

The superior salinity tolerance of bread wheat cultivar Shanrong No. 3 is unlikely to be caused by elevated Ta-sro1 poly-(ADP-ribose) polymerase activity

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Letters

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Dear Editor,

This letter deals with a 2014 publication in *The Plant Cell* by Liu et al. (2014) suggesting that the superior salinity tolerance of bread wheat (*Triticum aestivum*) cultivar Shanrong No. 3 (SR3) can be attributed to poly (ADPribose) polymerase (PARP) activity of the SIMILAR TO RCD-ONE (SRO) protein encoded by the SR3 allele of *sro1*, *Ta-sro1*. We present a structural analysis of the Ta-sro1 PARP domain at 2.1 Å resolution together with *in vitro* and *in vivo* assays of biochemical function, providing strong evidence that Ta-sro1 is unlikely to have PARP activity.

Protein ADP-ribosylation is a post-translational protein modification that is conserved in most eukaryotes and has attracted a growing interest in plant responses to biotic and abiotic stress (Liu et al., 2014; Feng et al., 2015; Kong et al., 2021; Yao et al., 2021). Protein-modifying ADP-ribosyltransferases use nicotinamide adenine dinucleotide (NAD⁺) as a co-substrate to transfer the ADP-ribose moiety of NAD⁺ to an amino acid side chain. In mammals and plants, enzymes of the PARP family act as the main

“writer” enzymes of intracellular ADP-ribosylation events although some members of the sirtuin family also catalyze protein ADP-ribosylation (Hottiger, 2015). The human genome codes for 17 PARP proteins. Out of these, 2 proteins lack apparent ADP-ribosyltransferase activity, 11 enzymes are restricted to mono-ADP-ribosylation (i.e. the transfer of a single ADP-ribose onto the target residue), and only 4 family members can catalyze multiple rounds of ADP-ribosylation leading to the formation of poly(ADP-ribose) (PAR) chains on substrate proteins (Hottiger, 2015). The type of enzymatic activity is closely correlated with the conservation of residues in the catalytic PARP domain that are either required for NAD⁺ binding or catalysis. A conserved His hydrogen bonds to the adenine ribose of NAD⁺ and, together with a conserved Tyr that stacks with the nicotinamide ribose ring, forms the basis of the co-substrate binding site (Wahlberg et al., 2012). A catalytic Glu residue acts as a general base to facilitate the nucleophilic attack by the acceptor on the donor ribose (Marsischky et al., 1995). Notably, the Glu residue is

dispensable for the initial ADP-ribosylation of an Asp or Glu residue because their side chains can function as intrinsic nucleophiles (Kleine et al., 2008). In contrast, during PAR chain elongation, the Glu is required as a general base to polarize the 2'-hydroxyl group of the acceptor's adenine ribose. Therefore, the Glu is conserved in all human enzymes with poly-ADP-ribosylation activity, whereas its replacement by other amino acids can limit certain PARP family members to mono-ADP-ribosylation (Kleine et al., 2008; Hottiger, 2015).

Plant genomes typically code for three different PARP proteins with occasional duplication of PARP genes leading to expression of different isoforms in several species (Vainonen et al., 2016). In *Arabidopsis* (*Arabidopsis thaliana*), PARP1 and PARP2 have polymerase activity and this is consistent with complete conservation of the His–Tyr–Glu triad in these enzymes (Feng et al., 2015). In contrast, PARP3 has a Cys–Val–Glu triad, does not bind NAD⁺, and is inactive with respect to catalyzing mono- or poly-ADP-ribosylation reactions (Gu et al., 2019). In addition to canonical PARP enzymes, plants have evolved a second group of proteins that share sequence and structural homology with the catalytic domains of PARPs. Transcriptional co-regulators of the SRO group are characterized by a conserved PARP domain and a C-terminal RST (RCD1 SRO TAF4) domain that binds transcription factors (Jaspers et al., 2010). Although the PARP domain in SRO proteins is strictly conserved, residues of the His–Tyr–Glu triad deviate from the consensus sequence in SRO proteins (Jaspers et al., 2010). In *Arabidopsis* RADICAL-INDUCED CELL DEATH 1 (RCD1), the founding member of the plant SRO protein family, the catalytic triad is replaced by Leu–His–Asn. RCD1 does not bind NAD⁺ and thus cannot catalyze canonical ADP-ribosyltransferase reactions (Jaspers et al., 2010). Moreover, mutations that alter the cleft of the RCD1 PARP domain that corresponds to the active site of canonical PARPs, do not compromise the biological function of RCD1 in development and oxidative stress tolerance (Wirthmueller et al., 2018). However, a recent report showed that the *Arabidopsis* SRO2 protein is an NAD⁺-dependent mono-ADP-ribosyltransferase (Kong et al., 2021). In SRO2, the His and Tyr residues that coordinate NAD⁺ binding are swapped and this may explain why the protein retains enzymatic activity. Liu et al. (2014) proposed that the bread wheat (*T. aestivum*) Ta-SRO1 protein can catalyze poly-ADP-ribosylation reactions although the His–Tyr–Glu is replaced by Leu–His–His in this protein. Moreover, the study by Liu et al. (2014) identified a Ta-SRO1 proteoform (Ta-sro1) that reportedly exhibits elevated PARP activity and renders wheat cultivar SR3 more tolerant to oxidative and high salinity stress. Ta-sro1 differs from its parental gene in three single nucleotide polymorphisms, two of which alter the amino acid sequence. Based on protein homology modeling, Liu et al. (2014) predicted that the polymorphism Ala/Thr³⁴³ maps to the Ta-SRO1 NAD⁺-binding

site and could alter its PARP activity. We aimed to understand (1) why Ta-SRO1 retains PARP activity despite nonconservation of the catalytic triad and (2) how the two polymorphisms enhance the enzymatic activity of the Ta-sro1 proteoform that confers elevated salinity tolerance in wheat.

