Plant Communications, Volume *3*

Supplemental information

Engineering drought and salinity tolerance traits in crops through CRISPR-mediated genome editing: Targets, tools, challenges, and perspectives

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Supplementary information 1

Introgression of drought and salinity tolerance traits in crops through CRISPR-based precision genome engineering

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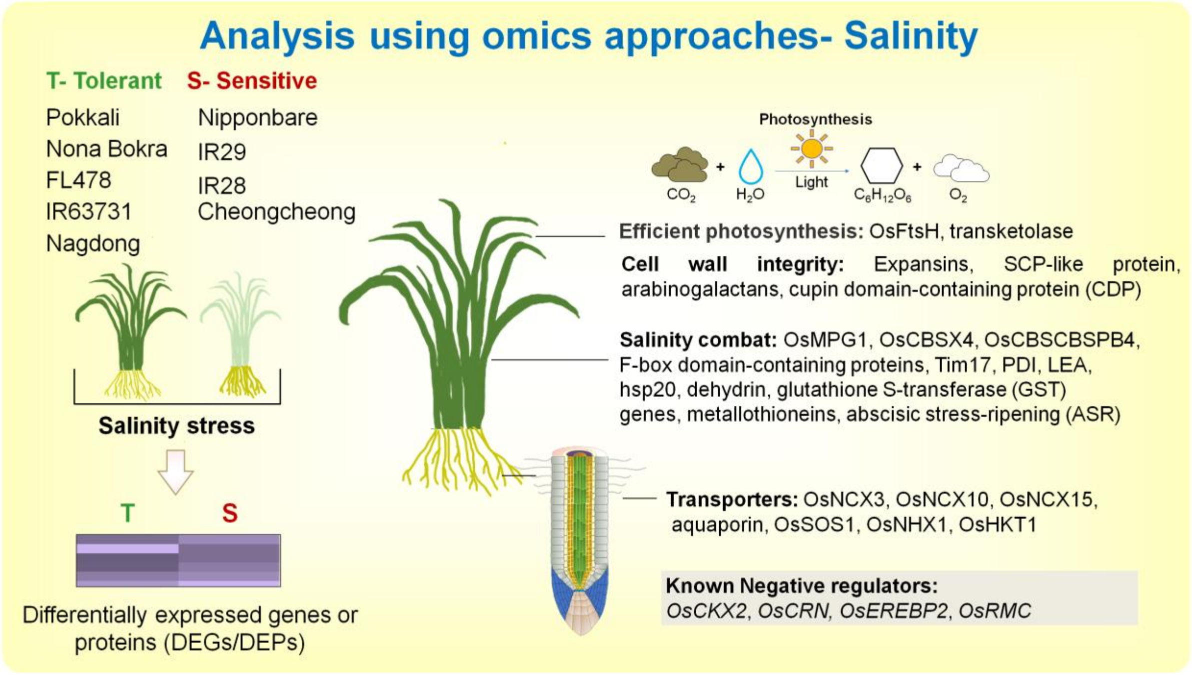

