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Supplementary information

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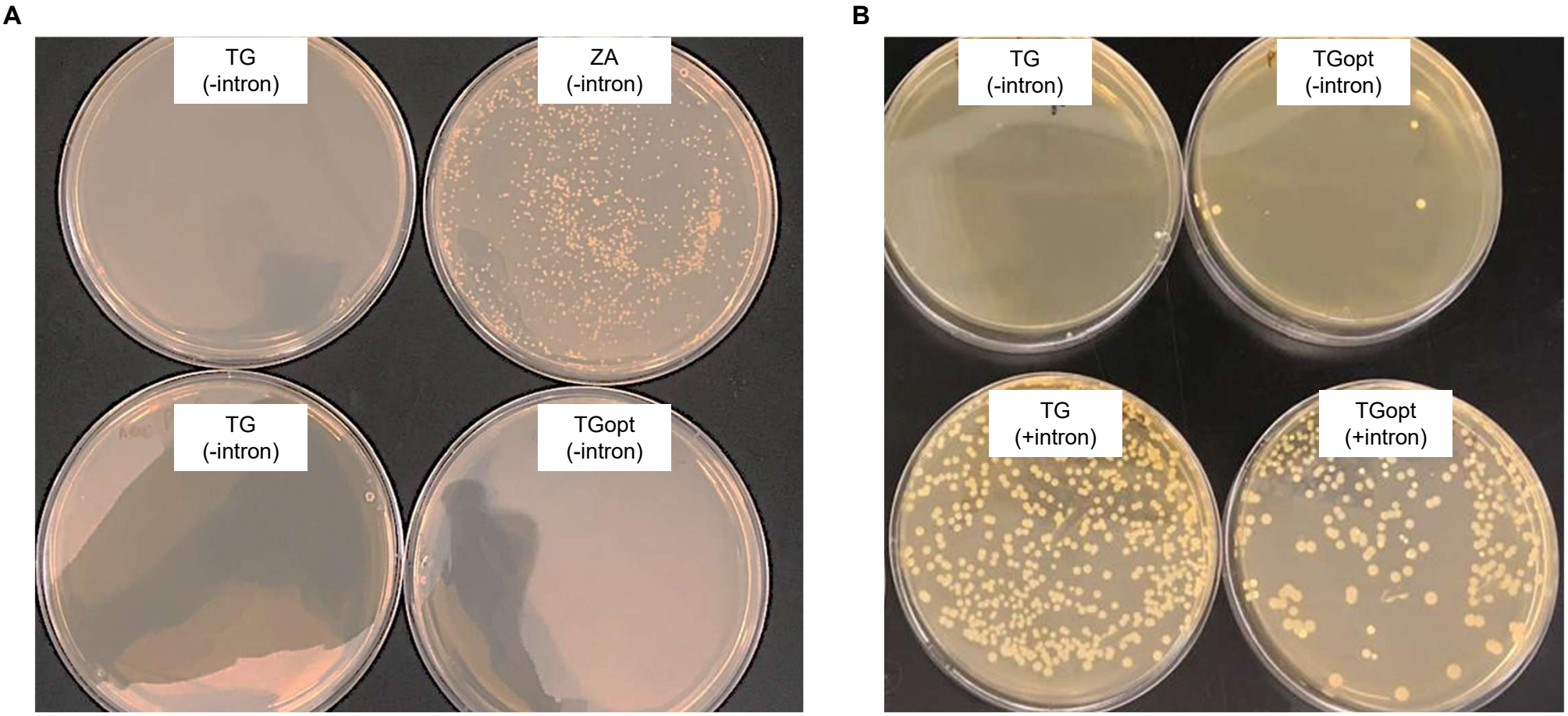
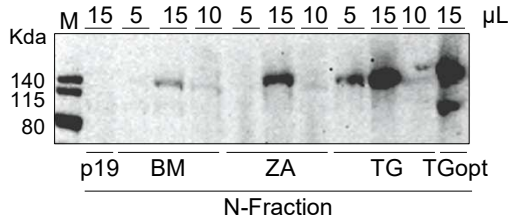
Efficient site-specific gene addition using R2 retrotransposons in tobacco and rice

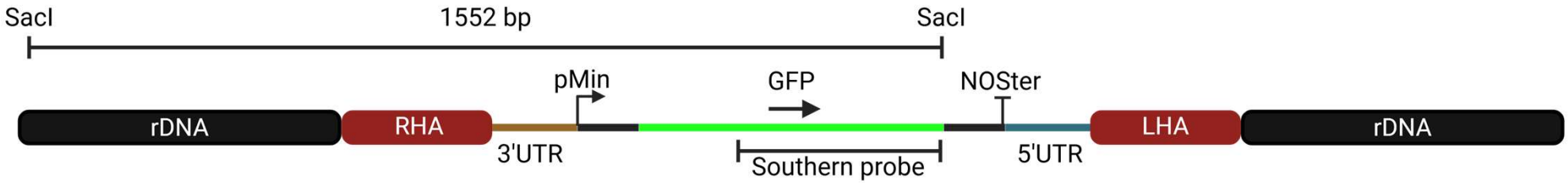
In the format provided by the authors and unedited



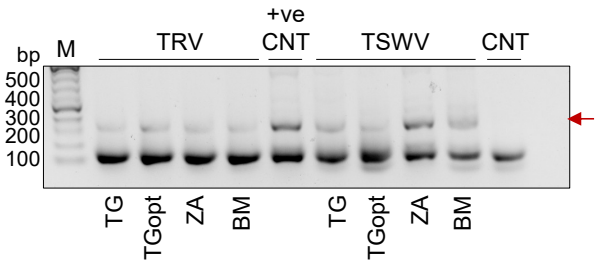
Supplementary Figure 1. Transformation of the Agrobacterium with R2 effectors.
(A) The R2 effector clones without introns. TG and TGopt clones transformed to *Agrobacterium* did not produce colonies.
(B) The addition of the intron blocked leaky expression and led to successful transformation and produced normal colonies of *Agrobacterium.*



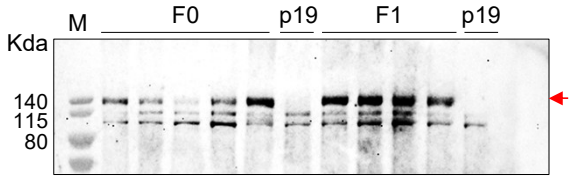
Supplementary Figure 2. Immunoprecipitation analysis for R2 effectors expression confirmation in *Nicotiana benthamiana*.
Proteins from the nuclear fraction were separated on 12 % SDS-PAGE, and the blot was developed with anti-Flag antibody. The TGopt clone from the previous experiment was used as a positive control.
The multiple lanes shown under each annotation represent independent replicates of the experiment.



Supplementary Figure 3. Schematic of genomic DNA digestion with SacI, yielding a 1,552-bp fragment for Southern blot analysis. The SacI site is within both the donor repair template and the rDNA target sequence.

