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Author Manuscript

Nat Biotechnol. Author manuscript; available in PMC 2014 August 01.

Published in final edited form as:

Nat Biotechnol. 2013 August ; 31(8): 688–691. doi:10.1038/nbt.2654.

Multiplex and homologous recombination-mediated plant genome editing via guide RNA/Cas9

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To the Editor:

Elucidation and manipulation of human, animal and plant genomes is key to basic biology research, medical advances and crop improvement. The development of targeted genome editing, particularly the homologous recombination-based gene replacement, is of great value in all organisms. Recent advances in engineered nucleases with programmable DNA binding specificities, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have provided valuable means in creating targeted mutations in metazoan and plant genomes with high specificity^{1–6}. However, these technologies demand elaborate design and assembly of individual DNA-binding proteins for each DNA target site^{1–6}. Recently, a simple, versatile and efficient genome engineering technology has been developed based on the bacterial CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR-associated (Cas) adaptive immune systems⁷. In type II CRISPR/Cas system from *Streptococcus pyogenes*, a single Cas9 endonuclease guided by a duplex of mature CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) could cleave trespassing DNA from bacteriophage or plasmids in a sequence-specific manner⁷. By reconstitution of the *S. pyogenes* Cas9 (SpCas9) and an artificial chimera of crRNA and tracrRNA called guide RNA (gRNA) in eukaryotic cells, including yeast, zebrafish, mouse and human, targeted genome editing could be readily achieved via either error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) of the intended cleavage site^{7–14}. However, the feasibility and efficacy of the gRNA/Cas9 technology in plants have not been examined.

To explore the gRNA/Cas9 technology for plant genome engineering, we first co-expressed the plant codon-optimized *SpCas9* (*pcoCas9*) and a gRNA targeting the *Arabidopsis*

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Note: Supplementary information is available in the online version of the paper.

AUTHOR CONTRIBUTIONS

J.F.L. and J.S. designed experiments; J.F.L. and D.Z. performed experiments; J.A., J.E.N., M.M and G.M.C. conducted bioinformatics analyses; J.B. supplied plant materials; J.F.L. and J.S. wrote the manuscript.

*thaliana PDS3 (PHYTOENE DESATURASE) gene (Fig. 1a,b) in *Arabidopsis* mesophyll protoplasts, which are freshly isolated leaf cells without cell walls. The protoplast transient expression system supports highly efficient DNA co-transfection and protein expression¹⁵. The *pcoCas9* was expressed under the hybrid constitutive 35SPPDK promoter¹⁵, while the gRNA was transcribed from the *Arabidopsis U6* polymerase III promoter (Fig. 1a). Notably, *pcoCas9* was expressed at a significantly higher level compared to the humanized *SpCas9*⁹ using the same expression vector in *Arabidopsis* protoplasts (Supplementary Fig. 1). In addition, *pcoCas9* encodes nuclear localization sequences (NLSs) at both protein termini (Fig. 1a) for optimal protein nuclear localization⁸. A potato IV2 intron (Fig. 1a) was inserted to minimize adverse effects on bacterial growth¹⁶ due to potential leaky expression and nuclease activities of *pcoCas9* in *E. coli* during cloning.*

To determine the mutagenesis efficiency of the gRNA/*pcoCas9* system in *Arabidopsis* protoplasts, genomic PCR (gPCR) amplicons using total genomic DNA (gDNA) from transfected protoplasts as templates were cloned and randomly selected for Sanger sequencing. With a DNA ratio of Cas9:gRNA at 1:1 during co-transfection, we detected 10 mutated *AtPDS3* target alleles among 180 randomly sequenced amplicons, reaching an approximate mutagenesis frequency of 5.6% (Fig. 1c). Interestingly, a ratio of Cas9:gRNA at 19:1 failed to induce any mutation in 180 sequenced amplicons, and no mutation was detected among 93 sequenced amplicons when *pcoCas9* was expressed alone (Fig. 1c). For a second tested gene, *AtFLS2 (FLAGELLIN SENSITIVE 2)*, in *Arabidopsis* protoplasts, the gRNA/*pcoCas9*-mediated mutagenesis also only occurred with a DNA ratio of Cas9:gRNA at 1:1 but not at 19:1 (Fig. 1d). In this case, a lower mutagenesis frequency (1.1%) was observed (Fig. 1d). It seemed that gRNA expression is the limiting factor for optimal targeting and mutagenesis in plant cells as in human cells¹².