Figure S1. Deciphering uniquely expressed genes in salt-stress tolerance using omics approaches is summarized for rice studies. Comparative omics studies between salinity tolerant and sensitive varieties have been performed to find the differentially expressed genes or proteins (DEGs or DEPs) [Cotsaftis et al., 2011- Pokkali (T), Nona Bokra (T), Nipponbare (S); Li et al., 2018- Pokkali (T), IR-29 (S),; Farooq et al., 2021- Pokkali (T), Nagdong (T), IR-28 (S), Cheongcheong (S)]. Comparative transcriptomics and translatome data predicted several unique DEGs/DEPs that could be combined precisely to improve the salinity tolerance in salt-sensitive commercial rice cultivars. In the above-ground part of the plant, photosynthesis is mainly affected by salinity stress. FtsH-like chloroplast proteins (Os02g43350 and Os05g38400) were highly accumulated during the salinity stress, and these proteins are associated with the photosynthetic electron transport chain (Li et al., 2018). Similarly, transketolase (Os04g19740) was highly expressed. Transketolase enzyme transfers a residue with two carbon atoms from fructose-6-phosphate to glyceraldehydes-3-phosphate, resulting in ribulose-1,5-bisphosphate (RuBP) formation. Both FtsH-like chloroplast proteins and transketolase could be modified and expressed differentially to mitigate the effects of salinity stress on photosynthesis. Cell wall integrity is essential in the cell defense from high salt accumulation. Many genes were upregulated in the salt-tolerant Pokkali land race, which includes arabinogalactan proteins, expansins, SCP-like proteins, and cupin domain-containing proteins (CDPs). Ion transporters are involved in the intrusion and extrusion of Na^+ during elevated salinity stress. Many transporters have been explicitly accumulated in the Pokkali. For instance, transporters like *OsNCX3*, *OsNCX10*, *OsNCX15*, *Aquaporins*, *OsSOS1*, *NHX1*, *and* OsHKT1 are the best candidates for reorganizing the Na^+ transport (Singh et al., 2015; Li et al., 2018). Specific transporters could be targeted to increase the extrusion or block the Na^+ intrusion. Many known downstream targets help in maintaining cellular homeostasis during salinity stress. The transcripts of the genes like *OsMPG1* (Kumar et al., 2012), *OsCBSX4* (Singh et al., 2012), and *OsCBSCBSPB4* (Kumar et al., 2018), were highly accumulated during salinity stress in Pokkali as compared to IR64 and their overexpression in tobacco has shown tolerance to the salinity stress. Further detailed study of the mode of action of these genes could help in exploring novel genes or pathways to alleviate the salinity stress. Similarly, Pokkali-specific upregulation of F-box domain-containing proteins, Tim17, PDI, LEA, hsp20, dehydrin, Glutathione S-transferase (GST) genes, metallothioneins, and Abscisic stress-ripening (ASR) was found in comparison to salt-sensitive IR29 variety (Li et al., 2018). These genes could be a strong candidate for tackling salinity stress. DNA methylation modulates the pre-transcriptional changes during stress. Analysis of methylation profile in salt-tolerant and susceptible rice varieties showed specific changes in Pokkali (Farooq et al., 2021). The DNA demethylases and methyltransferases were highly induced in the Pokkali compared to IR29. Further, the plasticity of DNA methylation could be tamed to generate the salt-tolerance traits in the salt-sensitive varieties. Most targets mentioned above are positive regulators and need overexpression or activation by CRISPRa-type systems. On the other hand, CRISPR/Cas9-mediated knockouts of negative regulators like *OsCKX2* (Joshi et al., 2018), *OsCRN* (Kojonna et al., 2022), *OsEREBP2* and *OsRMC* (Serra et al., 2013) could assist in producing salt-tolerant rice genotypes.

Supplementary Table S1. Abbreviations of transcription factors (TFs), genes, and protein families.

Abbreviation	**Description**
ABRE	ABA-responsive element family
ACQOS	*Acquired osmotolerance*
AITR	*ABA-induced transcription repressors*
AIW1, AIW2	*ABA-induced WD40-repeat 1*and *2*
AP2/ERF	APETALA2/ethylene-responsive TF factor family
AREB/ABF	ABRE-binding protein/ABRE binding factor family
AREB1	ABA-responsive element-binding protein 1 family
ARF4	Auxin response factor 4
ARGOS8	*Auxin-regulated gene involved in organ size 8*
AVP1	*Arabidopsis* vacuolar H^+-pyrophosphatase
BBS1	Bilateral blade senescence 1
BG3	*Big grain3*
bHLH	Basic helix-loop-helix family
bZIP	Basic leucine zipper protein family
C/VIF1	*Cell wall/vacuolar inhibitor of fructosidase 1*
CDF	Cycling DOF Factor family
CDPK	Calcium-dependent protein kinase family
CIPK	Calcinuerin B-like-interacting protein kinase family
CLCg	*Voltage-gated chloride channel* family
DOF15	DNA-binding with one finger 15
DPA4	*Development-Related PcG Target in the APEX4*
DREB	Dehydration responsive element binding protein family
DREB	*Dehydrin, 1,8-dihydroxy naphthalene* family
DST	*drought and salt tolerance* family
ELF4	*Early flowering 4*
ENA	Na^{2+}-extrusion family
ERA1	Enhanced Response to ABA1
ERF83	*Ethylene-responsive factor83*
FLN2	Fructokinase-like2
GI	*GIGANTEA* family
GID1a	*Gibberellin-insensitive dwarf1a*
GNC	*GATA, nitrate-inducible, carbon-metabolism-involved* family
GST	*Glutathione S-transferase* family
GTγ-2	Trihelix transcription factor γ-2