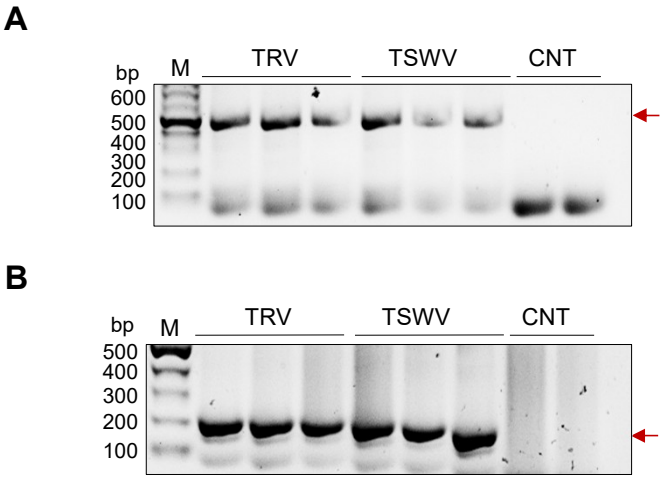


Supplementary Figure 4. PCR confirmation of non-LTR R2-mediated landing-pad integration into rDNA. Leaves agroinfiltrated with R2 effector constructs were subsequently infected with enriched recombinant RNA viruses (TRV and TSWV) expressing the landing-pad donor RNA template. Junction PCR was performed using primer pairs 1F/1R and 2F/2R to specifically amplify the 3′ integration junctions of R2-mediated landing-pad insertion, as indicated. Red arrowheads denote the expected PCR products corresponding to correctly integrated donor sequences. +ve CNT; positive control in which both the R2 effector and donor RNA template were expressed from a binary DNA system; CNT; negative control consisting of TG-expressing leaves infected with empty TRV.
The multiple lanes shown under each annotation represent independent replicates of the experiment.

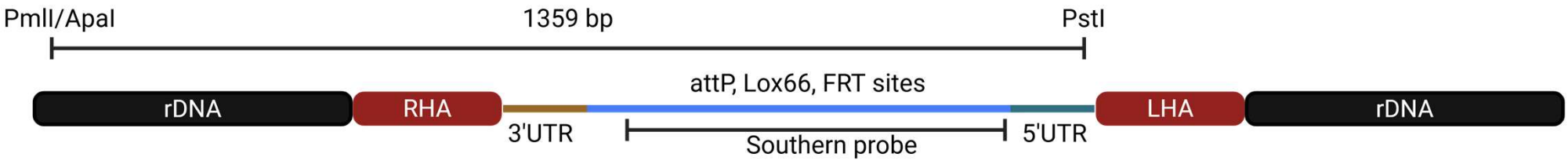


Supplementary Figure 5. Confirmation of ZA effector protein expression in transgenic *Nicotiana benthamiana*. Immunoblot analysis confirming stable expression of the ZA effector protein in regenerated F0 plants and in the subsequent F1 generation. Representative blots show ZA protein levels in independent transgenic lines. p19; RNAi silencing suppressor serves as a negative control.

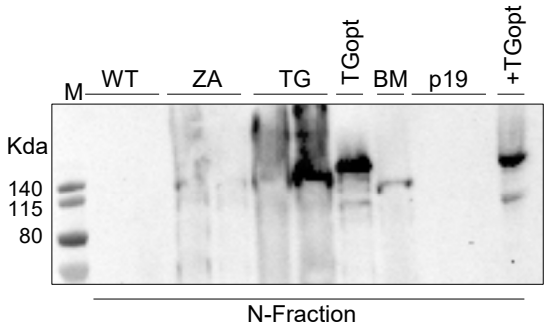
The multiple lanes shown under each annotation represent independent replicates of the experiment.



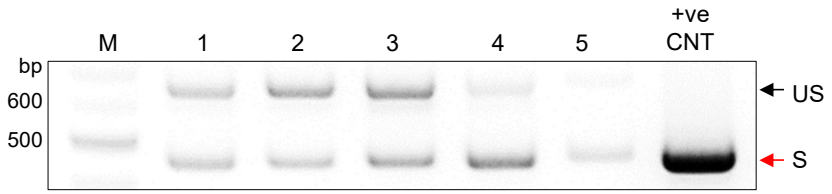
Supplementary Figure 6. PCR confirmation of non-LTR R2–mediated landing-pad integration into rDNA. Leaves of ZA effector expressing (ZA OE) plants were infected with enriched recombinant RNA viruses (TRV and TSWV) expressing the landing-pad donor RNA template. Junction PCR was performed using primer pairs 1F/1R (panel A) and 2F/2R (panel B) to specifically amplify the 3′ and 5′ integration junctions of R2-mediated recombinases landing pads cassette insertion, respectively. Red arrowheads denote the expected PCR products corresponding to correctly integrated donor sequences. CNT; negative control consisting of ZA OE leaves infected with empty TRV.
The multiple lanes shown under each annotation represent independent replicates of the experiment.



Supplementary Figure 7. Schematic of genomic DNA digestion with Pst1, PmlI, and ApaI, yielding a 1,359-bp fragment for Southern blot analysis. The PstI site is within the donor repair template, and the PmlI and ApaI sites are within the rDNA target sequence.