The presumed active site of Ta-sro1 differs in several key features from canonical PARPs

To understand the structural basis for the noncanonical PARP activity of Ta-sro1, we produced Selenomethionine-labeled, His₆-tagged Ta-sro1 PARP domain in *Escherichia coli*, crystallized the protein and solved its structure by single anomalous diffraction phasing (Adams et al., 2010) at 2.1 Å resolution (Protein Data Bank (PDB) identifier 7PLQ; Supplemental Table S1). The domain adopts the typical PARP fold with a β–α–loop–β–α signature at the donor site that binds NAD⁺ (Figure 1A). The donor site loop (D-loop) occludes the proposed active site of Ta-sro1 and is only partially resolved in the structure with little interpretable electron density for amino acids Met³³⁴–Gly³³⁶ (Figure 1A). The structure confirms that the His–Tyr–Glu triad is not conserved in Ta-sro1 (Figure 1A). The position of the conserved His residue is taken by Ta-sro1 Leu³¹². Strict conservation of a His residue at this position in active ADP-ribosyltransferases is explained by its function in forming a hydrogen bond to the 2'-OH of the adenine ribose (Ruf et al., 1996; Steffen et al., 2013). Consequently, His in this position is essential for HsPARP1 activity (Marsischky et al., 1995) and a corresponding His²¹ to Leu exchange in the structurally related diphtheria toxin abolishes ADP-ribosylation (Johnson and Nicholls, 1994; Bell and Eisenberg, 1997). The Tyr that stacks with the nicotinamide ribose ring in canonical PARP enzymes is replaced by His³⁴⁴ in Ta-sro1. In contrast, a second Tyr that is positioned on the opposite side of the cleft, stacking with the nicotinamide ring, is conserved (Tyr³⁵⁷) (Figure 1B). The catalytic Glu that is specifically required for the PAR chain elongating polymerase activity of PARPs is replaced by Ta-sro1 His⁴⁰⁷. Overall, the PARP domain structure reveals that the presumed active site of Ta-sro1 substantially differs in several key residues from canonical PARP domains of plants and mammals (Wahlberg et al., 2012; Vainonen et al., 2016; Gu et al., 2019). Despite the altered identity of amino acids that form the NAD⁺-binding site, Ta-sro1 apparently can still utilize NAD⁺ as a co-substrate (Liu et al., 2014). To understand how Ta-sro1 makes contact to NAD⁺ we superimposed the Ta-sro1 PARP domain onto the structure of the catalytic domain of human PARP1 crystallized in complex with the NAD⁺ analog benzamide adenine dinucleotide (PDB identifier 6BHV). Figure 1B shows the active site of HsPARP1 (gray) with the bound benzamide adenine dinucleotide molecule (pink) in comparison to the presumed Ta-sro1 active site (beige). In addition to the altered identity of several residues that make contact with