HAG1	*Histone acetyltransferase*
HAK20	*High-affinity K*$^+$ *transporter*
HD-Zip	Homeodomain-leucine zipper family
HKT	High-affinity K$^+$ transporter family
HKT1	*High-affinity K*$^+$ *transporter1*
HVP10	*Vacuolar H*$^+$*-pumping pyrophosphatase 10*
HyPRP1	*Hybrid proline-rich protein 1*
ITPK1	Inositol trisphosphate 5/6 kinase 1
KCO	TPK/K$^+$ channel outward channels family
KT/HAK/KUP	K$^+$ transporter (KT)/high-affinity K$^+$ (HAK)/ K$^+$ uptake permease (KUP) family
LBD40	*Lateral organ boundaries domain 40*
LRR-XII	Leucine-rich repeat receptor-like kinase subfamily XII
MAPK	Mitogen-activated-protein-kinase family
MOCA1	Monocation-induced (Ca^{2+}) increases 1
MYB	Myeloblastosis family
NAC	NAM, ATAF1-2, and CUC2 family
NCA1a/*OsNCA1b*	*No catalase activity 1a* and *b*
NHX	Na$^+$/H$^+$ or K$^+$/H$^+$ exchanger family
NPR1	*Non-expressor of pathogenesis-related gene 1*
OSCA1	*reduced hyperosmolality-induced Ca*$^{2+}$ *increase 1*
OST2	*OPEN STOMATA 2*
PdNF-YB21	*Root-specific nuclear factor Y*
PIL14	Phytochrome-interacting factor-like14
PQT3	*Paraquat tolerance 3*
RAV2	*Related to ABI3/VP1*
RBOHD	*Respiratory burst oxidase homolog D*
RR22	*B-type response regulator 22*
RR9, RR10	*Type-A response regulator 1* and *2*
SAPK1, SAPK2	*stress/ABA-activated protein kinase 1* and *2*
SAUR41	*Small auxin up RNA 41*
SnRK	SNF1-related protein kinase family
SOD7	*Suppressor of da1-1*
SOS	*Salt overly sensitive* antiporter family
SPL10	*Squamosa promoter-binding protein-like 10*
SRL1, 2	Semi-rolled leaf 1 and 2
UGT	*UDP-glycosyltransferase*
VGICs	Non-voltage-gated (tandem pore K$^+$) channels
WDR	(WD40-repeat) family

WHY	WHIRLY family

Supplementary Table S2. The Cas orthologs adopted for CRISPR-based tool development and used to plot the Figure 3.