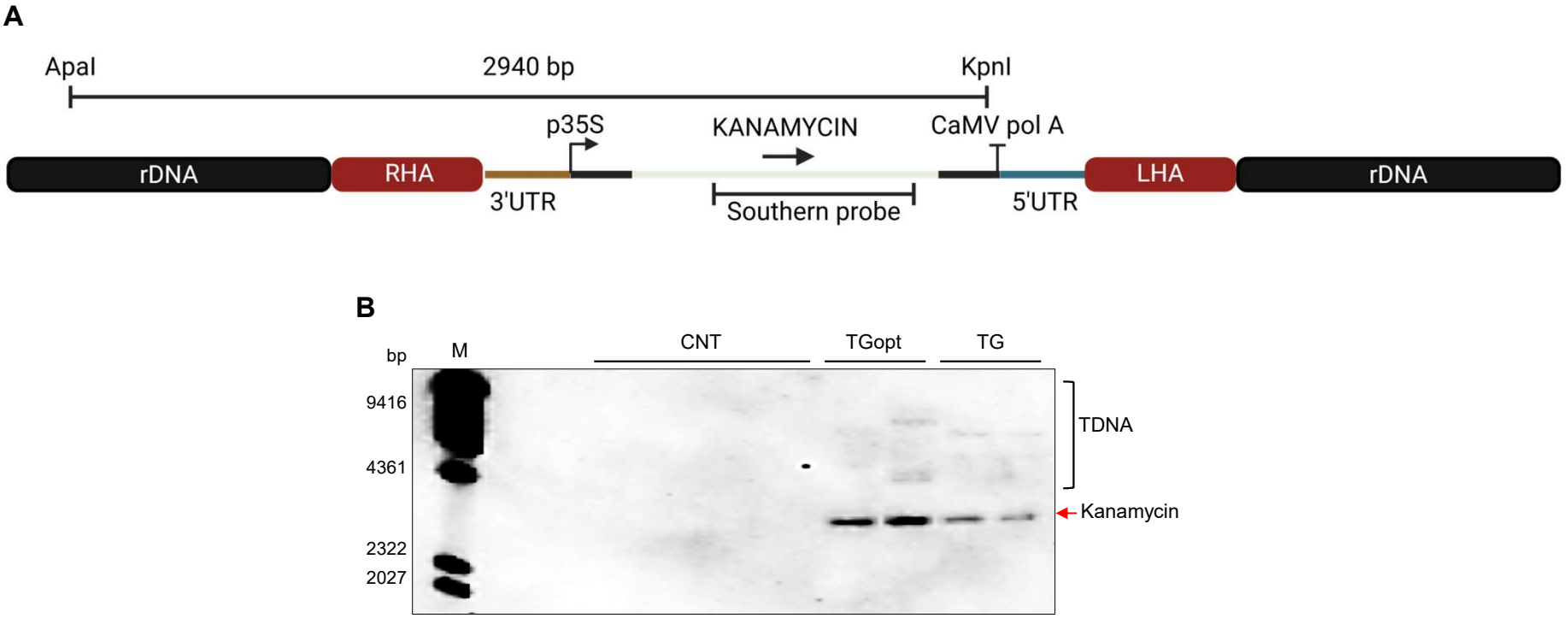
Supplementary Figure 8. Western blot analysis of R2 effectors expression confirmation of rice clones. Proteins from the nuclear fraction were separated on 12 % SDS-PAGE, and the blot was developed with anti-Flag antibody. The TGopt clone from the previous experiment was used as a positive control. p19; RNAi silencing suppressor serves as a negative control.
The multiple lanes shown under each annotation represent independent replicates of the experiment.



Supplementary Figure 9. RT-PCR-based confirmation of the donor RNA transcript production and splicing. RT-PCR confirms the successful production of the donor unspliced (US) RNA transcript and spliced (S) final RNA donor template ready for R2-mediated insertion. +ve CNT; a plasmid DNA serves as a PCR fragment size control.

R2 effector	No. of calli	Positive calli	Editing percentage
TGopt	90	11	12.22 %
TG	90	16	17.78 %
BM	90	0	0 %
ZA	90	0	0 %

Supplementary Figure 10. Comparison Table for rice calli transformed with R2 effectors. Total genomic DNA from the rice calli was subjected to PCR to confirm the presence of the genetic cassettes at rDNA.

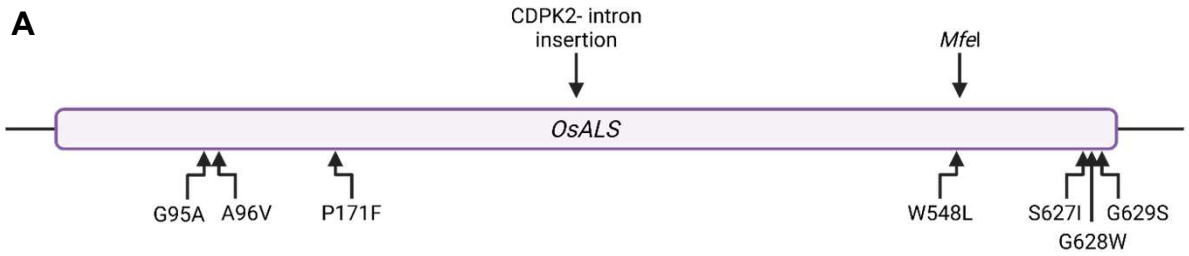


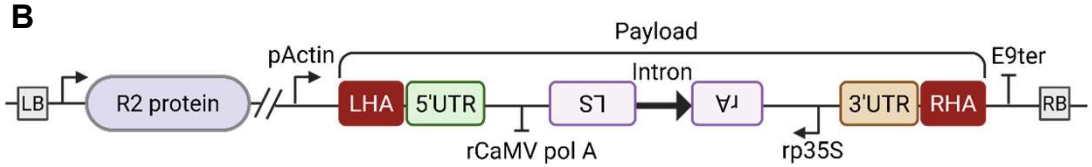
Supplementary Figure 11. Southern blot analysis for confirmation of the targeted integration of the kanamycin ORF into rDNA loci in rice.

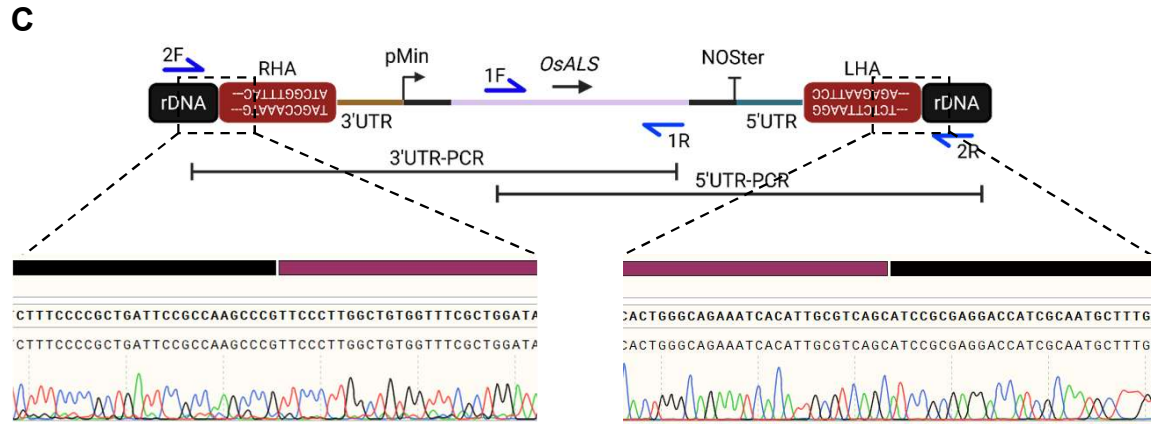
(A) Schematic of genomic DNA digestion with ApaI and KpnI, yielding a 2,940-bp fragment for Southern blot analysis. The KpnI site is within the donor repair template, and the ApaI site is within the rDNA target sequence.