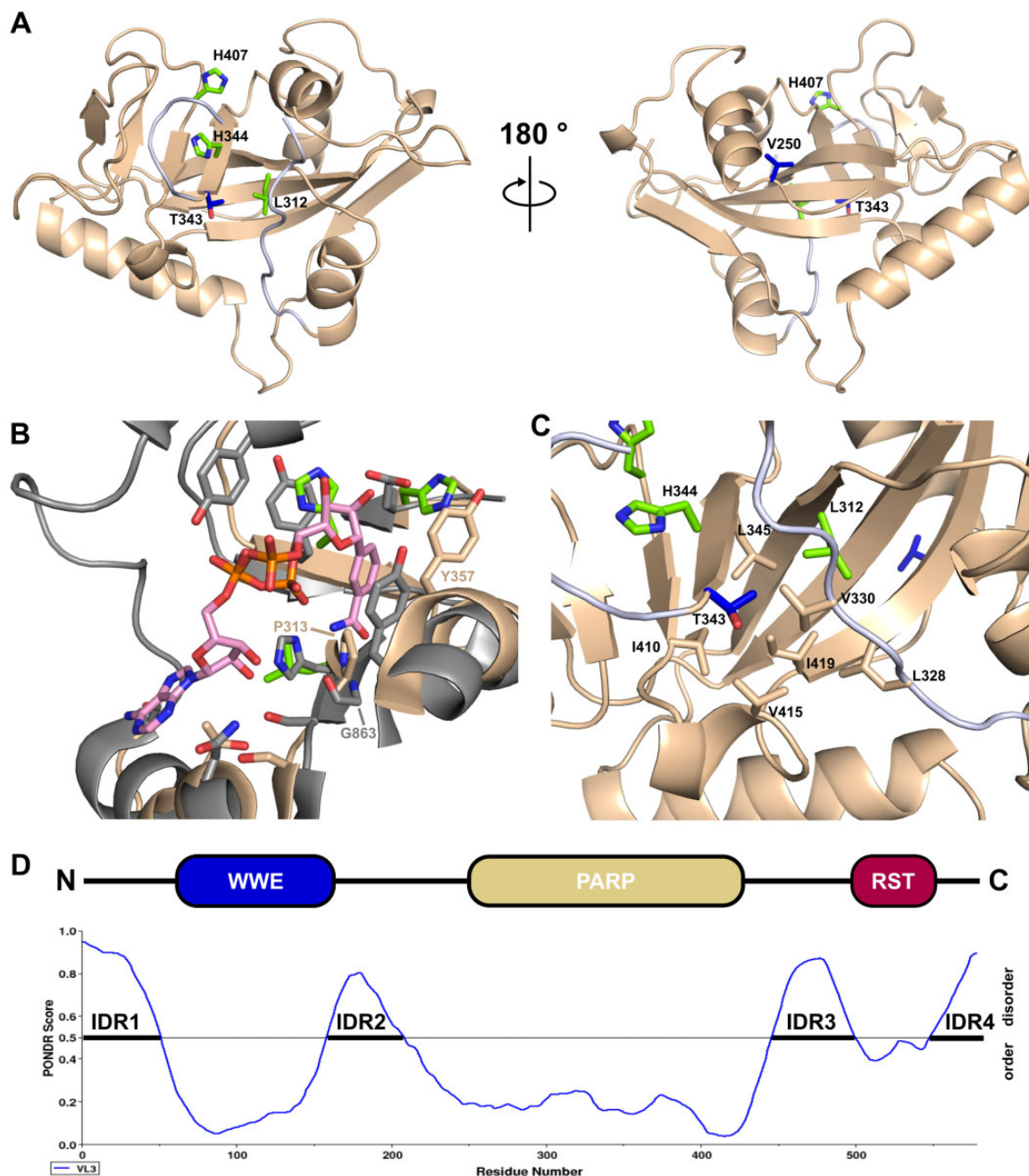


Figure 1 Crystal structure of the Ta-sro1 PARP domain. A, Structure of the Ta-sro1 PARP domain (residues 247–429) in cartoon representation. The postulated catalytic Leu–His–His triad is shown in green. Blue indicates the two polymorphic residues Val²⁵⁰ and Thr³⁴³. The partially disordered D-loop is shown in gray. B, Superposition of the Ta-sro1 PARP domain (beige) and the HsPARP1 PARP domain (gray) co-crystallized in complex with the NAD⁺ analog benzamide adenine dinucleotide (pink) (PDB identifier 6BHV). The position taken by Ta-sro1 residue Pro³¹³ is an invariant Gly in catalytically active PARPs. C, The side chain of the polymorphic residue Thr³⁴³ does not contribute to the postulated NAD⁺-binding site but points towards the hydrophobic core of the PARP domain. D, Ta-sro1 domain architecture and protein disorder profile as predicted by POND VL3-BA (Xue et al., 2010). IDRs are labeled IDR1–4. Reflection data and the Ta-sro1 PARP domain structure have been deposited at the Protein Data Bank with identifier 7PLQ. Diffraction images have been deposited at www.proteindiffraction.org under doi:10.18430/M37PLQ. 3D visualizations of protein structures were prepared using PyMol software version 1.7.2 (<https://sourceforge.net/projects/pymol/>).

NAD⁺ (described above), it is apparent that the side chain of Pro³¹³ would sterically interfere with NAD⁺ binding if Ta-sro1 would bind the co-substrate in the same orientation as canonical PARPs. The corresponding residue in catalytically active mono- and poly-ADP-ribosyltransferases is

an invariant Gly that accommodates the amide group of the nicotinamide moiety and stabilizes it by forming two hydrogen bonds (Wahlberg et al., 2012). Consistently, a corresponding Gly to Trp mutation in HsPARP10 abolishes ADP-ribosyltransferase activity (Yu et al., 2005).

Side chains of the polymorphic amino acids in Ta-sro1 do not directly contribute to the presumed NAD⁺-binding site

The Ta-sro1 PARP domain structure pinpoints the locations of the two amino acid polymorphisms that distinguish the hypermorphic proteoform Ta-sro1 from Ta-SRO1. Thr³⁴³ (Ala in Ta-SRO1) is located close to the proposed active site and its side chain is positioned toward the hydrophobic core of the protein formed by residues Leu³⁴⁵, Val³³⁰, Leu³²⁸, Ile⁴¹⁹, Val⁴¹⁵, and Ile⁴¹⁰ (Figure 1, A and C). In HsPARP1, the corresponding amino acid has been identified as a “gatekeeper” residue that, when mutated, widens the NAD⁺-binding site for access to bulkier NAD⁺ derivatives (Gibson et al., 2016). Based on protein homology modeling, Liu et al. (2014) proposed that the Ala/Thr³⁴³ side chain interacts with the co-substrate NAD⁺. However, our Ta-sro1 PARP domain crystal structure reveals that the Ta-sro1 Thr³⁴³ side chain points in the opposite direction and toward a hydrophobic area forming the basis of the postulated NAD⁺-binding site (Figure 1C). Conceivably, exchange of a hydrophobic to a polar amino acid at this site could slightly alter the position of strand β3 that forms one side of the pocket and this might affect the enzymatic activity of Ta-SRO1. The second polymorphic amino acid, Val²⁵⁰ (Gly in Ta-SRO1) is positioned on the opposite side of the domain and located close to the N-terminus of the crystallized construct (Figure 1A). In the context of the full-length protein, Val²⁵⁰ would form part of the transition from the predicted intrinsically disordered region 2 (IDR2) to the PARP domain (Figure 1D). Understanding the functional relevance of the Gly²⁵⁰ to Val exchange, therefore, may require further structural information in the context of the WWE-PARP domains. We noticed that the NESmapper algorithm for predicting putative nuclear export signals (NES) identifies the Ta-sro1 peptide GQPVD₂₄₇SAVRK₂₅₀L₂₅₃L₂₅₄L₂₅₅E (247–260) as a likely NES with a score of 15.1 (Kosugi et al., 2014). The exchange of Val²⁵⁰ to Gly lowers the NESmapper score to 2.5 indicating that the two proteoforms could have different nuclear export rates. However, when we expressed green fluorescent protein (GFP)-tagged variants of the two Ta-SRO1 proteoforms in *Nicotiana benthamiana*, both proteins appeared entirely nuclear localized suggesting that at least steady-state transport kinetics over the nuclear envelope are not substantially different (Supplemental Figure S1).