Ortholog	PAM (5'-3')	Source	Feature	Reference
SpCas9	NGG	*Streptococcus pyogenes*	Most used Cas ortholog	(Jinek et al., 2012)
St1Cas9	NNAGAAW	*Streptococcus thermophilus*	Cas ortholog	(Cong et al., 2013)
St3Cas9	NGGNG	*Streptococcus thermophilus*	Cas ortholog	(Cong et al., 2013)
NmeCas9	NNNNGMTT	*Neisseria meningitidis*	Cas ortholog	(Hou et al., 2013)
TdCas9	NAAAAN	*Treponema denticola*	Cas ortholog	(Esvelt et al., 2013)
FnCas9	NGG	*Francisella novivida*	Cas ortholog	(Fonfara et al., 2014; Price et al., 2015)
SaCas9	NNNRRT	*Staphylococcus aureus*	Cas ortholog	(Ran et al., 2015)
BlatCas9	NNNNCND	*Brevibacillus laterosporus*	Cas ortholog	(Karvelis et al., 2015)
FnCpf1	TTV/TTTV/KYTV	*Francisella tularensis*	Cas ortholog	(Zetsche et al., 2015)
LbCpf1	TTTV	*Lachnospiraceae bacterium* ND2006	Cas ortholog	(Zetsche et al., 2015)
AsCpf1	TTTV	*Acidaminococcus* sp. BV3L6	Cas ortholog	(Zetsche et al., 2015)
BpCpf1	-	*Butyrivibrio proteoclasticus*	Cas ortholog	(Zetsche et al., 2015)
SsCpf1	-	*Smithella* sp. *SC_K08D17*	Cas ortholog	(Zetsche et al., 2015)
Cas13a	-	*Leptotrichia shahii*	RNA targeting	(Abudayyeh et al., 2016)
CjCas9	NNNVRYAC	*Campylobacter jejuni*	Smallest Cas ortholog	(Kim et al., 2017)
ThermoCas9	NNNNCNR	*Geobacillus thermodenitrificans T12*	Cas ortholog	(Mougiakos et al., 2017)
Cas13b	-	*Bergeyella zoohelcum*	RNA targeting	(Smargon et al., 2017)
ScCas9	NNG	*Streptococcus canis*	Cas ortholog	(Chatterjee et al., 2018)
MbCpf1	TTV/TTTV	*Moraxella bovoculi* 237	Cas ortholog	(Tóth et al., 2018)
Cas13d	-	*Eubacterium siraeum*	RNA targeting	(Konermann et al., 2018)
CasX	TTCN	Uncultured microbial communities	Cas ortholog	(Liu et al., 2019)
Cas12f (Cas14) homologs	5' T- or C-rich	Different species	Cas ortholog	(Karvelis et al., 2020)
SmacCas9	NAAN	*Streptococcus macacae*	Cas ortholog	(Chatterjee et al., 2020a)
ErCpf1 (MAD7)	YTTN	*Eubacterium rectale*	Royalty-free ortholog	(I. Inscripta, 2020)
CeCpf1	TTTV	*Coprococcus eutactus*	Cas ortholog	(Chen et al., 2020)
BfCpf1	TTTV	*Butyrivibrio fibrisolvens*	Cas ortholog	(Chen et al., 2020)

CasΦ (Cas12j)	TBN	Bacteriophage	Cas ortholog	(Pausch et al., 2020)
ShyCas9	NNARMM	*Staphylococcus hyicus*	Cas ortholog	(Schmidt et al., 2021)
SluCas9	NNGG	*Staphylococcus lugdunensis*	Cas ortholog	(Schmidt et al., 2021)
SmiCas9	NNGG	*Staphylococcus microti*	Cas ortholog	(Schmidt et al., 2021)
SpaCas9	NNGG	*Staphylococcus pasteuri*	Cas ortholog	(Schmidt et al., 2021)

N:any nucleotide, R:A/G, M:A/C, W:A/T, V:G/C/A, Y:C/T, H:A/C/T, B:G/T/C.

Supplementary Table S3. The Cas variants adopted for CRISPR-based tool development and used to plot the Figure 3.