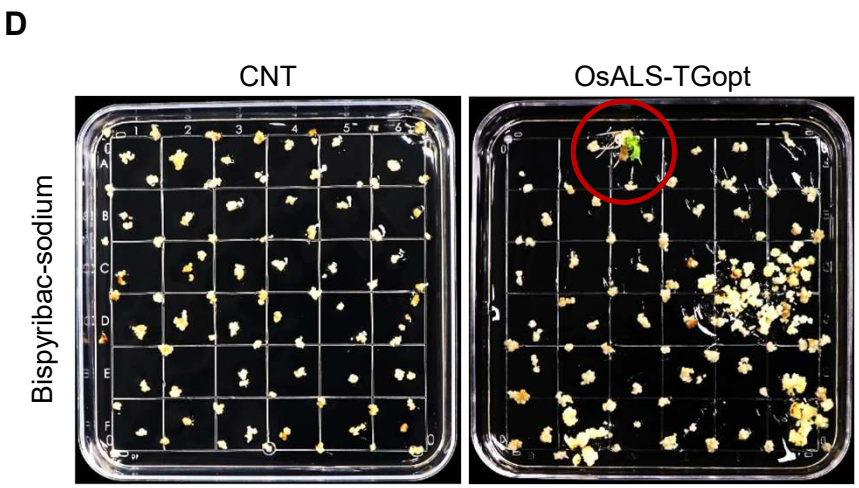
(B) Restriction enzymes digested the total genomic DNA, which was resolved on an agarose gel and blotted to a nylon membrane. DIG-labeled DNA probes specific to the donor template were applied to detect the integration of the kanamycin donor RNA template into rDNA. The red arrowhead indicates the corresponding 3176 bp DNA fragment at the rDNA in the rice genome. CNT; wild-type rice genomic DNA serves as a negative control.

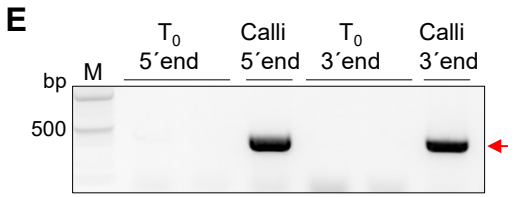
The multiple lanes shown under each annotation represent independent replicates of the experiment.

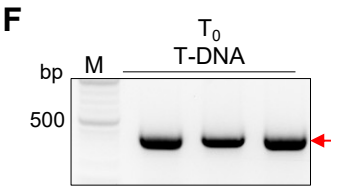

A
CDPK2- intron insertion
MfeI
OsALS
G95A
A96V
P171F
W548L
S627I
G629S
G628W

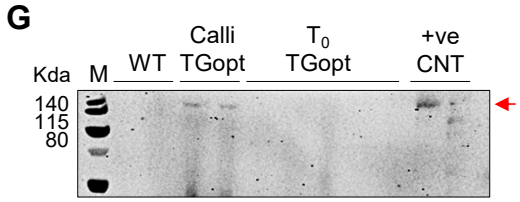

B
Payload
pActin
Intron
E9ter
LB
R2 protein
LHA
5'UTR
3'UTR
RHA
RB
rCaMV pol A
rp35S


C
2F
RHA
pMin
1F
OsALS
NOSter
LHA
rDNA
3'UTR
5'UTR
1R
2R
3'UTR-PCR
5'UTR-PCR


D
CNT
OsALS-TGopt
Bispyribac-sodium


E
bp
M
T0 5´end
Calli 5´end
T0 3´end
Calli 3´end
500


F
bp
M
T0 T-DNA
500


G
Kda
M
WT
Calli TGopt
T0 TGopt
+ve CNT
140
115
80

Supplementary Figure 12.

R2-mediated targeted gene insertion for trait engineering in rice.

(A) An engineered variant of *OsALS* (*LOC_Os02g30630*) is synthesized with multiple substitutions that confer resistance to the herbicide. These substitutions are G95A (GGC>GCC), A96V (GCG>GTC), P171F (CCC>TTC), W548L (TGG>TTG), S627I (AGT>ATC), G628W (GGG>TGG), and G629S (GGC>AGC). The substitution of W548L also creates an *MfeI* restriction digestion site, which is used for genotyping of targeted insertion in the genome. An *OsCDPK2_1* intron (291 bp) from rice was introduced into the ALS variant ORF to stop the expression from the T-DNA. Silent substitutions L400L (CTA>TTA) and Y561Y (TAC>TAT) were introduced to disrupt putative splice acceptor or splice donor sites, respectively.

(B) Codon-optimized R2 effector Tgopt was expressed under the Ubiquitin promoter (pUBI). The RNA donor template of 3613 bp is expressed from the rice Actin promoter. The modified payload with homology arms of 100 bp on each side of the donor template was used. The gene expression of the ALS mutant variant from the CaMV35S promoter is possible only after precise integration into the rice genome. The RNA donor template comprises reverse sequences of the ALS mutant variant, rCaMV35Ster-rALS-intron-rpCaMV35S. The pCaMV35S promoter can express the ALS allele only after integration of the spliced transcript into the genome. Arrows indicate the directionality of the intron splicing in the initial transcript and expression of integrated ALS-ORF from pCaMV35S. The ends of the donor insert site at the target site are shown in red boxes.

(C) Schematic of the junction amplification and sequence confirmation of the OsALS integration in the rice genome. Sanger sequencing of 3' and 5' junctions confirms the successful integration of the OsALS mutant variant in the rice rDNA.

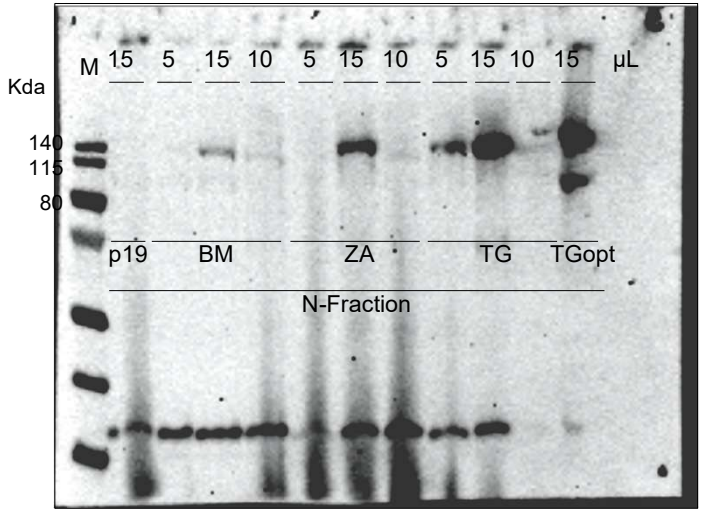
(D) *Agrobacterium*-mediated transformation of rice calli was performed using the binary plasmid with R2-Tgopt and RNA donor template. The transformed calli were selected and regenerated on 0.75 μM bispyribac sodium (BS). A non-specific RNA donor template with R2-Tgopt was transformed as a BS selection control. Regeneration was only detected with the ALS mutant variant donor template. The red circle indicates the regenerated BS-resistant shoot.

(E) Junction PCR confirmation of *OsALS* integration in hygromycin-selected regenerated plants. 5′ and 3′ junction amplicons from calli were included as positive controls.

