Homology-directed repair using transient CRISPR/Cpf1-geminiviral replicon in tomato

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Genome editing via homology-directed repair (HDR) pathway in somatic plant cells was very inefficient compared to illegitimate repair by non-homologous end joining (NHEJ). Here, compared to a Cas9-based replicon system, we improved approximately 3-fold in the HDR-based genome editing efficiency via transient geminiviral replicon system equipping with CRISPR-LbCpf1 in tomato and obtained replicon-free, but with stable HDR alleles. Our work may pave a way for transgene-free rewriting of alleles of interest in asexually as well as sexually reproducing plants.

In plant somatic cells, DNA double strand breaks (DSBs) are efficiently repaired by a NHEJ mechanism, which dominates over the HDR pathway[1,2]. However, to our knowledge, HDR is the major way to precisely edit a gene of interest regardless the mutation types, lengths, and locations of DNA sequences. Therefore, there is a practical demand to develop an efficient HDR-based genome editing system for crop breeding. Here, we report an efficient homology-directed repair using transient CRISPR-associate protein (Cas12a or Cpf1)-geminiviral replicon in tomato.

Previously, geminiviral replicons combined with the Cas9 or TALEN were successfully used to increase HDR efficiency in tomato[3]. It was suggested that Cpf1 might have an advantage in HDR-based genome editing compared to Cas9, because the cutting site of Cpf1 is located distal to the protospacer adjacent motif (PAM), allowing recutting even after indel mutations introduced during NHEJ-mediated repair[4]. To explore the possibility to use this to improve HDR-based genome editing, we re-engineered a *Bean Yellow Dwarf Virus* (*BeYDV*) replicon to supply high doses of homologous donor templates, and used a CRISPR/*Lachnospiraceae bacterium* Cpf1 (LbCpf1) system[4] for DSB formation (**Fig. 1**). Selection of HDR events was supported by a double selection/ screening system using kanamycin resistance and anthocyanin overproduction (**Fig. 1a**).

To validate our system, the LbCpf1 expression cassette driven by a CaMV 35S promoter and 5'UTR with AtUBI10 intron I, guide RNA scaffolds and donor templates were cloned into a *de novo* engineered geminiviral DNA amplicon (**Fig. 1b**,

Supplementary Fig. 1) and transformed via Agrobacteria into tomato cotyledon explants. The LbCpf1 system using two guide RNAs for targeting the ANT1 gene, a key transcription factor controlling anthocyanin pathway, showed the highest HDR efficiency, at 4.51±0.63 %, visible as purple calli and/or shoots (**Fig. 1c,d**), compared to the other constructs including a "minus Rep" ($pRep^-$), "minus gRNA" ($pgRNA^-$) and a CRISPR/SpCas9-based construct (pTC217)[3]. The data revealed that functional geminiviral replicons were crucial for the enhancement of HDR efficiencies (**Fig. 1c**) as shown in other works[3,5]. This is the first report showing highly efficient HDR in plants using Cas12a expressing from a geminiviral replicon.

Boyko and coworkers[6] showed the strong impact of short-day conditions on intrachromosomal recombination repair (ICR) repair in *Arabidopsis*. We tested if the same could be true in tomato somatic cells. Using various lighting regimes, including complete darkness (DD), short (8-h light/16-h dark; 8L/16D) and long (16L/8D) day conditions, we found that HDR efficiencies achieved under short and long day conditions were higher in the case of LbCpf1, but not SpCas9, and reached up to 6.62±1.29% ($p<0.05$, **Fig. 1e**).

Temperature is an important factor controlling ICR[6] and CRISPR/Cas9-based targeted mutagenesis in plants[7]. We compared HDR efficiencies of pHR01 and PTC217 systems at various temperature treatments, since the two nucleases, SpCas9 and LbCpf1, may respond differently. Our data revealed that within the temperature range of 19-31°C, somatic HDR increased with increasing temperature (**Fig. 1f**). Notably, at

31°C, LbCpf1 showed more than 2-fold higher HDR efficiency compared to SpCas9 ($p<0.05$).

Next, we tested several additional approaches for HDR improvement in tomato. The first was to activate nine HDR pathway genes (**Supplementary Table 1**) using the dCas9-sun tag/scFv-VP64 activation system[8]. A single construct system (pHR01-Activ, **Supplementary Fig. 2a**) showed negative effects on HDR (data not shown), which may be due to its large size (~32 kb as T-DNA and ~27 kb as circularized replicon).