Variants	**PAM (5’-3’)**	**Mutation**	**Feature**	**Reference**
SpCas9(D1135E)	NAG, NGA	D1135E	Relaxed PAM	(Kleinstiver et al., 2015)
SpCas9(VQR)	NGA	D1135V/R1335Q/T1337R	Relaxed PAM	(Kleinstiver et al., 2015)
SpCas9(EQR)	NGAG	D1135E/R1335Q/T1337R	Relaxed PAM	(Kleinstiver et al., 2015)
SpCas9(VRER)	NGCG	D1135V/G1218R/R1335E/T1337R	Relaxed PAM	(Kleinstiver et al., 2015)
SaCas9 KHH	NNNRRT	E782K/N968K/R1015H	Relaxed PAM	(Kleinstiver et al., 2015)
SpCas9(K855A)	NGG	K855A	Enhanced specificity	(Slaymaker et al., 2016)
eSpCas9(1.0)	NGG	K810A/K1003A/R1060A	Enhanced specificity	(Slaymaker et al., 2016)
eSpCas9(1.1)	NGG	K848A/K1003A/R1060A	Enhanced specificity	(Slaymaker et al., 2016)
SpCas9-HF1	NGG	N497A/R661A/Q695A/Q926A	Enhanced specificity	(Kleinstiver et al., 2016)
SpCas9 QQR1	NAAG	G1218R/N1286Q/I1331F/D1332K/R1333Q/R1335Q/T1337R	Relaxed PAM	(Anders et al., 2016)
HeFSpCas9	NGG	N497A/R661A/Q695A/K848A/Q926A/K1003A/R1060A	Enhanced specificity	(Kulcsár et al., 2017)
HypaCas9	NGG	N692A/M694A/Q695A/H698A	Enhanced specificity	(Chen et al., 2017)
AsCpf1(RR)	TYCV, CCCC	S542R/K607R	-	(Gao et al., 2017)
AsCpf1(RVR)	TATV	S542R/K548V/N552R	-	(Gao et al., 2017)
LbCpf1(RR)	TYCV, CCCC	G532R/K595R	Relaxed PAM	(Gao et al., 2017)
LbCpf1(RVR)	TATV	G532R/K538V/Y542R	Relaxed PAM	(Gao et al., 2017)
evoCas9	NGG	M495V/Y515N/K526E/R661Q	Enhanced specificity	(Casini et al., 2018)
HiFi Cas9	NGG	R691A	Enhanced specificity	(Vakulskas et al., 2018)
Sniper Cas9	NGG	F539S/M763I/K890N	Enhanced specificity	(Lee et al., 2018)
eHF1-Cas9	NGG	N497A/R661A/Q695A/K848A/Q926A/K1003A/R1060A	Enhanced specificity	(Liang et al., 2018)
eHypaCas9	NGG	N692A/M694A/Q695A/H698A/K848A/K1003A/R1060A	Enhanced specificity	(Liang et al., 2018)
xCas9 3.7	NG, GAA, GAT	A262T/R324L/S409I/E480K/E543D/M694I/E1219V	Relaxed PAM	(Hu et al., 2018)
SpCas9-NG	NG	R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R	Relaxed PAM	(Nishimasu et al., 2018)
FnCpf1(RVR)	TYCV, TCTV	N607R/K671R	-	(Tóth et al., 2018)
FnCpf1(RR)	TWTV	N607R/K613V/N617R	-	(Tóth et al., 2018)
MbCpf1(RR)	TYCV, TCTV	N576R/K637R	Relaxed PAM	(Tóth et al., 2018)
MbCpf1(RVR)	TWTV	N576R/K582V/N586R	Relaxed PAM	(Tóth et al., 2018)

enAsCpf1	VTTV, TTTT, TTCN, TATV	E174R/S542R/K548R	Temperature insensitive	(Kleinstiver et al., 2019)
iSpmacCas9	NAA	R221K/N394K	Relaxed PAM	(Chatterjee et al., 2020a)
SpCas9-NRRH	NRRH	R1114G/D1135N/V1139A/D1180G/E1219V/Q1221H/A1320V/R1333K	Relaxed PAM	(Miller et al., 2020)
SpCas9-NRTH	NRTH	R1114G/D1135N/D1180G/G1218S/E1219V/Q1221H/P1249S/E1253K/P1321S/D1332G/R1335L	Relaxed PAM	(Miller et al., 2020)
SpCas9-NRCH	NRCH	R1114G/D1135N/E1219V/D1332N/R1335Q/T1337N/S1338T/H1349R	Relaxed PAM	(Miller et al., 2020)
ScCas9-Sc^{++}	NNG	T1227K	Relaxed PAM	(Chatterjee et al., 2020b)
HiFi-Sc^{++}	NNG	R701A/T1227K	Relaxed PAM	(Chatterjee et al., 2020b)
SpG	NGN	D1135L/S1136W/G1218K/E1219Q/R1335Q/T1337R	Relaxed PAM	(Walton et al., 2020)
SpY	NRN, NYN	A61R/L1111R/D1135L/S1136W/G1218K/E1219Q/N1317R/A1322R/R1333P, R1335Q/T1337R	Relaxed PAM	(Walton et al., 2020)
enLbCpf1	TTTV	D156R/G532R/K538R	Temperature insensitive	(Schindele and Puchta, 2020)
ttLbCpf1	TTTV	D156R	Temperature tolerance	(Schindele and Puchta, 2020)
enCjCas9	NNNVRYAC	L58Y/D900K	Enhanced specificity	(Nakagawa et al., 2021)

N:any nucleotide, R:A/G, M:A/C, W:A/T, V:G/C/A, Y:C/T, H:A/C/T, B:G/T/C.

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