To extend the application of gRNA/*pcoCas9*-mediated genome editing to other plant systems, we carried out a parallel study using tobacco (*Nicotiana benthamiana*) protoplasts. Surprisingly, by targeting *NbPDS* (ortholog of *AtPDS3*) at two different sites, we obtained substantially higher mutagenesis frequencies than in *Arabidopsis*, namely 37.7% for the target 1 (Fig. 1e) and 38.5% for the target 2 (Fig. 1f). Interestingly, the gRNA/*pcoCas9*-induced mutagenesis frequently led to significant DNA deletions or insertions but rare single nucleotide (nt) substitutions in tobacco cells (Fig. 1e,f and Supplementary Fig. 2a,b) as in animal and human cells displaying relatively high mutation rates (e.g., 37.6% in K562 cells and 24.6% in 293T cells)^{7–14}. In contrast, single nt deletions, insertions or substitutions were most frequently detected in *Arabidopsis* cells with relatively low mutation rates from 1.1 to 5.6% (Fig. 1c,d). Our results clearly demonstrated that the gRNA/*pcoCas9* system is effective in plant cells. Whether the different genome mutagenesis frequencies and patterns between *Arabidopsis* and tobacco by gRNA/*pcoCas9* are due to distinct plant genotypes or physiological states requires future investigation.

To validate the occurrence of the gRNA/*pcoCas9*-induced targeted mutagenesis in the *PDS* gene in *planta*, we transiently co-expressed *pcoCas9* and *AtPDS3*- or *NbPDS*-targeting gRNA via a single binary plasmid (Fig. 1g) in intact leaves of two-week-old *Arabidopsis* seedlings or five-week-old tobacco plants through *Agrobacterium* leaf infiltration. Biallelic disruption of *PDS* in the *Arabidopsis* or tobacco genome would abolish carotenoid

biosynthesis and promote chlorophyll oxidation leading to a photobleached phenotype. We did not observe any visible albino spot on agroinfiltrated leaves from *Arabidopsis* or tobacco plants 7 days post infiltration. There was either no cell with biallelic disruption of *PDS* or the population of photobleached cells was too small, as the cell division might have ceased in the infiltrated leaves. Careful screens at the single cell level after the degradation of existing chlorophyll is necessary for further characterization using fluorescent microscopy. Importantly, by sequencing target gPCR amplicons, we did detect precise genomic mutations in the *AtPDS3* or *NbPDS* target sequence in cells from agroinfiltrated leaves with a mutagenesis frequency of 2.7% for *AtPDS3* and 4.8% for *NbPDS* (Fig. 1g). Considering that agroinfiltration exhibits lower efficiency and higher variability in gene transfer than the protoplast transfection¹⁵, these mutagenesis frequencies might reflect dilution of the targeted mutations by wild-type gDNA from leaf cells without successful DNA delivery. Notably, the different gRNA/pcoCas9-induced mutagenesis patterns in *Arabidopsis* and tobacco protoplasts were also observed in corresponding whole plants. While targeted mutations in *Arabidopsis* seedlings were frequently single nt substitutions (Fig. 1h), those in tobacco plants often involved significant DNA deletions (Fig. 1i). The leaves infiltrated with *Agrobacterium* expressing *pcoCas9* alone did not induce mutations in the target regions (Fig. 1g). These data established that the gRNA/pcoCas9 system is also effective *in planta*.