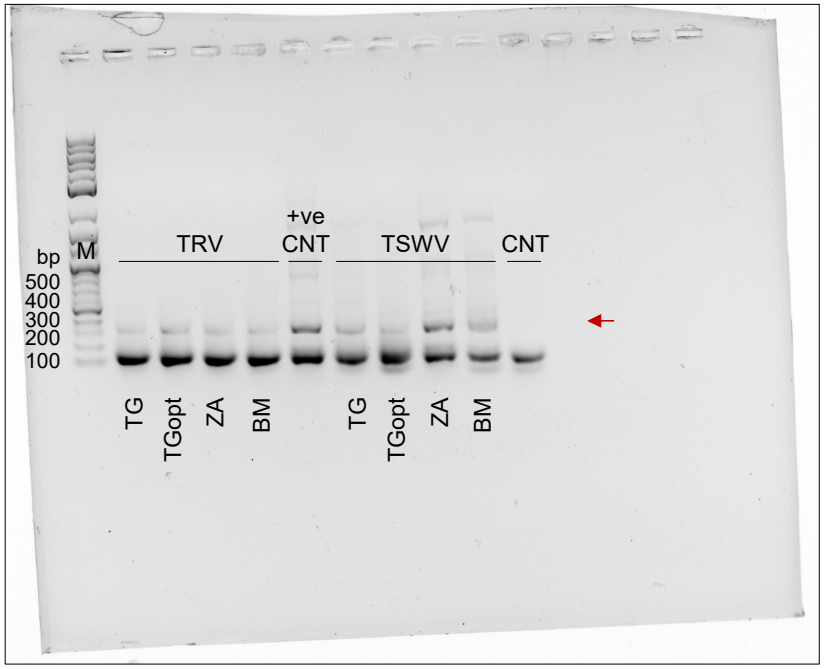
(F) PCR confirmation of T-DNA sequences encoding the R2 machinery in regenerated tissue.

(G) Immunoprecipitation-based detection of R2 effector expression. R2-TGopt protein was detected in calli but not in tissue from T_0 plants. +ve CNT; TGopt protein expressed in *N. benthamiana* leaves serves as positive control.

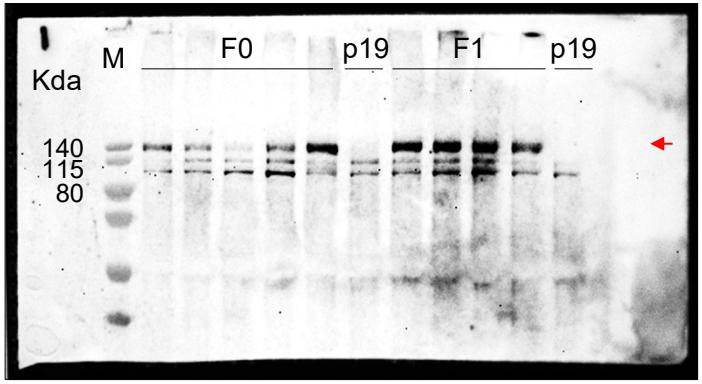
The multiple lanes shown under each annotation represent independent replicates of the experiment.



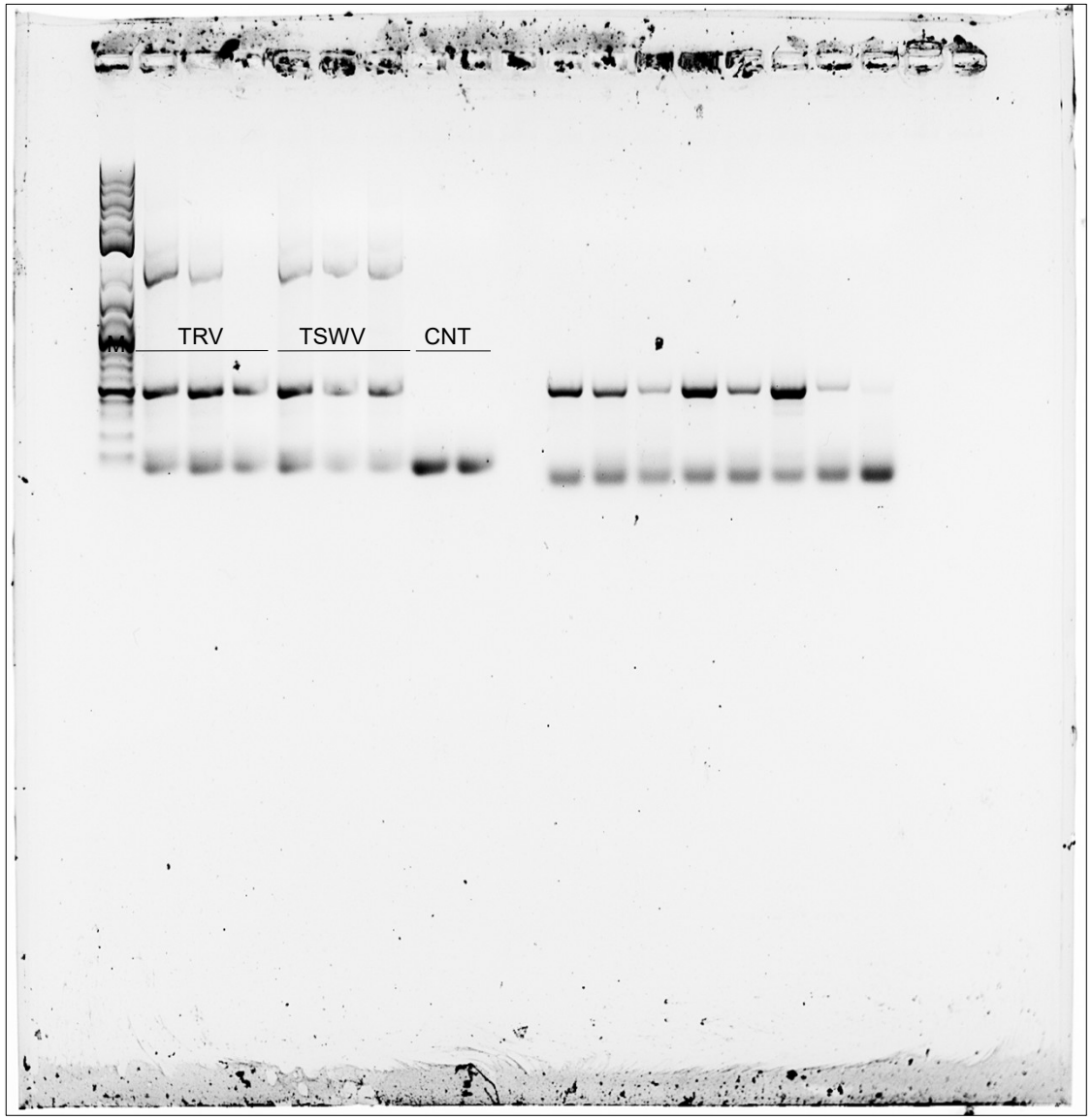
Supplementary Fig. 2: Unprocessed gel images