Ta-sro1 is catalytically inactive with respect to canonical ADP-ribosylation

The observed structural divergence from the consensus motif at the active site of the Ta-sro1 PARP domain prompted us to test whether Ta-sro1 can indeed bind NAD⁺ and is able to perform ADP-ribosylation reactions. For mammalian PARP domains, thermal stabilization of the catalytic domain by small molecule inhibitors that mimic nicotinamide can serve as a proxy for NAD⁺ binding (Wahlberg et al., 2012). We determined the thermal stability of the Ta-sro1 PARP

domain with increasing concentrations of the nicotinamide analog 6(5H)-phenanthridinone that was previously shown to stabilize ten catalytically active mammalian PARP domains (Wahlberg et al., 2012). We did not observe a thermal stabilization of the Ta-sro1 PARP domain by 6(5H)-phenanthridinone, even at concentrations in the millimolar range (Figure 2A). In contrast, the L713F variant of the HsPARP1 catalytic domain showed increased thermal stability (approximately +4°C) at 6(5H)-phenanthridinone concentrations above 2 μM (Figure 2A). We then performed *in vitro* binding assays with ³²P-labeled NAD⁺ to test if the Ta-sro1 PARP domain can bind the presumed co-substrate. We also produced full-length Ta-sro1 protein using the expression plasmid from Liu et al. (2014) to test if flanking sequences of the PARP domain or other domains are required for NAD⁺ binding. As shown in Figure 2B, neither full-length Ta-sro1 nor the isolated PARP domain bound detectable amounts of ³²P-NAD⁺. In contrast, the same assay detected ³²P-NAD⁺ binding to HsPARP10 that was used as a positive control (Kleine et al., 2008). Many ADP-ribosyltransferases of the PARP family auto-ADP-ribosylate in the presence of NAD⁺ (Feng et al., 2015). Unlike the catalytic domain of HsPARP10 that attaches a single ADP-ribose onto Glu residues in an intermolecular reaction (Kleine et al., 2008), neither the Ta-sro1 PARP domain nor the full-length protein showed auto-ADP-ribosylation *in vitro* when incubated with ³²P-labeled NAD⁺ (Figure 2C). The postulated PARP activity of Ta-sro1 is based on a commercially available colorimetric assay (PARP Universal Colorimetric Assay Kit, Trevigen, Gaithersburg, MD, USA) that uses histones as substrates for ADP-ribosyltransferases. We repeated this assay according to the manufacturer’s manual (see “PARP Inhibitor Assay Protocol”) with 0.5 μg of protein per well and each reaction performed in triplicate for full-length Ta-sro1, the isolated PARP domain, and a protein fragment that also includes the N-terminal Trp-Trp-Glu (WWE) domain of the protein (Ta-sro1 WWE-PARP) (Figure 2D). Compared to the positive control included in the assay kit (full-length HsPARP1) and the variant HsPARP1 L713F catalytic domain produced in our laboratory, all three Ta-sro1 constructs were inactive with respect to PARP activity (Figure 2D). Values for relative PARP activities of the negative control BSA and a reaction without protein were in the same range as those of the Ta-sro1 constructs. The nicotinamide analog 3-aminobenzamide (3AB) inhibited ADP-ribosylation of histones by HsPARP1, demonstrating that the assay truly reflects ADP-ribosyltransferase activity (Figure 2D). Therefore, the standardized assay on which the previously reported Ta-sro1 PARP activity is based, is not reproducible under our conditions.

Ta-sro1 does not auto-ADP-ribosylate when expressed in *N. benthamiana*

Several PARP enzymes require other proteins for catalytic activity or depend on a binding partner to modify specific amino acids (Yang et al., 2017; Suskiewicz et al., 2020). Given