To test whether the gRNA/pcoCas9 system allows multiplex genome editing in *Arabidopsis* protoplasts, we first identified an identical gRNA target site (target candidate No.2, Supplementary Fig. 3) for both *AtRACK1b* and *AtRACK1c*, two members of the *Arabidopsis RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1)* family (Fig. 2a). By co-expressing *pcoCas9* and the cognate gRNA, we observed mutations in both target genes with a comparable mutagenesis frequency (2.5–2.7%, Fig. 2b). Similarly, only single nt substitutions or insertions were detected in these *Arabidopsis* genes (Fig. 2b). Notably, no mutation was detected in a homologous sequence from *AtRACK1a* (Fig. 2b), which contains a valid PAM but two mismatches to the 12-bp seed sequence governing the gRNA specificity^{7–9,13,16,17} (Fig. 2a), illustrating the high specificity of the gRNA/pcoCas9-directed genome editing in plant cells. We further co-expressed *pcoCas9* and tandem gRNAs aiming for two juxtaposed targets in *AtPDS3* with a 24-bp spacer (Fig. 2c). Interestingly, this simultaneous targeting with two gRNAs led to deletions of up to 48 bp genomic segments between these two target sites by gRNA/pcoCas9 with a mutation frequency of 7.7% (Fig. 2d and Supplementary Fig. 2c). Taken together, these results demonstrated that the gRNA/pcoCas9 system could facilitate multiplex genome editing in plants.

We next addressed whether the presence of a DNA donor upon gRNA/pcoCas9-mediated generation of a double-strand break would lead to gene replacement by HDR, which could precisely integrate an intended mutation from the DNA donor into the target site. We co-expressed *pcoCas9* and the gRNA aiming for the *NbPDS* target 1 in tobacco protoplasts and concurrently supplied a double-stranded DNA donor that contains a unique *AvrII* site flanked by a 533-bp left homology arm and a 114-bp right homology arm to the *NbPDS* locus (Fig. 2e). *AvrII* digestion of gPCR amplicons spanning the *NbPDS* target site revealed *AvrII* incorporation in the target locus with a frequency of 10.7%, and this incorporation

strictly relied on both gRNA and the DNA donor (Fig. 2f). Sanger sequencing further verified the anticipated creation of the AvrII site in the target sequence without additional modifications and indicated an HDR-mediated gene replacement at a frequency of 9.0% (Fig. 2g). In addition, we detected NHEJ-mediated targeted mutagenesis at the *NbPDS* locus with a frequency of 14.2% (Supplementary Fig. 4). As mesophyll protoplasts are isolated from differentiated leaves without active cell division, we tested the possibility of enhancing HDR by triggering ectopic cell division. Co-expression of *Arabidopsis CYCD3 (CYCLIN D-TYPE 3)*, a master activator of cell cycle, hardly promoted the HDR in tobacco protoplasts (Fig. 2f). Exploration of HDR in *Arabidopsis* protoplasts was unsuccessful, presumably due to intrinsically low efficiency of HDR in *Arabidopsis*¹⁸.

To facilitate genome-wide application of the gRNA/pcoCas9 technology in *Arabidopsis*, we bioinformatically generated a database for a total of 1,466,718 unique gRNA target sequences in *Arabidopsis* exons (Supplementary Database), which cover >99% (26,942 out of 27,206) of nuclear protein-encoding genes defined by TAIR10 (The *Arabidopsis* Information Resource 10). Targeting efficacy and specificity of selected gRNA target candidates from this database need to be experimentally determined each time during future implementation. We also introduced a facile method to manually design a shared gRNA target site specific for multiple homologous target genes by aligning their coding sequences and blasting for off-targets (Supplementary Fig. 3). The gRNA/pcoCas9 technology enables an easy reprogramming of DNA targeting specificity by changing the 20-nt guide sequence in the gRNA without modifying the pcoCas9 protein. We have established a simple and rapid procedure to create a custom gRNA through overlapping PCR (Supplementary Fig. 5). Thus, it is feasible to use a single or tandemly expressed gRNAs (Fig. 2c) to simultaneously target multigene families, which is not easily achievable with ZFNs and TALENs.