The size of viral replicons is inversely correlated with their copy numbers[5,9]. In this work we also tested a novel idea to use a T-DNA producing multiple replicons (pHR01-MR, **Fig. 2a**, and **Supplementary Fig. 2b**). Compared to pHR01, the construct showed HDR efficiencies with 39% increase. We also confirmed the release of three replicons from a single vector (pHR01-MR) used in this work (**Fig. 2b**). To our best knowledge, this is the first report that multiple replicons can be used for efficient genome editing via HDR pathway.

The NHEJ repair pathway is the dominant DNA repair pathway in somatic plant cells and in competition with the HDR pathway. SCR-7, an inhibitor of mammalian ligase IV, was successfully used to enhance HDR up to about 19-fold in mice, 5 times in human cell line[10,11]. However, nothing has been reported about how it affects plant HDR, so we tested the effect of different SCR-7 concentrations during incubation times of 0, 1, 2, and 3 days. As expected, SCR-7 enhanced HDR efficiency several fold at 1 or 10 μM up and up to 50 μM concentrations (**Supplementary Fig. 3**). However,

SCR-7 treatment reduced dramatically plant regeneration efficiency and is therefore not readily applicable.

To verify the HDR repair events in the study, PCR analyses were conducted using primers specific for the right (UPANT1-F1/NptII-R1) and left (ZY010F/TC140R) (**Fig. 1a**; **Supplementary Table 2** and **3**) junctions, using genomic DNAs extracted from derived HDR events (independently regenerated purple plants or Genome Edited generation 0 (GE0)) (**Fig 2c**, **Supplementary Fig. 4**). For pHR01, all (16/16) of the independent events the expected band for right junction integration and 10/16 independent events showed the expected band for left junction repair (**Fig. 2b**). More importantly, 15 out 16 events showed no amplification of circularized forms (**Supplementary Fig. 5a**) of the DNA replicon, and even the replicon-carrying event lost it after long-term growth in greenhouse conditions (data not shown), indicating those plants were free of replicon (**Fig. 2d**). The PCR products were Sanger sequenced to identify junction sequences. A majority of the events (11/16) showed sequences corresponding to perfect right arm integration by HDR repair, 5/16 events showed a combination of HDR and NHEJ repair with NHEJ fingerprint at the 5' terminal of pNOS sequence (**Supplementary Fig. 6a**), highlighted in blue in the event C1.8). All of the sequences amplified from left junctions showed perfected DNA sequence exchange by HDR pathway (**Supplementary Fig. 6b**).

To confirm stable heritable edits, we grew Genome Edited generation 1 (GE1) plants (**Fig. 2e**) obtained from self-pollination of LbCpf1-based HDR GE0 events, and found

segregating population in purple phenotype (**Supplementary Table 4**). PCR analyses of the segregated plants showed inheritance of the edited allele (**Fig. 2f** and **Supplementary Fig. 7**). Offspring segregated from the #C1.4 event were analyzed in detail. Five dark-purple plants (C1.4.1-C1.4.5, were homozygous for the ANT1 HDR edited allele, **Supplementary Fig. 8**), six pale purple plants (C1.4.30-C1.4.35, were heterozygous for the ANT1 HDR edited allele, **Supplementary Fig. 8**), and two wild-type like plants did not contain the HDR edited allele, as expected (Fig. 2f, predicted results correlating to their phenotypes). Dark-purple plants showed the PCR amplification from the replaced allele, but no amplification of wild-type allele when PCRs were performed using primers flanking outside the editing site (**Fig. 1a**). In contrast, heterozygous and wild-type plants showed a band corresponding to the wild-type allele. Sanger sequencing revealed perfect inheritance of the HDR edited allele from GE0 generation of event C1.4 (**Supplementary Fig. 9**) to its homozygous offspring. These data also showed no amplification of circular forms of the DNA replicon (**Fig. 2f** and **Supplementary Fig. 7**) indicating that GE1 plants were also free of the replicons. By contrast, Several GE1 plants obtained from the pTC217 showed amplification of circularized replicons (data not shown). Recently, we also succeeded to have a perfect HDR GE0 event to produce salt tolerant allele (N217D)[12] (**Supplementary Table 5**) using our system with a *HKT1;2* gene donor template which contains neither antibiotic selection marker nor ANT1 color marker (data not shown). It should be noted that the mutated nucleotide (A to G) of *HKT1;2* is not accessible by

any currently known base editor (BE) including xCas9-ABE[13], underlining the significance of HDR-based genome editing.