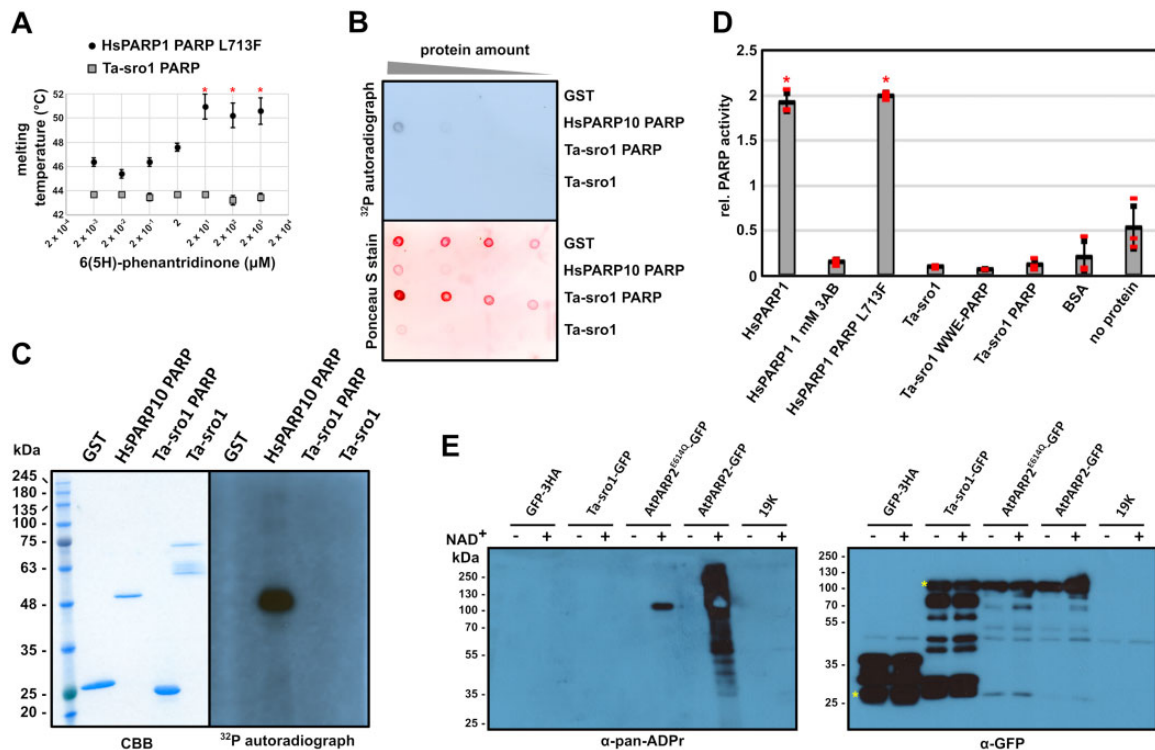


Figure 2 The Ta-sro1 PARP domain does not bind NAD^+ and does not auto-ADP-ribosylate *in vitro* or in plant cells. A, Thermal stability profiles of the Ta-sro1 PARP domain and the HsPARP1 PARP domain determined by Differential Scanning Fluorimetry. Calculated melting temperatures were averaged from three independent experiments, each consisting of three technical replicates, $n = 3$. Melting temperatures are plotted against concentrations of the NAD^+ analog 6(5H)-phenantridinone. Error bars represent standard deviations. Asterisks indicate significant differences compared to the lowest ligand concentration (one-way analysis of variance (ANOVA), Tukey's honestly significant difference (HSD) test, $P < 0.01$). B, *In vitro* ^{32}P - NAD^+ -binding assay with full-length Ta-sro1 or the Ta-sro1 PARP domain, the HsPARP10 PARP domain, and GST. The Ponceau S stain indicates protein amounts. C, To assess the proteins from (B) for auto-ADP-ribosylation activity, they were incubated with [^{32}P]- $\beta\text{-NAD}^+$ at 30°C for 30 min. and the incorporated radioactivity was detected by exposure of the dried gel to X-ray film. CBB, Coomassie Brilliant Blue stain. D, Colorimetric PARP activity assay with histones as substrates. HsPARP1 refers to full-length HsPARP1 protein included in the assay kit as positive control. Error bars represent standard deviations, red bars show individual data points of technical replicates, $n = 3$. Asterisks indicate significant differences compared to the BSA control (one-way ANOVA, Tukey's HSD, $P < 0.01$). Expression and purification procedures for proteins in (A–D) are described in Supplemental Methods. E, The indicated proteins were transiently expressed in *N. benthamiana* and extracted in the presence (+) or absence (–) of $300 \mu\text{M}$ $\beta\text{-NAD}^+$. Proteins were purified via the GFP-tag and analyzed by $\alpha\text{-GFP}$ and $\alpha\text{-pan-ADP-ribose}$ binding reagent immunoblots. Leaves expressing only the viral silencing suppressor 19K or GFP-3HA served as negative controls. Asterisks indicate the expected molecular weights of GFP-3HA and GFP-fusions of Ta-sro1 and AtPARP2. Results are representative of two (B and C) or three (D and E) independent experiments.

that the previously reported PARP activity of Ta-sro1 is based on an *in vitro* assay with recombinantly expressed protein, dependence on another plant factor to gain catalytic activity appears unlikely. Nevertheless, we assessed whether Ta-sro1 has mono- or poly-ADP-ribosyltransferase activity in plant cell extracts. We used transient *Agrobacterium*-mediated expression to produce Ta-sro1 and several control proteins as GFP fusions in *N. benthamiana*. We extracted the proteins in the absence or presence of $300 \mu\text{M}$ NAD^+ and immunoprecipitated them using a GFP-nanobody coupled to magnetic beads (Chen et al., 2018). After separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), we probed soluble protein extracts with anti-pan-ADP-ribose binding reagent that binds to mono- and poly-(ADP-ribose) (Figure 2E). Ta-sro1-