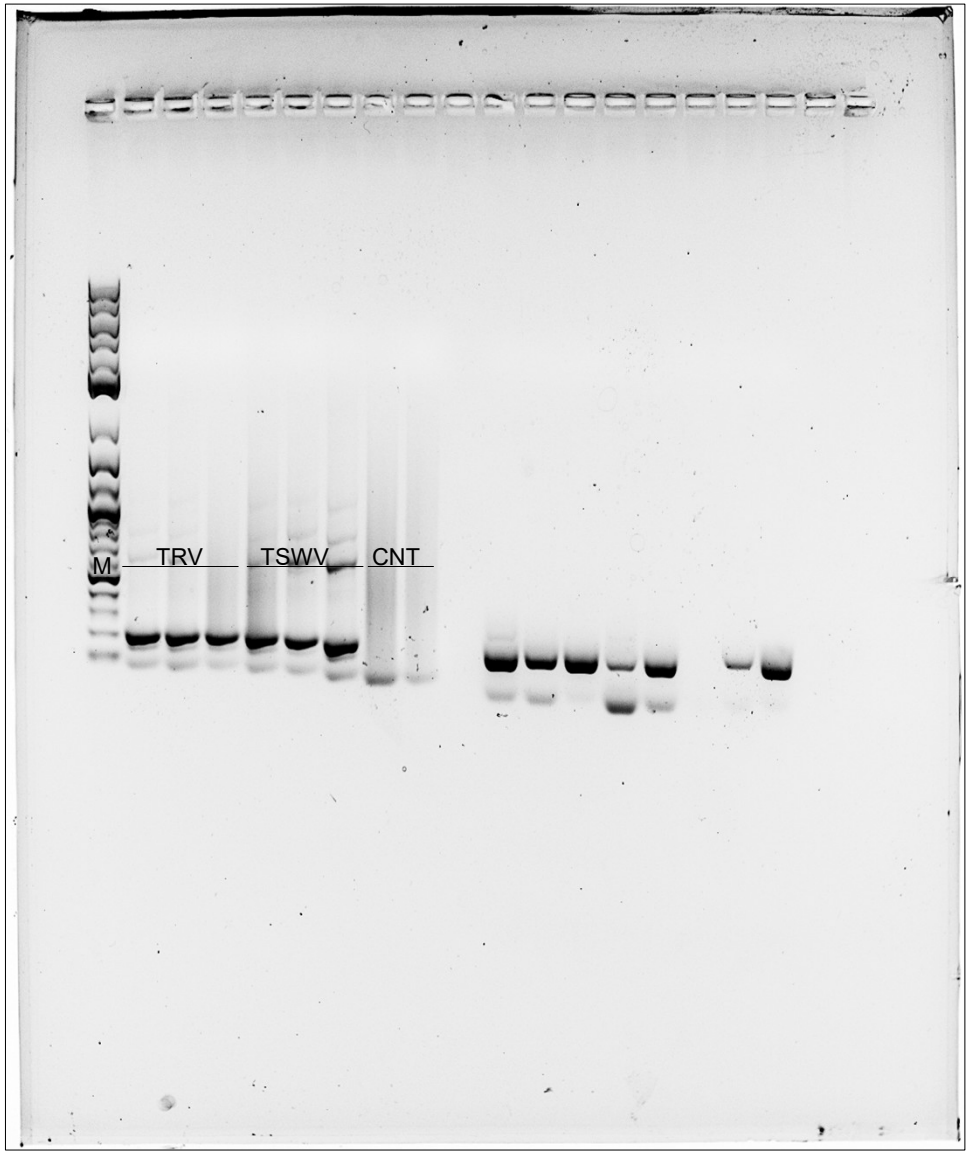
Supplementary Fig. 4: Unprocessed gel images



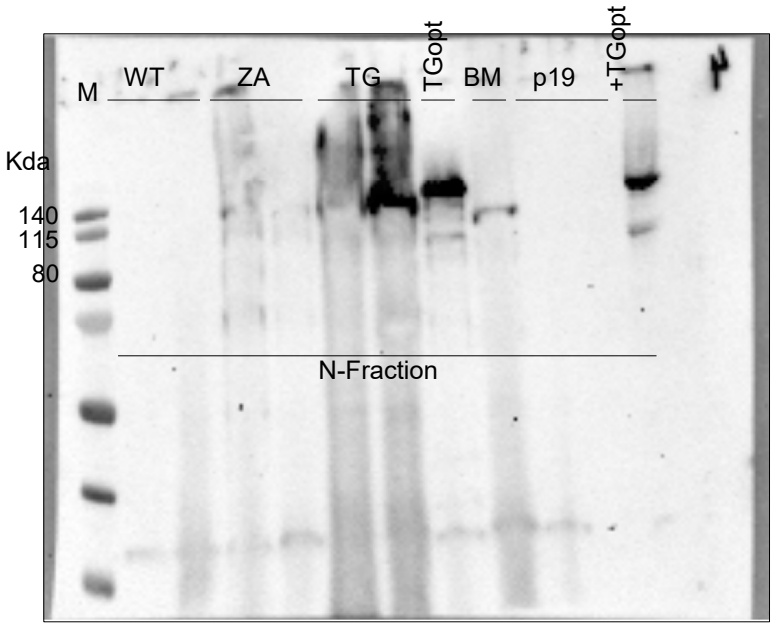
Supplementary Fig. 5: Unprocessed gel images