Taken together, through applications of various approaches, our study showed a high improvement of HDR efficiency in tomato somatic cells. The HDR allele exhibited similar inheritance to natural allele as it was transferred to next generation following Mendelian rules. The advancement of HDR in somatic cells and obtaining replicon-free HDR-edited plants in GE0 generation will open a door for practical applications of the technique to genetically improve crop traits, with special interest in asexually reproducing crops.

ONLINE METHODS

Construction and cloning of HDR testing systems. The entire design principle and cloning works followed MoClo[14] and Golden Gate[15] protocols. The pLSL.R.Ly was designed by amplifying the long intergenic region (LIR), short intergenic region (SIR) and lycopene marker from pLSLR plasmid[3] and cloned following the order shown in the **Supplementary Fig. 1a**. Level 2 Golden Gate BpiI restriction sites flanking the pink marker gene (lycopene) were also integrated inside the replicon for cloning of HDR expression cassettes. The release of circularized DNA replicons was validated in tomato leaves (**Supplementary Fig. 1b**) as well as tomato cotyledon explants (data not shown). pTC147 and pTC217 plasmids[3] were obtained from Addgene and was used as a reference. The LbCpf1-based HDR replicons were similarly designed and cloned (as the SpCas9-based constructs) with two guide RNAs (LbCpf1_gRNA1 and

LbCpf1_gRNA2, **Fig. 1a**). Donor DNAs were constructed for integration of an antibiotic selection marker (NptII) and insertion of a CaMV 35S promoter for driving over-expression of ANT1 gene (**Fig. 1a**). The dual guide RNA construct was designed by multiplexing the LbCpf1 crRNAs as a tandem repeat of scaffold RNA followed by 23nt guide RNA sequences. The crRNAs were driven by an AtU6 promoter (Kamoun Lab, Addgene #46968) and terminated by a 7-T chain sequences.

Tomato transformation. Our research study on HDR improvement was conducted using tomato (Hongkwang cultivar, a local variety) as a model plant. All the binary vectors were transformed into *Agrobacterium tumefaciens* GV3101 (pMP90) using electroporation. Agrobacterium-mediated transformation was used to deliver editing tools into tomato cotyledon fragments (**Supplementary Fig. 10**). Explants for transformation were prepared from 7-day-old cotyledons. Sterilized seeds of the Hongkwang cultivar were grown in MSO medium (half-strength MS medium containing 30 g/L of sucrose, pH 5.8) at 25±2ºC under 16 h/8 h light/dark conditions. Seven-day-old seedlings were collected, and their cotyledonary leaves were sliced into 0.2-0.3 cm fragments. The fragments (explants) were pre-treated on PREMC medium [MS basal salts, Gamborg B5 vitamins, 2.0 mg/L of Zeatin trans-isomer and 0.2 mg/L of indolyl acetic acid (IAA), 1 mM of putrescine and 30 g/L of glucose, pH 5.7] for 1 day. The pre-cultured explants were then pricked and transformed using *A. tumefaciens* GV3101::pMP90 cells carrying HR construct(s).

A. tumefaciens GV3101::pMP90 cells were grown in primary culture overnight (LB containing suitable antibiotics) in a shaking incubator at 30ºC. Agrobacteria were then collected from the culture (OD 0.6-0.8) by centrifugation. The cells were re-suspended in liquid ABM-MS (pH 5.2) and acetosyringone 200 µM. Transformation was carried out for 25 min at RT. The explants were then transferred to co-cultivation medium containing all of the components in the ABM-MS medium and acetosyringone 200 µM, pH 5.8. The co-cultivation plates were kept in the darkness at 25ºC for 2 days, and the explants were shifted to non-selection medium (NSEL) for 5 days and then sub-cultured to selection medium (SEL5). The non-selection and selection media contained all of the components in the pre-culture medium, as well as 300 mg/L of timentin and 80 mg/L of kanamycin. Sub-culture of the explants was carried out at 14-day-interval to achieve the best regeneration efficiency. Explants containing purple calli or shoots were then transfer onto SEL5R medium (similar to SEL5 but reduced zeatin trans-isomer to 1.0 mg/L) for further regeneration and/or elongation. When the shoots were sufficiently long (1.5-3.0 cm), they were transferred to rooting medium (containing all of the components in the elongation medium except zeatin trans-isomer and plus 1.0 mg/L IBA) to generate intact plants. The intact plants from the rooting medium were transferred to vermiculite pots to allow them to harden before shifting them to soil pots in a greenhouse with a temperature of 26±2ºC and under a 16 h/8 h photoperiod. Experimental treatment of physical conditions and data collection were conducted as described in **Supplementary Fig. 10**.