GFP did neither show signals for mono- nor for poly-ADP-ribosylation activity in this assay. In contrast, the canonical Arabidopsis PARP2 enzyme formed PAR chains in a NAD^+ -dependent manner. A PARP2 variant, in which the Glu residue that is essential for chain elongation is replaced by a Gln, produced a defined band of ~ 100 kDa that could correspond to mono-ADP-ribosylated PARP2-GFP. These results suggest that even at relatively high concentrations of NAD^+ and in presence of other plant co-factors that might be required for catalytic activity, Ta-sro1 does not exhibit mono- or poly-ADP-ribosyltransferase activity. Collectively, our reassessment of the proposed Ta-sro1 PARP activity revealed that the previously reported catalytic activity could not be reproduced under our conditions and with appropriate controls. Therefore, the elevated tolerance of wheat cultivar SR3

to high salinity and oxidative stress conditions cannot be explained by an altered PARP activity of the Ta-sro1 proteoform and other possible mechanisms need to be considered. SR3 plants exhibit higher H_2O_2 levels under salinity stress but also under control conditions (Liu et al., 2014). Given the role of reactive oxygen species in signal transduction and cell-to-cell communication in response to salinity stress (Evans et al., 2016), the altered redox homeostasis of wheat cultivar SR3 may underlie its enhanced salt tolerance phenotype. Arabidopsis RCD1 lacks ADP-ribosyltransferase activity and *rcd1* mutants also exhibit an altered sensitivity to reactive oxygen species (Shapiguzov et al., 2019). This suggests that redox imbalances in *sro* mutants are not necessarily caused by an altered ADP-ribosyltransferase activity of SRO proteins, but more likely relate to their function as transcriptional co-regulators.

Are plant SRO proteins noncanonical ADP-ribosyltransferases?

The comparably low number of canonical PARPs in plants, paired with the structural conservation of PARP(-like) domains in SRO proteins, have fueled the hypothesis that SROs may represent an additional plant-specific family of noncanonical ADP-ribosyltransferases (Lamb et al., 2012; Kong et al., 2021). Given that a His–Tyr–[Glu/Asp/Gln] triad is conserved in ADP-ribosyltransferases from diphtheria toxin to mammalian and plant PARPs, we explored the level of conservation of the corresponding residues in plant SRO proteins. We used JACKHMMER (Potter et al., 2018) and the Ta-sro1 PARP domain as a query to identify sequence-related PARP domains within the *Viridiplantae*. This search identified 1,399 homologs until first members of canonical

PARPs started to appear in the second iteration. The sequence logo in Figure 3 is representative of 1,084 *bona fide* SRO PARP domains from proteins with a (WWE-)PARP-RST domain structure, 312 homologs without additional annotated domains, and three proteins with a domain structure representative of canonical PARPs. As shown in Figure 3, in SRO PARP domains, there is no strong conservation of the His and Tyr that coordinate NAD^+ binding and the same holds true for the Glu residue required for polymerase activity. Notably, in a subset of 33 PARP domains from 14 different plant families the His and Tyr residues are conserved and in all of these the His is followed by a Gly (Supplemental Data Set 1). In contrast, there is no conservation of the Glu in these SRO family members and the equivalent position is often taken by a Trp or Arg. This indicates that structural features of the NAD^+ -binding site are conserved in a few SRO family members and consequently these proteins might mono-ADP-ribosylate their substrates by substrate-assisted catalysis. From an evolutionary perspective, the plant SRO protein family therefore represents a valuable resource of natural variation in the otherwise highly conserved PARP domain. As reported by Kong et al. (2021), at least one SRO protein retained the ability to catalyze mono-ADP-ribosylation reactions. In Arabidopsis SRO2, the positions of the His and Tyr residues are swapped suggesting that certain deviations from the consensus motif are compatible with ADP-ribosyltransferase activity. The position of the conserved Gly is taken by Ala¹¹⁹ in SRO2. Unlike the bulky pyrrolidine ring of the Pro residues in the Arabidopsis RCD1 and Ta-sro1 structures at this position, the smaller Ala side chain is less likely to interfere with accommodation of the nicotinamide moiety and the Ala backbone retains the capacity to form two hydrogen bonds with the co-