We have tested a total of seven target sequences in five target genes in *Arabidopsis* or tobacco, and obtained targeted mutagenesis in all cases. The variation in mutagenesis efficiency among different genes in *Arabidopsis* may stem from distinct gRNA binding strength to individual target sequences or distinct chromatin structure and epigenetic state at individual target loci, which requires future investigation. We have demonstrated that plant protoplasts provide an excellent system to rapidly evaluate the efficiency of the gRNA/pcoCas9-mediated genome editing at a specific genomic locus. Our data also suggested that targeting an *Arabidopsis* gene with multiple gRNAs could improve the success rate of targeted mutagenesis and generate deletions to ensure gene knockout. Significantly, gRNA/pcoCas9 achieved high efficiency of HDR-mediated gene replacement in tobacco protoplasts. The superb potency, simplicity, versatility and specificity of the gRNA/pcoCas9 technology demonstrated in this work promise marker gene-independent and antibiotic selection-free genome engineering with high precisions in diverse plant species to advance basic science and biotechnology.

Supplementary Material

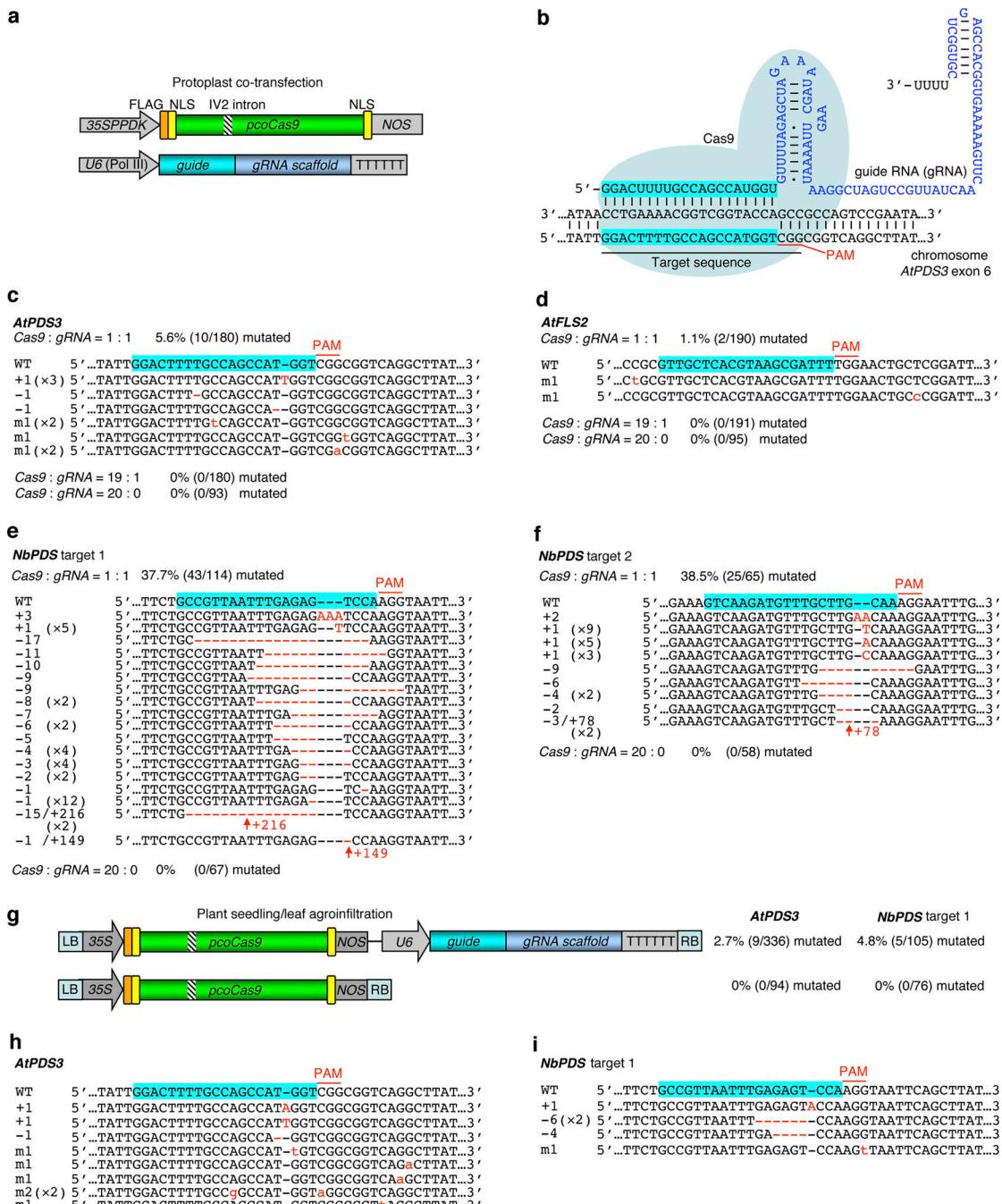
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Frederick Ausubel for critical reading of the article, Daniel Voytas for discussion on the HDR strategy, Yan Xiong for the CYCD3 expression plasmid. J.F.L. is supported by the MGH ECOR Postdoctoral Fellowship for Medical Discovery. The Research is supported by the DOE grant DE-FG02-02ER63445 to G.M.C., the NSF grant IOS-0843244 and the NIH grants R01 GM60493 and R01 GM70567 to J.S.