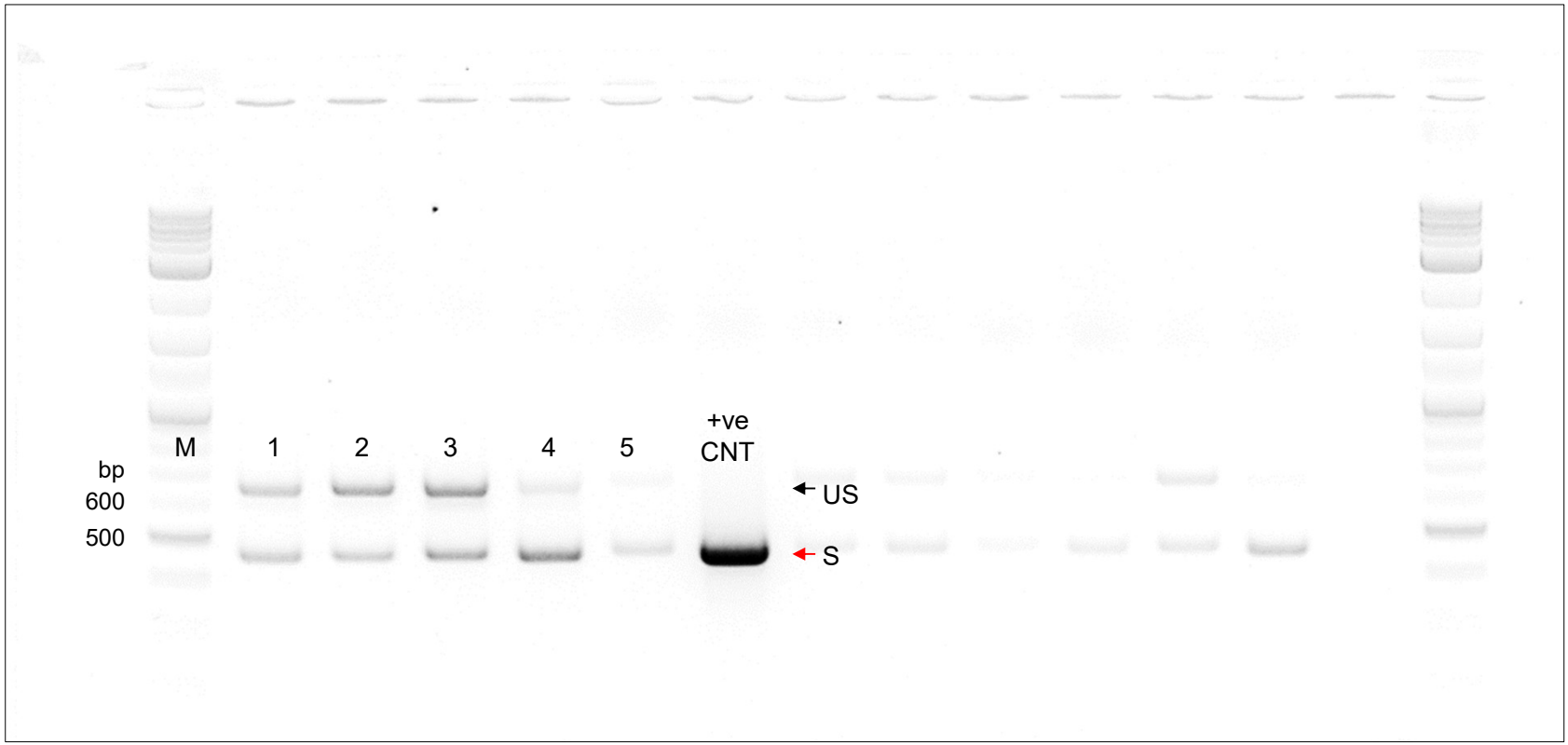
Supplementary Fig. 6A: Unprocessed gel images



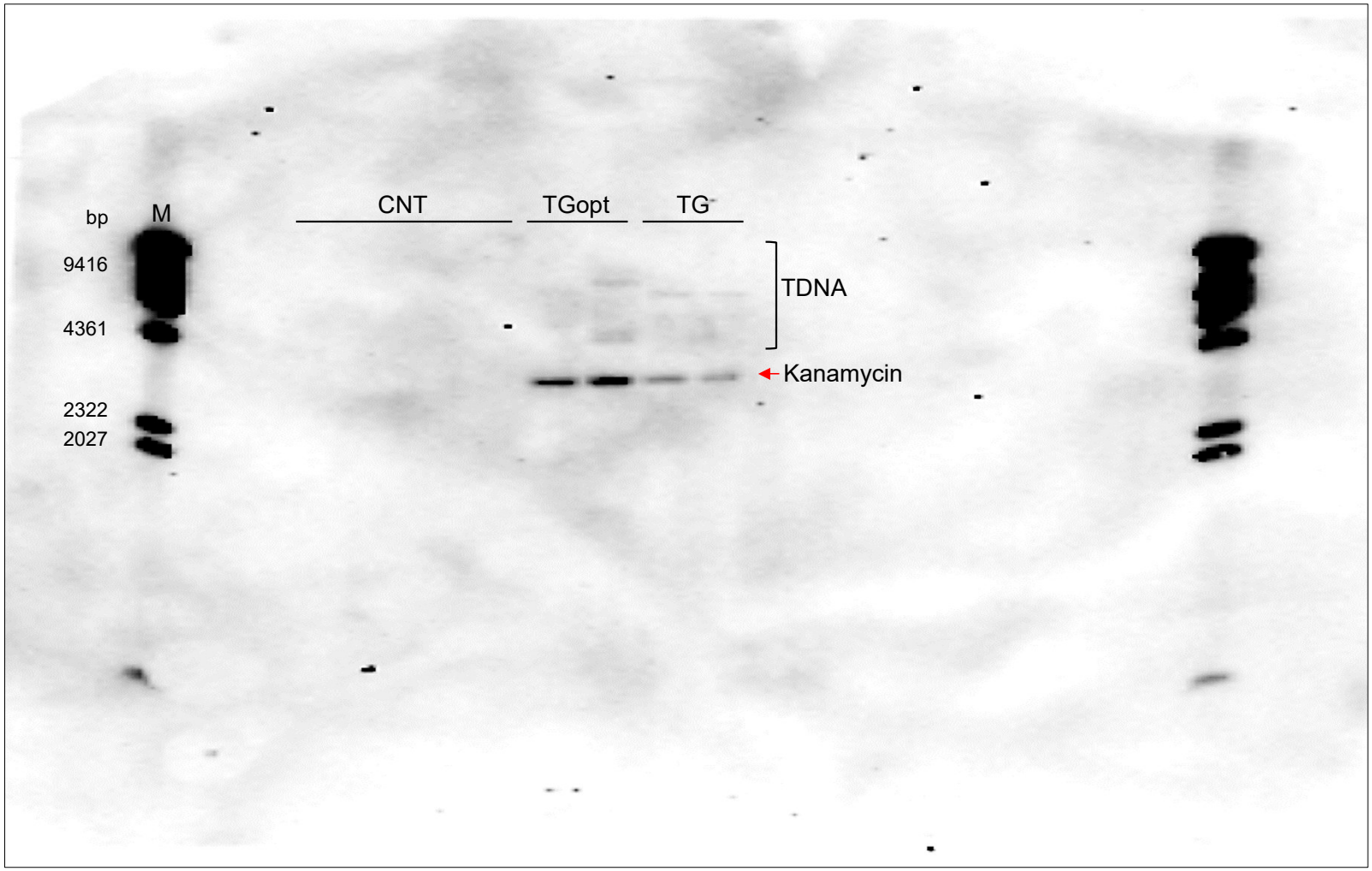
Supplementary Fig. 6B: Unprocessed gel images