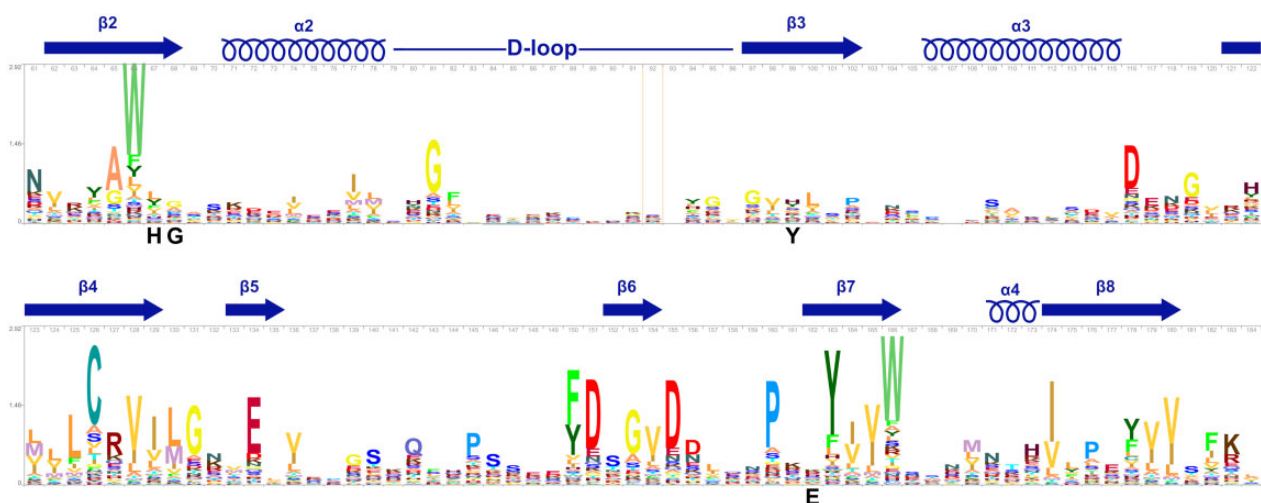


Figure 3 The His–Tyr–Glu triad is not conserved in the SRO protein family. SRO proteins were identified using JACKHMMER (Potter et al., 2018) with the Ta-sro1 PARP domain as a query and the following settings: HmmerWeb version 2.41.2, database Reference proteomes, restricted to *Viridiplantae* (taxid:33090), E-values Sequence = 0.0001, Hit = 0.0003. The sequence conservation logo was generated with Skylogn (Skylogn.org by Jody Clements, Travis Wheeler & Robert Finn, Interactive logos for alignments and profile HMMs, <http://skylogn.org/>, CC BY 3.0 license) and corresponds to Ta-sro1 amino acids 306–429. The positions of the conserved His, Gly, Tyr, and Glu are indicated. Conserved secondary structure elements are depicted above the sequence logo.

substrate. It will be informative to correlate the level of divergence in key residues of the NAD⁺-binding site with ADP-ribosyltransferase activity in other members of the SRO family. Future analyses should further explore how SRO proteins influence transcription factor activity in abiotic and biotic stress responses and address the question of why PARP domains are strictly conserved in this group of transcriptional co-regulators. However, we argue that, given the non-conservation of the His–Tyr–Glu triad in the majority of SRO proteins, the biochemical basis for reported noncanonical ADP-ribosyltransferase activities should be investigated to substantiate their mode of catalytic activity. Many PARP domains can be readily produced as soluble proteins in *E. coli* and assays to test for NAD⁺ (analog) binding, as well as mono- and/or poly-ADP-ribosyltransferase activity are outlined in this letter and well documented for mammalian PARPs (Kleine et al., 2008; Wahlberg et al., 2012; Glumoff et al., 2022). Analyses of noncanonical plant ADP-ribosyltransferases should include site-directed mutagenesis of residues predicted to coordinate NAD⁺ binding combined with enzyme assays and, where feasible, complementation studies of the respective plant mutant phenotypes as well as evidence for ADP-ribosylation of substrates by protein mass spectrometry.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. The Ta-sro1 and Ta-SRO1 proteoforms do not show differences in subcellular localization when transiently expressed in *N. benthamiana*.

Supplemental Table S1. X-ray data collection, refinement, and validation statistics of the Ta-sro1 PARP domain structure.

Supplemental Table S2. Statistical analysis of experimental data presented in Figure 2A.

Supplemental Table S3. Statistical analysis of experimental data presented in Figure 2D.

Supplemental Table S4. List of oligonucleotides used in this work.

Supplemental Methods. Experimental procedures for protein expression, purification, and crystallization as well as the activity and binding assays.

Supplemental File S1. Protein Data Bank X-Ray Validation Report.

Supplemental Data Set 1. The His, Gly, and Tyr residues that coordinate NAD⁺ binding are conserved in several plant PARP domains beyond canonical PARPs.

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References

- Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**: 213–221
- Bell CE, Eisenberg D (1997) Crystal structure of diphtheria toxin bound to nicotinamide adenine dinucleotide. *Adv Exp Med Biol* **419**: 35–43
- Chen C, De Masi R, Lintermann R, Wirthmueller L (2018) Nuclear import of Arabidopsis poly(ADP-Ribose) polymerase 2 is mediated by importin- α and a nuclear localization sequence located between the predicted SAP domains. *Front Plant Sci* **9**: 1581
- Evans MJ, Choi WG, Gilroy S, Morris RJ (2016) A ROS-assisted calcium wave dependent on the AtRBOHD NADPH oxidase and TPC1 cation channel propagates the systemic response to salt stress. *Plant Physiol* **171**: 1771–1784
- Feng B, Liu C, de Oliveira MV, Intorne AC, Li B, Babilonia K, de Souza Filho GA, Shan L, He P (2015) Protein poly(ADP-ribosylation) regulates Arabidopsis immune gene expression and defense responses. *PLoS Genet* **11**: e1004936
- Gibson BA, Zhang Y, Jiang H, Hussey KM, Shrimp JH, Lin H, Schwede F, Yu Y, Kraus WL (2016) Chemical genetic discovery of PARP targets reveals a role for PARP-1 in transcription elongation. *Science* **353**: 45–50
- Glumoff T, Sowa ST, Lehtiö L (2022) Assay technologies facilitating drug discovery for ADP-ribosyl writers, readers and erasers. *Bioessays* **44**: e2100240
- Gu Z, Pan W, Chen W, Lian Q, Wu Q, Lv Z, Cheng X, Ge X (2019) New perspectives on the plant PARP family: Arabidopsis PARP3 is inactive, and PARP1 exhibits predominant poly (ADP-ribose) polymerase activity in response to DNA damage. *BMC Plant Biol* **19**: 364
- Hottiger MO (2015) Nuclear ADP-ribosylation and its role in chromatin plasticity, cell differentiation, and epigenetics. *Annu Rev Biochem* **84**: 227–263
- Jaspers P, Overmyer K, Wrzaczek M, Vainonen JP, Blomster T, Salojärvi J, Reddy RA, Kangasjärvi J (2010) The RST and PARP-like domain containing SRO protein family: analysis of protein structure, function and conservation in land plants. *BMC Genomics* **11**: 170
- Johnson VG, Nicholls PJ (1994) Histidine 21 does not play a major role in diphtheria toxin catalysis. *J Biol Chem* **269**: 4349–4354
- Kleine H, Poreba E, Lesniewicz K, Hassa PO, Hottiger MO, Litchfield DW, Shilton BH, Lüscher B (2008) Substrate-assisted