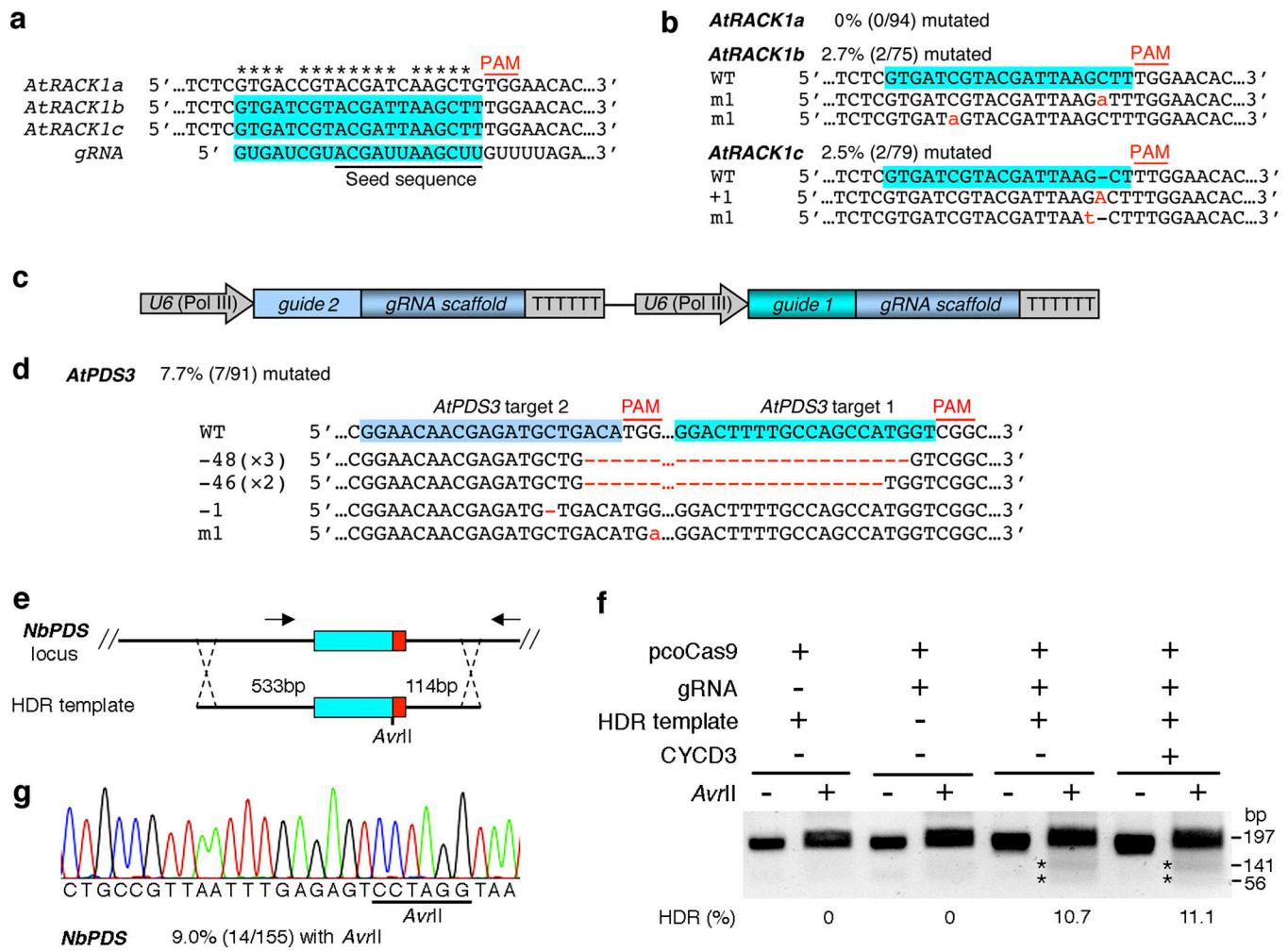
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**Figure 1.**