HDR efficiency calculation. HDR efficiencies were calculated by normalization of purple spot numbers per cotyledon fragment obtained using genome editing constructs to purple spots numbers per cotyledon fragment counted in case of SlANT1 over-expression cassette (pTC147[3] and pANT1^{ox}, **Fig. 1b**) transformed in the same conditions.

Plant genomic DNA isolation. Tomato genomic DNA isolation was performed using DNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol. Around 200 mg of leaf tissue was crushed in liquid nitrogen using ceramic mortar and pestle and processed with the kit. Genomic DNA was eluted from the mini spin column with 50-80µl of TE or nuclease free water.

HDR event evaluation. Assessment of gene targeting junctions was performed by conventional PCR using primers flanking left (UPANT1-F1/NptII-R1) and right (ZY010F/TC140R[3], **Supplementary Table 2** and **3**) junctions and a high fidelity taq DNA polymerase (Phusion taq, Thermo Fisher Scientific, USA) and Sanger sequencing (Solgent, Korea). DNA amplicons were evaluated by semi-quantitative PCRs and qPCRs (using KAPA SYBR FAST qPCR Kits, Sigma-Aldrich, USA). Analyses of inherited behaviors of HDR edited allele were performed with genome edited generation 1 (GE1) by PCRs and Sanger sequencing. Circularized replicons were detected using PCR with the respected primers for either pHR01 (**Supplementary Table 2**) or pTC217 (**Supplementary Table 3**).

Statistical analyses. HDR efficiencies were recorded in at least three replicates and statistically analyzed and plotted using PRISM 7.01 software. In **Fig. 1c**, multiple comparisons of the HDR efficiencies of the other constructs with that of pRep$^-$ were done by one-way ANOVA analysis (Uncorrected Fisher LSD test, n=3, df=2, t=4.4; 4.4 and 1.5 for pTC217; pHR01 and pgRNA$^-$, respectively). In **Fig. 1e**, pairwise comparisons of the HDR efficiencies of pTC217 and pHR01 in the three lighting conditions were done by Student t-test (DD: t=1.222, df=4; 8L/16D: t=2.424, df=7 and 16L/8D: t=3.059, df=4). In **Fig 1f**, comparisons of the HDR efficiencies of pTC217 and pHR01 in the various temperature conditions were done by Student t-test (19°C: t=2.656, df=2; 25°C: t=3.346, df=2; 28°C: t=2.099, df=5; 31°C: t=4.551, df=2). The bars in **Fig. 1c, 1e** and **1f** represent for mean values with standard error of the mean (SEM) shown as error bars.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (Grant NRF 2017R1A4A1015515) and by the Next-Generation BioGreen 21 Program (SSAC, Grant PJ01322601), Rural Development Administration (RDA), Republic of Korea. We thank MinWoo Park for providing Hongkwang tomato seeds.

AUTHOR CONTRIBUTIONS

T.V.V., V.S. and J.Y.K. designed the experiments; T.V.V., V.S., E, J. K., M.T.T., J.K., Y.W.S., and D.T.H.D performed the experiments; T.V.V. and J.Y.K. analyzed the results; T.V.V. and J.Y.K. wrote the manuscript.

COMPETING INTERESTS

The authors have submitted a Korean patent application (application no. 10-2018-0007579) based on the results reported in this paper.

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References

1. Jiang, W. et al. Nucleic Acids Res 41(20), e188 (2012).

2. Puchta, H. The Plant Journal 13(3), 331–339 (1998).

3. Čermák, T. et al. Genome Biol 16, 232 (2015).

