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Prime Editing in the model plant *Physcomitrium patens* and its potential in the tetraploid potato

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ABSTRACT

Since its discovery and first applications for genome editing in plants, the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology has revolutionized plant research and precision crop breeding. Although the classical CRISPR-Cas9 system is a highly efficient tool for disruptive targeted mutagenesis, this system is mostly inefficient for the introduction of precise and predictable nucleotide substitutions. Recently, *Prime Editing* technology has been developed, allowing the simultaneous generation of nucleotide transitions and transversions but also short defined indels. In this study, we report on the successful use of *Prime Editing* in two plants of interest: the plant model *Physcomitrium patens* and the tetraploid and highly heterozygous potato (*Solanum tuberosum*). In both cases editing rates were lower than with other CRISPR-Cas9 based techniques, but we were able to successfully introduce nucleotide transversions into targeted genes, a unique feature of *Prime Editing*. Additionally, the analysis of potential off-target mutation sites in *P. patens* suggested very high targeting fidelity in this organism. The present work paves the way for the use *Prime Editing* in *Physcomitrium patens* and potato, however highlighting the limitations that need to be overcome for more efficient precision plant breeding.

1. Introduction

Reverse genetic-based study is dominated today by CRISPR-Cas (clustered regularly interspaced short palindromic repeat) gene editing techniques [1,2]. The large natural prokaryotic Cas gene pool coupled with the development of laboratory variants such as the nickase Cas9 [3] allows their use in virtually all biological systems provided an established experimental transformation procedure is available [4]. CRISPR-Cas9, the best understood and most used system, has served as the basis for the development of many molecular tools [2]. This system's functional unit is a large multi-domain ribonucleoprotein formed of an apoprotein, Cas9, and the so-called sgRNA (for short guide RNA). Together, they can bind to DNA, find a specific sequence and generate a double stranded DNA break (DSB) at a targeted genome site. Specificity for the targeted DNA sequence is coded by the sgRNA [5]. This approach is very precise in term of targeting and is highly mutagenic because the repair of the DSBs is mediated by the error-prone repair machinery of the transformed organism, which generates unpredicted insertion or deletions (indels) of different sizes. In some cases, CRISPR-Cas can also generate unwanted off-target mutations elsewhere in the genome that need to be carefully analyzed [6]. Several approaches have been developed to address these problems of unpredictable mutations at the target site and off-target mutations by modifying the initial system to edit the genome more specifically and predictively [7,8]. The *Prime Editing* system represents such a successful endeavour [9]. In this system, the apoenzyme or Prime editor is a fusion protein composed of a *Streptococcus pyogenes* Cas9 nickase (SpnCas9 H840A) and an optimized Moloney murine leukemia virus reverse transcriptase (M-MLV RT) [9]. This apoprotein possesses the same Cas9 property of targeting DNA sequence complementary to the RNA guide but, due to its nickase

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modification, generates a single-stranded DNA (ssDNA) cut that should not engage the DSB mutagenic repair mechanism, thereby avoiding the introduction of unwanted mutations. The apoprotein also contains a RT domain that can reverse-transcribe a specified modification (single or multiple nucleotide changes as well as short indels) into DNA using the RNA guide RT-template. The RNA guide, referred as the *Prime Editing* guide RNA (pegRNA), has a double role: it mediates recognition of the targeted locus and, following the ssDNA cut, its 3' extension harbors both a primer binding site (PBS) and a reverse transcription sequence (RT sequence) that templates introduction of the desired edition at the target site [9]. Inspired by available online sgRNA design tools (e.g. CRISPOR [10]), the complexity of pegRNA design has seen the development of specific online pegRNA design tools including Peg-finder [11], PE-Designer/PE-Analyzer [12], pegIT [13], PrimeDesign [14] and, finally, PlantPegDesigner [15], which is more specifically tailored for *Prime Editing* in plants.