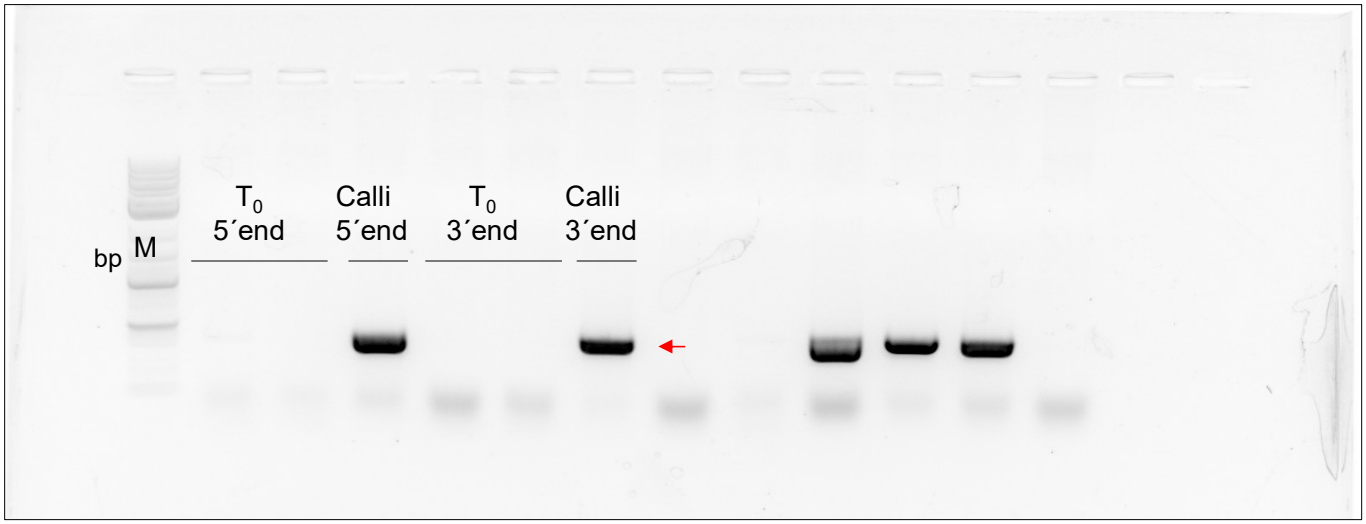
Supplementary Fig. 8: Unprocessed gel images



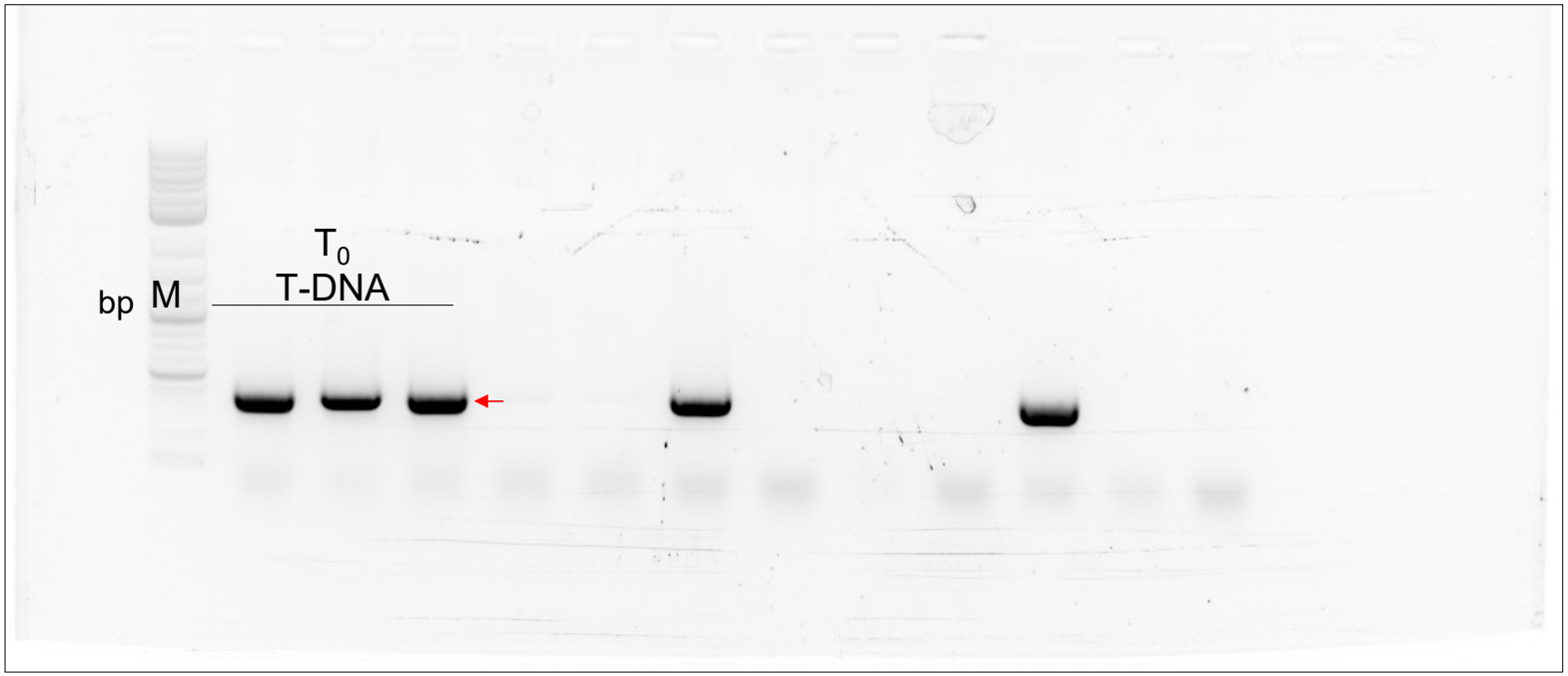
Supplementary Fig. 9: Unprocessed gel images



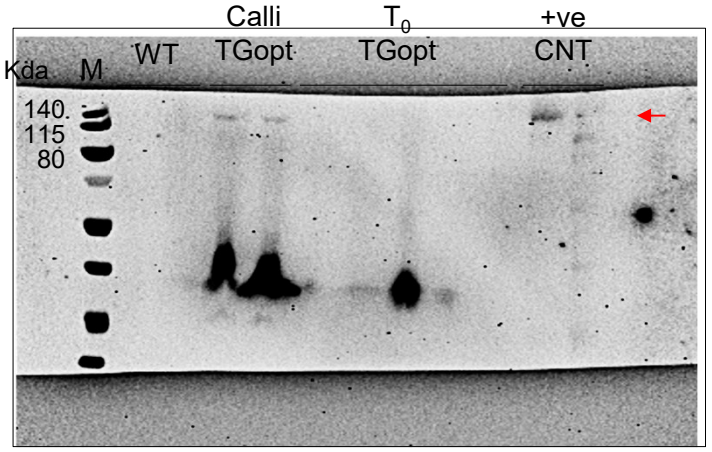
Supplementary Fig. 11B: Unprocessed gel images



Supplementary Fig. 12E: Unprocessed gel images



Supplementary Fig. 12F: Unprocessed gel images



Supplementary Fig. 12G: Unprocessed gel images