4. Zetsche, B. et al. Cell 163(3):759-771 (2015).

5. Baltes, N.J. et al. Plant Cell 26(1), 151–163 (2014).

6. Boyko, A. et al. Mutat Res 572(1-2), 73-83 (2005).

7. LeBlanc, C. et al. Plant J 93(2), 377-386 (2018).

8. Tanenbaum, M. E. et al. Cell 159(3), 635-646 (2014).

9. Suárez-López, P., Gutiérrez, C. Virology 227: 389–399 (1997). 10. Maruyama, T. et al. Nat Biotechnol 33(5), 538-542 (2015).

10. Maruyama, T. et al. Nat Biotechnol 33(5), 538-542 (2015).

11. Chu, V.T. et al. Nat Biotechnol 33(5), 543-8 (2015).

12. Ali, A. et al. Plant Physiol 171(3):2112-26 (2016).

13. Hu, J.H. et al. Nature 556(7699):57-63 (2018).

14. Weber, E. et al. PLoS One 18;6(2), e16765 (2011).

15. Engler, C. et al. ACS Synth Biol 21;3(11), 839-843 (2014).

Figure 1 HDR-based genome editing of ANT1 locus.

(**a**) Representatives of ANT1 targeting sites and homologous DNA donor template construction. The upstream sequence of ANT1 locus (middle panel) was selected for targeting by HDR. Two guide RNAs were used (depicted by two vertical arrows on the middle panel and sequence details in bottom panel). Kanamycin expression cassette (pNOS-NptII-tOCS) and CaMV 35S promoter was designed to be inserted at a position 142 bp upstream of ANT1 start codon. (**b**) T-DNA constructs used for HDR improvement experiments. The dual guide RNA scaffold (2x1gRNA^{ANT1}) was driven by Arabidopsis U6 promoter core element (75bp). LbCpf1 expression cassette was re-engineered to contain Arabidopsis Ubiquitin 1 intron I downstream of CaMV 35S promoter and upstream of LbCpf1 and to be terminated by CaMV 35S terminator (35S-Cpf1I-t35S). Red and orange boxes show long intergenic region and short intergenic region of geminivirus DNA. (**c**) HDR efficiency comparison among

different constructs. (**d**) Representative photograph of HDR edited T0 events indicating as purple calli (red arrows) or direct HDR shoot formation (purple arrow). (**e**) Impact of photoperiod on HDR. Tomato cotyledon fragments transformed were incubated under different lighting regimes for the first 10 days post-washing. DD: continuous darkness; 8L/16D: 8 hours-lighting/16 hours-darkness; 16L/8D: 16 hours-lighting/8 hours-dark. (**f**) HDR efficiencies of pTC217 and pHR01 construct obtained in various temperatures. HDR efficiencies were recorded in at least triplicates, calculated and plotted using PRISM 7.01 software (details of statistical analyses are described in Online Methods part). The bars represent for mean values with standard error of the mean (SEM) shown as error bars. *: significantly different ($p<0.05$); ns: not significantly different; p values are showing on the top of the bars of (**f**) for comparisons.

Figure 2 Novel approaches for HDR improvement and analyses of the HDR edited plants.

(**a**) Multi-replicon construct tested for improvement of HDR over NHEJ. Red and orange boxes show long intergenic region and short intergenic region of geminivirus DNA. (**b**) PCR detection of circularized replicons simultaneously released from multiple replicon vector (pHR01-MR). 0d, 3d, 6d and 9d: samples collected at 0, 3, 6 and 9 days post transformation with *Agrobacterium* carrying pHR01-MR. (**c**) Representative HDR edited plant in greenhouse conditions and their fruits. (**d**) PCR analysis data of some representative HDR independent events. (**e**) Generation 1 of the

HDR edited events (GE1). GE1 plant (left) germinated in soil pot in comparison with wild-type plant (right). (**f**) PCR analysis data of some GE1 offspring of C1.4 event. P: pHR01 plasmid isolated from Agrobacteria; L: 1kb ladder; N: water control; WT: wild-type tomato Hongkwang; C1.1, C1.2, C1.3, C1.8: Independent LbCpf1-based HDR GE0 events; C1.4.1, C1.4.2, C1.4.3, C1.4.4 and C1.4.5: GE1 plants, showing dark purple color, obtained from self-pollination of the event C1.4.