After its initial development in yeast and human cell lines [9], *Prime Editing* has been shown to be effective in several different animals (see [16] for a short review) such as mice [17] and *Drosophila* [18]. *Prime Editing* has been employed successfully in plants, first in rice ([19], followed by [20–24]). Not unsurprisingly since *Prime Editing* holds great promises for precision breeding, [8] as several traits of interest can be conferred by point mutations rather than gene loss-of-function [25], reports of its use in other cereals such as wheat [19] or maize [26], but also in Arabidopsis, *Nicotiana benthamiana* [27] and tomato [28] have followed, all recently reviewed in detail by Molla and collaborators [7].

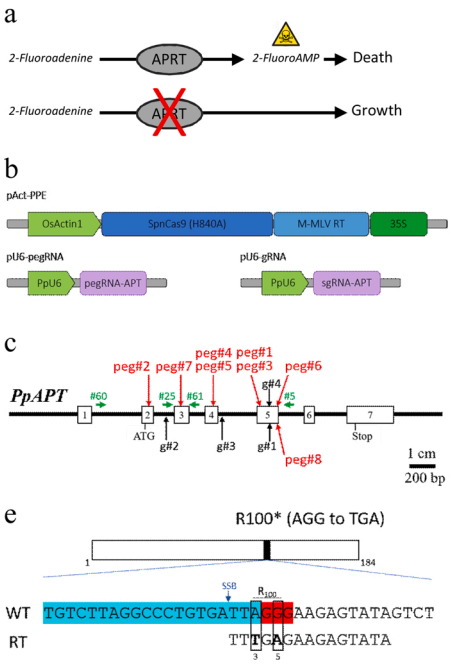
In the present study we aimed to evaluate the potential of *Prime Editing* in two different plants, the moss model *Physcomitrium patens,* as it has already been used successfully to study different CRISPR-Cas9-mediated genome editing techniques [29,30], and the important crop plant *Solanum tuberosum* (potato). To favour the rapid identification of editing events, we used a different reporter system for each plant. For *P. patens*, targeting of the gene *PpAPT* has already been used to evaluate gene targeting efficiency in different mutant backgrounds (see notably [31,32]) or efficiency of gene modulation [33]. In brief, the normal function of Adenine phosphoribosyltransferase (APRT) in the plant is to convert adenine into AMP. APRT can also convert 2-Fluoroadenine (2FA) into 2-Fluoro-AMP, a lethal compound for plants usable for counter selection [34]. Hence, CRISPR-Cas null and strong mutation events in the *PpAPT* gene will confer viability on 2FA-containing medium (Fig. 1a) and plants can thus can be isolated, grown and their mutation pattern analyzed (see for example [30,31]). In flowering plants, modification of the *acetolactate synthase* (*ALS*) gene offers a similar scheme. It has been long known that weed resistance to several herbicides have been generated by several specific amino acid changes in this gene [35], to a point that mutated *ALS* is used as a resistance marker in several plants, notably rice [36]. With respect to the present study, we used specific modification of the native potato *St*ALS Pro-187 which confers resistance to the herbicide chlorsulfuron as previously shown using a CRISPR-Cas9 mediated cytidine base editor [37].

2. Materials and methods

2.1. Molecular cloning

The *Prime Editing* enzyme used in this study was composed of the coding sequence of nCas9 (H840A) fused to M-MLV reverse transcriptase (D200 N, T306 K, W313 F, T330 P, L603W), similar to the construct used by Anzalone and collaborators [9]. Additionally, two SV40 NLS were added to the N- and C-terminal ends of the protein. The coding sequence was codon-optimized for dicotyledons, synthesized (Twist-Bioscience, San Francisco, Ca, USA) and subsequently cloned into an intermediate pTwistENTR plasmid through *Sac*I restriction followed by T4 DNA ligation (ThermoFisher Scientific, USA). Following sequence verification by Sanger sequencing, the coding fragment was cloned into pBS TPp-A [38] using the Gateway™ LR reaction (Invitrogen, USA) to generate pAct:PPE and used to transform moss protoplasts (Fig. S1a). Similarly, the same fragment was cloned into a modified pDeCas9 backbone [39] with *Asc*I restriction enzyme followed by T4 DNA ligation (ThermoFisher Scientific, USA) to create the pDePPE plasmid to transform potato (Fig. S1b). The previously described pAct:Cas9 [29] was used to evaluate the targeting efficiency of the pegRNA guide RNA in moss. To perform a similar evaluation in potato, pDeCas9-NPTII [39] was used.