Targeted plant genome editing by gRNA/pcoCas9. (a) Schematic of the gRNA/pcoCas9 constructs for protoplast co-transfection. (b) Diagram of the gRNA/pcoCas9 complex targeting the *Arabidopsis* AtPDS3 exon 6. (c–f) Targeted genome editing on AtPDS3 (c) and AtFLS2 (d) in *Arabidopsis* protoplasts and NbPDS (e,f) in tobacco (*Nicotiana benthamiana*) protoplasts. (g) Binary plasmids for genome editing of AtPDS3 and NbPDS in *Arabidopsis* and tobacco plants, respectively, via *Agrobacterium*-mediated transient gene expression. (h,i) Targeted genome editing on AtPDS3 in *Arabidopsis* seedlings (h) and NbPDS in

tobacco leaves (**i**). The mutation rate in **c–g** was calculated based on the mutant/total alleles of randomly selected clonal amplicons of the target locus. In **c–f**, **h** and **i**, blue shadow marks the target sequence recognized by cognate gRNA. PAM, the protospacer adjacent motif. DNA insertions, deletions and point mutations are shown in red as upper case letters, dashes and lower case letters, respectively. The upright arrow and number in red indicate a long insertion.

**Figure 2.**

Multiplex and HDR-mediated genome editing by gRNA/pcoCas9 in plant cells. (a) Diagram of a single gRNA targeting two genes. *AtRACK1b* and *AtRACK1c* but not *AtRACK1a* from the *Arabidopsis* RACK1 family are gRNA targets. The target sequence recognized by gRNA is shadowed in blue and the gRNA seed sequence is underlined. (b) Targeted mutations induced by gRNA/pcoCas9 in *AtRACK1b* and *AtRACK1c* but not *AtRACK1a* in *Arabidopsis* protoplasts. (c) Schematic of a tandem gRNA construct. (d) Large genomic deletions are induced by double gRNAs targeting the *AtPDS3* locus. In b and d, DNA insertions, deletions and point mutations are shown in red as upper case letters, dashes and lower case letters, respectively. (e) Diagram of the HDR strategy. Successful HDR creates an AvrII site in the target sequence of the *NbPDS* locus. The arrows represent the primers for gPCR amplification of the target region. (f) AvrII digestion products (marked by asterisks) of *NbPDS* target amplicons exist upon successful HDR in tobacco protoplasts. *Arabidopsis* cyclin D-type 3 (CYCD3), a master activator of cell cycle. (g) DNA sequencing evidence of successful HDR in the presence of pcoCas9, gRNA and HDR template.