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List of Supplementary Figures

Supplementary Figure 1 The *de novo* engineered geminiviral amplicon (named as pLSL.R.Ly) and its replication in tomato. (**a**) Map of pLSL.R.Ly. The DNA amplicon is defined by its boundary sequences (Long Intergenic Region, LIR) and a terminated sequence (Short Intergenic Region, SIR). The replication associated protein (Rep/RepA)

is expressed from the LIR promoter sequence. All of the expression cassettes of HDR tools were cloned into the vector by replacing the red marker (Lycopene) using a pair of type IIS restriction enzyme (BpiI, flanking ends are TGCC and GGGA). Left (LB) and right (RB) denote the borders of a T-DNA. (**b**) Circularized DNA detection in tomato leaves infiltrated with pLSL.R.Ly compared to that of pLSLR. Agrobacteria containing the plasmids were infiltrated into tomato leaves (Hongkwang cultivar) and infiltrated leaf were collected at 6, 8 and 11 dpi and used for detection of circularized DNAs. N: water; P1: positive control for pLSL.R.Ly; positive control for P2: pLSLR; Cx: Control samples collected at x dpi; Ixy: infiltrated sample number y collected at x dpi; I11V: sample collected from leaves infiltrated with pLSLR at 11 dpi. PCRs using primers specific to GAPDH were used as loading control.

Supplementary Figure 2 Novel systems for HDR improvement. (**a**). Single construct system for activation of HDR-related genes involving in HDR repair pathway. Long intergenic region (LIR) and short intergenic region (SIR) are depicted by color bars in the bottom. (**b**). Schematic system and released forms of multiple replicon system. General construction of multiple replicon complex is designed with 3 LIR and 3 SIR sequences (top panel). Donor template was cloned in one replicon and the other component for inducing DSBs were placed in the other replicon (middle panel). Three replicons would be formed from the construct (bottom panel).

Supplementary Figure 3 Enhancement of HDR efficiency using SCR7, a chemical inhibitor of NHEJ repair pathway. To test the different concentration of SCR7 with

different duration of treatment, cotyledons transformed with pTC217 were cultured on medium supplied with 0, 1, or 10 μM SCR7 for 1, 2, or 3-day period at 28 °C in the dark.

Supplementary Figure 4 Morphological appearance of GE0 plants

Supplementary Figure 5 Circularized DNA replicon released by HDR vectors. (**a**) pHR01 replicon. (**b**) pTC217 replicon.

Supplementary Figure 6 Sanger sequencing data to confirm donor exchanges. (**a**) Right junction. (**b**) Left junction. C1.1, C1.2, C1.3, C1.8, C1.11, C1.12, and C1.17: Independent LbCpf1-based HDR GE0 events