The *Prime Editing* guide RNAs (pegRNAs) designed to target and edit the moss *APT* gene (Pp3c8_16590, https://phytozome-next.jgi.doe.gov/ [40]) were all constructed with an identical expression structure but with variations present only in the targeting and editing (PBS and RT



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Name	Targeted mutation			RT template length	PBS length
	type	position	nature		
pegRNA#1	3 snp	6	G-to-C	18	13
		7	G-to-T		
		14	A-to-G		
pegRNA#2	ins4	2-5	ATAC	18	14
pegRNA#3	2 snp	3	A-to-T	15	14
		5	G-to-A		
pegRNA#4	2 snp	5	G-to-T	16	14
		7	T-to-A		
pegRNA#5	del1	5	- G	15	14
pegRNA#6	3 snp	2	G-to-T	16	13
		5	G-to-T		
		7	C-to-A		
pegRNA#7	3 snp	3	A-to-T	19	10
		9	G-to-T		
		11	C-to-G		
pegRNA#8	del1	5	- C	16	14
	snp	8	T-to-A		

Fig. 1. *Prime Editing* strategy for precise modifications of the *PpAPT* gene.
(**a**) Selection of *apt* mutants based on 2FA resistance. (**b**) Schematic representation of the prime editor pAct-PPE and pU6-pegRNA constructs used for transient co-transfection of moss protoplasts (PE2). For PE3, an additional construct, pU6-sgRNA, was co-transfected. (**c**) Structure of the *PpAPT* gene and pegRNA positions. Boxes in white represent the exons and black lines represent the introns. The eight pegRNA positions are indicated in red, the sgRNAs used for PE3 in black and the primers used for PCR and sequencing in green. (**d**) Information about the eight *APT*-pegRNAs. For each pegRNA, targeted mutations are indicated: type and nature of mutation, position on RT template, length of RT template and PBS (defined by the SSB site). (**e**) Schematic representation of the APRT protein with the wild-type genomic sequence (WT) and expected RT product (RT) using APT-pegRNA#3 (2 SNPs), leading to modification of Arg_{100} to a premature stop codon. The target sequence is highlighted in blue and the PAM in red, the blue arrow represents the SSB site and the relative positions of mutations are indicated below the expected RT product sequence.

template) sequences. Transcription of the pegRNAs was driven by the *Pp*U6 promoter and transcript termination was assured by the SUP4 [41] terminator. pegRNA expression constructs contained a common spacer sequence and a sgRNA scaffold (see an example in Fig. S2 for the pegRNA#1 vector map, in Fig. S3a for an illustration of the pegRNA#1/gDNA interaction at the *PpAPT* locus and Fig. S3b for the full pegRNA#1 expression module sequence) [9]. The sequences of the eight specific pegRNA targeting and editing sequences are listed in the Table S1. Complete expression units were synthetized (TwistBioscience, San Francisco, Ca, USA) and cloned into a pTwist Amp High Copy by the manufacturer. The RNA guides (sgRNA) used for PE3 and Cas9 target efficiency evaluation were built with the same structure (Table S2). The expression cassette consisted of the promoter of the *P. patens* U6 snRNA [42], the specific 5'-G-N(19)-3' guide sequences targeting the *APT* gene and the tracrRNA scaffold. The complete unit was synthesized by Twist Bioscience (San Francisco, California, USA) and sub-cloned into the pDONR207-NeoR vector [30] using the Gateway™ BP reaction (Invitrogen, USA) to create the final vector. PE3 pAPTgRNA-PE3#1 to pAPTgRNA-PE3#4 were designed to target the 26 – 272 bps of the eight different pegRNAs into the *PpAPT* gene. The pegRNA-gRNA-PE specific pairing is shown in Table S1 and their sequences in Table S2. The Cas9 control vectors psgRNA#C2, psgRNA#C3, psgRNA#C6 and psgRNA#C8 contain the same target sequences as pegRNA#2, pegRNA#3, pegRNA#6 and pegRNA#8, respectively (Table S2). Before use, plasmid DNA was ethanol precipitated to ensure sterility for protoplast transformation.