- catalysis by PARP10 limits its activity to mono-ADP-ribosylation. *Mol Cell* **32**: 57–69
- Kong L, Feng B, Yan Y, Zhang C, Kim JH, Xu L, Rack JG, Wang Y, Jang JC, Ahel I, et al.** (2021) Noncanonical mono(ADP-ribosylation) of zinc finger SZF proteins counteracts ubiquitination for protein homeostasis in plant immunity. *Mol Cell* **81**: 4591–4604.e8
- Kosugi S, Yanagawa H, Terauchi R, Tabata S** (2014) NESmapper: accurate prediction of leucine-rich nuclear export signals using activity-based profiles. *PLoS Comput Biol* **10**: e1003841
- Lamb RS, Citarelli M, Teotia S** (2012) Functions of the poly(ADP-ribose) polymerase superfamily in plants. *Cell Mol Life Sci* **69**: 175–189
- Liu S, Liu S, Wang M, Wei T, Meng C, Wang M, Xia G** (2014) A wheat SIMILAR TO RCD-ONE gene enhances seedling growth and abiotic stress resistance by modulating redox homeostasis and maintaining genomic integrity. *Plant Cell* **26**: 164–180
- Marsischky GT, Wilson BA, Collier RJ** (1995) Role of glutamic acid 988 of human poly-ADP-ribose polymerase in polymer formation. Evidence for active site similarities to the ADP-ribosylating toxins. *J Biol Chem* **270**: 3247–3254
- Potter SC, Luciani A, Eddy SR, Park Y, Lopez R, Finn RD** (2018) HMMER web server: 2018 update. *Nucleic Acids Res* **46**: W200–W204
- Ruf A, Mennissier de Murcia J, de Murcia G, Schulz GE** (1996) Structure of the catalytic fragment of poly(ADP-ribose) polymerase from chicken. *Proc Natl Acad Sci USA* **93**: 7481–7485
- Shapiguzov A, Vainonen JP, Hunter K, Tossavainen H, Tiwari A, Järvi S, Hellman M, Aarabi F, Alseekh S, Wybouw B, et al.** (2019) Arabidopsis RCD1 coordinates chloroplast and mitochondrial functions through interaction with ANAC transcription factors. *Elife* **8**: e43284
- Steffen JD, Brody JR, Armen RS, Pascal JM** (2013) Structural implications for selective targeting of PARPs. *Front Oncol* **3**: 301
- Suskiewicz MJ, Zobel F, Ogden TE, Fontana P, Ariza A, Yang JC, Zhu K, Bracken L, Hawthorne WJ, Ahel D, et al.** (2020) HPP1 completes the PARP active site for DNA damage-induced ADP-ribosylation. *Nature* **579**: 598–602
- Vainonen JP, Shapiguzov A, Vaattovaara A, Kangasjärvi J** (2016) Plant PARPs, PARGs and PARP-like proteins. *Curr Protein Pept Sci* **17**: 713–723
- Wahlberg E, Karlberg T, Kouznetsova E, Markova N, Macchiarulo A, Thorsell AG, Pol E, Frostell Å, Ekblad T, Öncü D, et al.** (2012) Family-wide chemical profiling and structural analysis of PARP and tankyrase inhibitors. *Nat Biotechnol* **30**: 283–288
- Wirthmueller L, Asai S, Rallapalli G, Sklenar J, Fabro G, Kim DS, Lintermann R, Jaspers P, Wrzaczek M, Kangasjärvi J, et al.** (2018) Arabidopsis downy mildew effector HaRxL106 suppresses plant immunity by binding to RADICAL-INDUCED CELL DEATH1. *New Phytol* **220**: 232–248
- Xue B, Dunbrack RL, Williams RW, Dunker AK, Uversky VN** (2010) PONDR-FIT: a meta-predictor of intrinsically disordered amino acids. *Biochim Biophys Acta* **1804**: 996–1010
- Yang CS, Jividen K, Spencer, A, Dworak N, Ni L, Oostdyk LT, Chatterjee M, Kušmider B, Reon B, Parlak M, et al.** (2017) Ubiquitin modification by the E3 ligase/ADP-ribosyltransferase Dtx3L/Parp9. *Mol Cell* **66**: 503–516.e5
- Yao D, Arguez MA, He P, Bent AF, Song J** (2021) Coordinated regulation of plant immunity by poly(ADP-ribosylation) and K63-linked ubiquitination. *Mol Plant* **14**: 2088–2103
- Yu M, Schreek S, Cerni C, Schamberger C, Lesniewicz K, Poreba E, Vervoorts J, Walsemann G, Grötzinger J, Kremmer E, et al.** (2005) PARP-10, a novel Myc-interacting protein with poly(ADP-ribose) polymerase activity, inhibits transformation. *Oncogene* **24**: 1982–1993