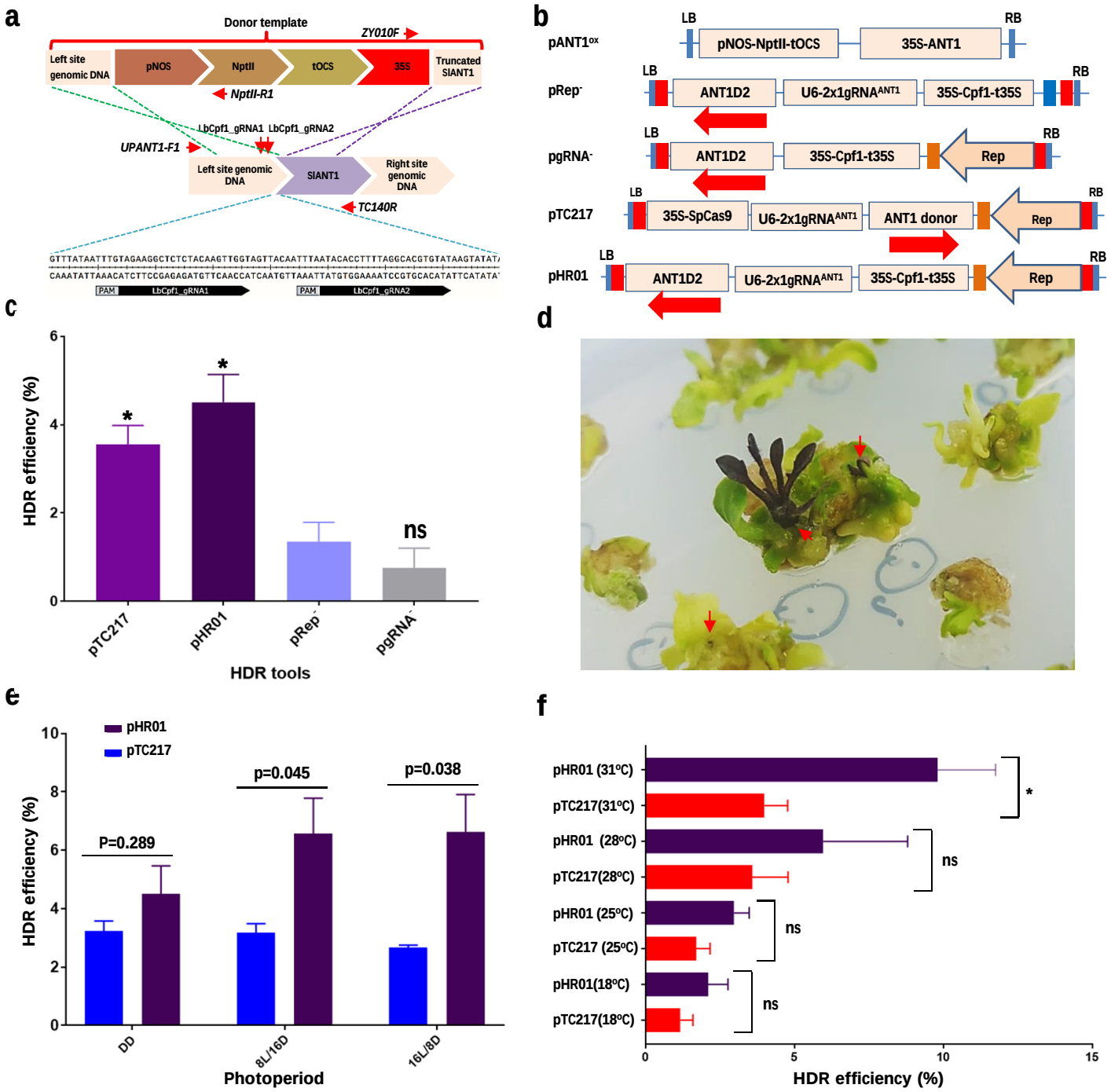
Supplementary Figure 7 PCR analyses of GE1 plants obtained from GE0 LbCpf1-based HR events. P: pHR01 plasmid isolated from Agrobacteria; L: 1kb ladder; N: Water control; WT: wild-type Hongkwang; C1.6.1-C1.6.5: GE1 offspring of event C1.6.; C1.9.1: GE1 offspring of event C1.9; C1.10.1 and C1.10.2: GE1 offspring of event C1.10; C1.11.1-C1.11.4: GE1 offspring of event C1.11; C1.12.1-C1.12.5: GE1 offspring of event C1.12; C1.14.1-C1.14.4: GE1 offspring of event C1.14; C1.15.1 and C1.15.2: GE1 offspring of event C1.15; C1.16.1-C1.16.4: GE1 offspring of event C1.16.

Supplementary Figure 8 Morphological appearance of GE1 plants

Supplementary Figure 9 Analyses of left and right junction sequences of GE1 plants. Sanger sequencing data to confirm donor exchanges for right (**a**) and left (**b**) junctions of the GE1 plants are presented

Supplementary Figure 10 Timeline and contents of *Agro*-mediated transformation protocol used in this work. Step by step protocol is presented with each number in the circles indicates number of days after seed sowing (upper panel) and treatments used in each steps are shown in below panel.

