins⁵⁰ could be a critical factor. Tissue-specific LRT2 expression is indicated by confocal microscopy studies of rice roots that showed highly localized expression of an LRT2-GFP fusion protein at the root tip, at lateral root primordia (where lateral roots initiate) and within lateral roots³⁰. Hence, the coordinated expression and localization of LRT2 with its OsIAA11 substrate could explain the unique and essential role of this molecular switch in the auxin circuit that regulates rice lateral root development.

By analogy with mechanisms established for LRT2 homologs, an additional role or alternative explanation of why LRT2 is essential for lateral root development could be through highly specific interactions with other proteins. Cyclophilins are known to undergo interactions that facilitate functions independent of their catalytic function. For example, cyclosporin A binding to hCypA induces complex formation with calcineurin, generating what was an entirely unexpected impact on immune system signaling, ultimately blocking the transcriptional activation of genes involved in the early stages of T cell response⁵¹. Another intriguing example is the developmental impact of a cyclophilin B (CypB) mutation in American quarter horses that inhibits collagen biosynthesis through altered interactions between catalytically active CypB and protein binding partners⁵². Similarly, deletion and truncation mutations in human CypB are associated with osteogenesis imperfecta phenotypes via a mechanism involving procollagen biosynthesis⁵³. With regard to LRT2, additional protein-protein interactions are indeed implicated. Notably, LRT2 is pulled down with immunoprecipitated SCF^{TIR1} (the cognate E3 ligase of OsIAA11) in plant extracts²⁰. Given the low affinity of LRT2 interaction with OsIAA11¹⁶ and the high affinity interaction between SCF^{TIR1} and the degron sequence²³, this suggests that LRT2 engages in additional interactions involving the E3 ligase machinery.

The studies presented here identify several LRT2 mutants with diminished activities and stable folds. Three single-residue mutants were further characterized with regard to their binding and catalysis. With CRISPR/Cas9 technology, these mutants can be incorporated into the endogenous LRT2 gene in rice to investigate the impact of protein dynamics on lateral root development. Moreover, plant cells with their cell walls removed (protoplasts) enable transient expression of OsIAA11 and LRT2 fused to fluorescent proteins, which will enable the effects of these LRT2 mutants on auxin circuit dynamics to be quantified at the single-cell level using confocal fluorescence microscopy. Overall, these mutants will enable the investigation of relationships between isomerization rate, auxin circuit dynamics, and phenotypic changes in lateral root development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Peptidyl-prolyl *cis-trans* isomerase reaction models used for characterizing LRT2 mutants and homologs

a) The four-state reaction scheme corresponding to the substrate perspective, with the microscopic reaction parameters illustrated. b) The overall observed exchange rate between free *cis* and free *trans* isomers obtained using ROESY or Nzz exchange spectroscopy, k_{ex}^{obs} , that includes both the binding and on-enzyme reaction steps. c) The binding reaction depicted from the perspective of the enzyme (E), where the substrate (S) is rapidly exchanged in the enzyme-bound state (represented by a pink circle) such that the binding reaction can be modeled as an apparent 2-state system (i.e., free and bound E) with an apparent dissociation constant, K_D^{App}

Fig. 2. Mutagenesis of LRT2.

a) Sequence alignment of LRT2 with cyclophilins from wheat (TaCypA), human (CypA) and *E. coli* (PPIB). b) The LRT2 homology model (green) aligned with the crystal structure of human Cyclophilin A (cyan, PDB:1awr.1) shows the conserved catalytic residues H61, R62, Q118, F120, W128 and H133 (LRT2 numbering). c) Residues in the loop preceding W121 in hCypA that are not conserved in LRT2. d) Corresponding residues in the loop preceding W128 in the LRT2 homology model

Fig. 3. Thermodynamic stability of LRT2 mutants.

Thermal melting of LRT2 WT and mutants was detected by thermal shift measurements. a) G72A LRT2 mutant shows a completely unfolded profile throughout the full temperature range examined, while H61Q, Q118A, and F120A each show a lower melting temperature (T_m) than WT. b) All remaining mutants show T_m values closer to that of WT LRT2.

Notably, the W128A LRT2 mutant has a slightly higher T_m, indicating a more stable protein

Fig. 4. Effects of mutations on the observed LRT2 catalysis of ^{104}W -p105 *cis-trans* exchange rate measured by ROESY.

a) Expanded region of 2D ^1H - ^1H ROESY spectrum of OsIAA11^{98–109} (mixing time of 120 ms) showing the ^{104}W -H_E^{cis} and ^{104}W -H_E^{trans} peaks. Exchange crosspeaks are observed only in the presence of P125K and VPCS LRT2 mutants, indicating detectable catalysis by these two mutants. b) Fitting (lines) of the normalized peak intensities (dots) as function of mixing time for the auto (I_{tt} and I_{cc}) and exchange (I_{tc} and I_{ct}) peaks of ^{104}W -He in the absence and presence of the indicated LRT2 mutants yielded the corresponding $k_{\text{ex}}^{\text{obs}}$ values (Table 2)

Fig. 5. Effects of mutations on the observed LRT2 catalysis of ^{104}W -p 105 *cis-trans* exchange rate measured by N_{ZZ} -exchange spectroscopy.

(a-d) Expanded region of 2D ^1H - ^{15}N N_{ZZ} -exchange spectra (mixing time = 0.55s) of ^{15}N -OsIAA11⁷²⁻¹²⁵ showing ^{104}W -NH ϵ peaks. Exchange peaks between the ^{104}W -NH ϵ^{cis} and ^{104}W -NH ϵ^{trans} auto peaks are observed for samples that contain LRT2 mutants P125K (a) and VPCS (b). Exchange peaks are not observed for samples that contain LRT2 mutants W128A (c) or H133Q (d). (e,f) Fitting (lines) of normalized peak intensities (dots) for auto (I_{tt} and I_{cc}) and exchange (I_{tc} and I_{ct}) peaks in the presence of LRT2 mutants P125K (e) and VPCS (f) yielded the corresponding $k_{\text{ex}}^{\text{obs}}$ values (Table 2)

Fig. 6. Apparent binding affinity, K_D^{App} , of P125K, W128A and H133Q mutants.

a) Plot of the fraction of ^{15}N -LRT2-P125K bound (from chemical shift perturbation) as a function of OsIAA11^{98-109} concentration. Experimental data (points) was fit to the 2-state binding model (line) to obtain the K_D^{App} value. b) Expanded region of ^{15}N - ^1H HSQC spectrum of ^{15}N -LRT2-W128A showing overlay of the A108 NH peak at different OsIAA11^{98-109} concentrations from apo (black) to the highest (red). The peak trajectory follows the same path as A108 in WT LRT2. c) Expanded region of ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}_{\text{GP}}/^{13}\text{C}_{\text{P}}$ -OsIAA11 $^{98-109}$ titrated with unlabeled LRT2-H133Q, showing overlay of the $^{104}\text{Gly}^{\text{cis}}$ peak at apo (black) and highest LRT2-H133Q concentration (red). Also shown is the $^{104}\text{Gly}^{\text{cis}}$ peak at the highest concentration in a titration with WT LRT2 (blue) and the corresponding fully bound position (x) as previously determined by lineshape analysis¹⁶

Fig. 7. Goodness of fit for the lineshape analysis of ¹⁵N-OsIAA11⁷²⁻¹²⁵ titrated with LRT2-P125K.

a) Theoretical plots of K_{eq}^b versus k_{ex}^b for the two independently measured k_{ex}^{obs} values for P125K (Table 2), showing possible pairings of K_{eq}^b and k_{ex}^b values corresponding to the ROESY (circles) and Nzz (squares) k_{ex}^{obs} values using the plus (filled) and minus (open) solutions of Eq. 10, and the K_{eq}^b , k_{ex}^b coordinate obtained by lineshape analysis (pink ellipse). (b-g) Overlays of experimental (gray) and simulated (pink) ¹⁵N-¹H HSQC spectra of ¹⁵N-OsIAA11⁷²⁻¹²⁵ showing the ¹⁰⁴W-NHe^{cis} and ¹⁰⁴W-HHe^{trans} peaks at each LRT2-P125K concentration (mM): (b) zero, (c) 0.13, (d) 0.25, (e) 0.50, (f) 1.0 and (g) 2.17.

Fig. 8. LRT2 homologs display comparable catalysis of $^{104}\text{W-p}^{105}$.

(a-c) Expanded region of the ^1H - ^{15}N NZZ exchange NMR spectrum (mixing time = 0.55s) of ^{15}N -OsIAA11⁷²⁻¹²⁵ in the presence of hCypA (a) or PPIB from *E. coli* (b), and of the ROESY spectrum (70 ms) of OsIAA11⁹⁸⁻¹⁰⁹ in the presence of TaCypA (c). Exchange peaks between the $^{104}\text{W-NH}\epsilon^{\text{cis}}$ and $^{104}\text{W-NH}\epsilon^{\text{trans}}$ auto peaks are observed in the presence of each homolog. (d-f) Fitting (lines) of normalized peak intensities for the auto (I_{tt} and I_{cc}) and exchange (I_{tc} and I_{ct}) peaks in the presence of hCypA (d), PPIB (e) and TaCypA (f) yielded the corresponding $k_{\text{ex}}^{\text{obs}}$ values (Table 2)

Table 1

Melting temperature of LRT2 WT and mutants measured by ThermoFluor assay

LRT2 Variant	Melting Temperature (C°)
WT	48.78 ±0.54
H61Q	34.98±1.18
R62A	45.86±0.33
G72A	Not Determined (unfolded at 25°C)
Q118A	41.75±0.59
F120A	40.28±2.21
P125K	46.46±2.77
W128A	49.03±0.17
V124A/P125K/C126T/S127E	41.13±1.10
H133Q	45.20±0.55

Table 2

Isomerization rates for LRT2 mutants and homologs determined by ^1H - ^1H ROESY and ^1H - ^{15}N ZZ exchange experiments.

Cyclophilin Variant	ROESY-measured k_{ex}^{obs} (s^{-1}) 120 μM LRT2 1.84 μM OsIAA11 ⁹⁸⁻¹⁰⁹	Nzz-measured k_{ex}^{obs} (s^{-1}) 16 μM LRT2 800 μM OsIAA11 ⁷²⁻¹²⁵
LRT2 (WT) ^a	3.63 $\pm$ 0.08 ^a	0.62 $\pm$ 0.01 ^a
LRT2 R62A	ND ^b	Not measured
LRT2 P125K	2.7 $\pm$ 0.3	0.47 $\pm$ 0.04
LRT2 W128A	ND ^b	0.0 $\pm$ 0.04
LRT2 H133Q	ND ^b	0.12 $\pm$ 0.04
LRT2 VPCS	3.3 $\pm$ 0.3	0.55 $\pm$ 0.04
hCypA	Not measured	0.69 $\pm$ 0.04
PPIB	Not measured	0.37 $\pm$ 0.04
TaCypA	1.8 $\pm$ 0.3	Not measured

^aData reported previously¹⁶.

^bCould not be determined due to the absence of exchange crosspeaks at all mixing times.