Figure 1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UPANT1-F1
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SlANT1
Right site genomic DNA
TC140R
PAM
LbCpf1_gRNA1
PAM
LbCpf1_gRNA2
b
pANT1ox
LB
pNOS-NptII-tOCS
35S-ANT1
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c
HDR efficiency (%)
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8L/16D
16L/8D
Photoperiod
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pHR01 (28°C)
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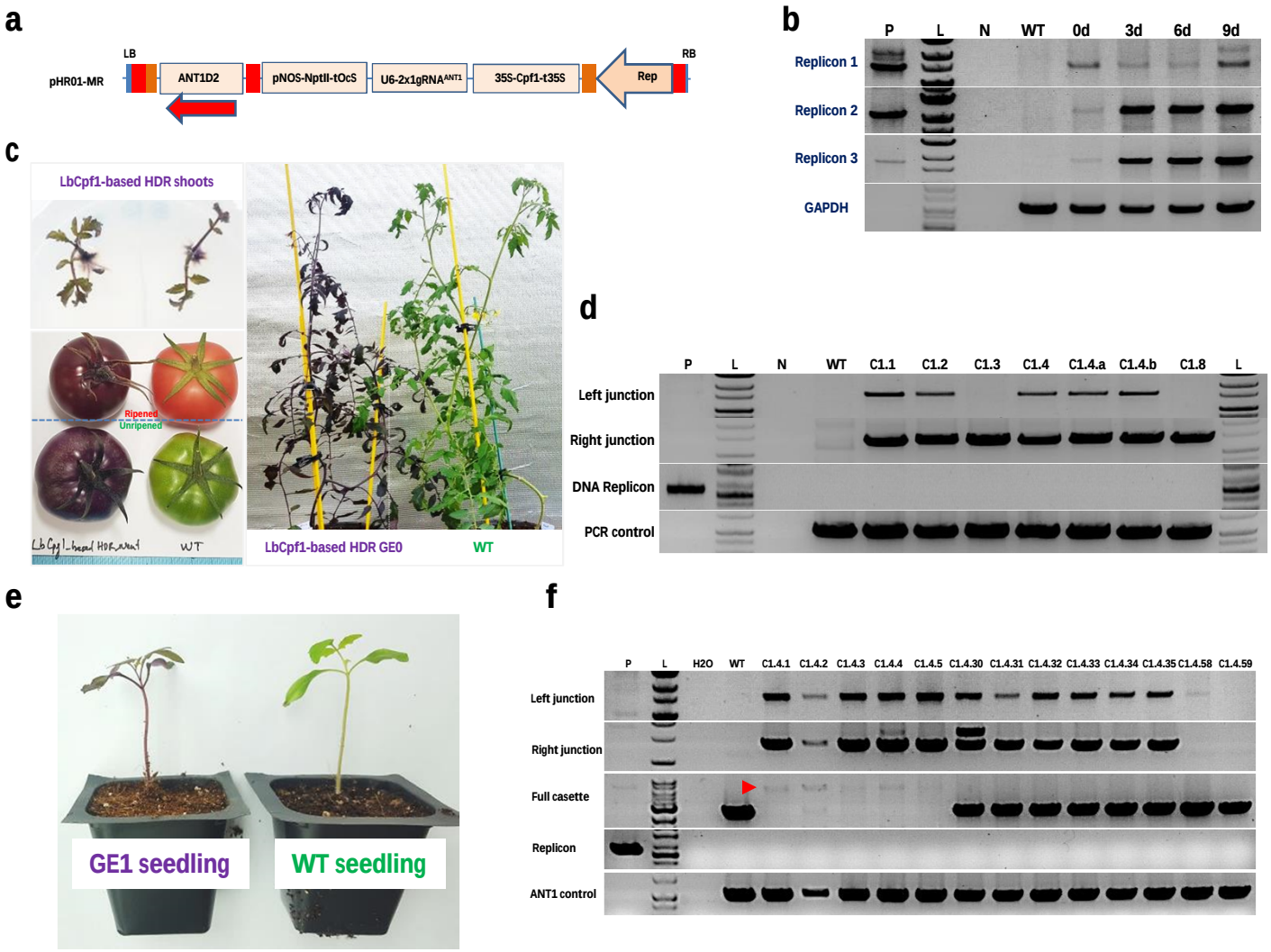
HDR efficiency (%)

Figure 2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ANT1D2
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Rep
b
P
L
N
WT
0d
3d
6d
9d
Replicon 1
Replicon 2
Replicon 3
GAPDH
c
LbCpf1-based HDR shoots
Ripened
Unripened
WT
LbCpf1-based HDR GE0
WT
d
P
L
N
WT
C1.1
C1.2
C1.3
C1.4
C1.4.a
C1.4.b
C1.8
L
Left junction
Right junction
DNA Replicon
PCR control
e
GE1 seedling
WT seedling
f
P
L
H2O
WT
C1.4.1
C1.4.2
C1.4.3
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C1.4.30
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C1.4.33
C1.4.34
C1.4.35
C1.4.58
C1.4.59
Left junction
Right junction
Full casette
Replicon
ANT1 control