The reference sequences for the potato *ALS* genes are PGSC0003DMG400034102 for *StALS1* and PGSC0003DMG400007078 for *StALS2* [43]. The pegRNA-StALS and sgRNA-StALS used in potato transformation were driven by the *At*U6-26 promoter and terminated with the T1 domain of the ScSUP4 terminator. Both constructs were synthesized by Twist Bioscience (San Francisco, Ca, USA) (sequence Tables S1 and S2 respectively). For the PPE2 strategy, pegRNA-StALS was cloned into the pDePPE (Fig. S1) plasmid through a LR Gateway reaction (ThermoFisher Scientific, USA), resulting in the binary plasmid pDePPE2-StALS (Fig. S4a). For the PPE3 strategy, pegRNA-StALS and sgRNA-StALS were assembled through *BstZI*/*Mlu*I restriction followed by T4 DNA ligation (ThermoFisher Scientific, USA), and then cloned into pDePPE through a LR Gateway reaction (ThermoFisher Scientific, USA), resulting in the binary plasmid pDePPE3-StALS (Fig. S4b). For classical editing to induce indels, pegRNA-StALS was cloned into the pDeCas9-NPTII [39] plasmid through a LR Gateway reaction (ThermoFisher Scientific, USA), resulting in the binary plasmid pDeCas9-StALS (Fig. S4c). The final constructs were checked by restriction digestion and Sanger sequencing.

2.2. Plant material and processing

Wild type (WT) *Physcomitrium patens* ecotype Gransden pedigree Versailles was used in the present study [44]. WT and mutant tissue propagation was routinely performed on $PpNH_4$ medium ($PpNO_3$ medium supplemented with 2.7 mM NH_4-tartrate, [45]) in growth chambers set at 60 % humidity at 23 °C with 16 h of light (quantum irradiance of 80 μmol m^{-2} s^{-1}) and 8 h of dark. Moss protoplast isolation and transfection were performed from six day-old blended protonemal tissue as previously described [46,47]. Protoplasts were transfected with a total of 15–20 μg of circular DNA consisting of equal amounts of co-transformed vectors as follow: 7.5 μg of the pAct-PPE plasmid and 7.5 μg of each pegRNA plasmid for PPE2 or 7 μg of the pAct-PPE plasmid, 7 μg of pegRNA plasmid and 7 μg of sgRNA for PPE3. Protoplasts were imbedded in alginate and spread on cellophane disks laid on $PpNH_4$ medium supplemented with 0.33 M Mannitol and left to regenerate for one week. Plants on cellophane disks were then transferred for selection directly onto $PpNH_4$ supplemented with 10 μM 2-FA (Fluorochem, Hadfield, United Kingdom) to select clones that were mutated at the *APT* locus and thus resistant to this chemical [42]. Growing plants after ten days were counted and individually sub-cultured on fresh $PpNH_4$ medium until harvesting for genotyping.

The tetraploid potato cultivar Desiree (ZPC, Joure, The Netherlands) was propagated *in vitro* in a controlled environmental chamber at 19 °C under a 16 h light/8 h dark photoperiod and transformed as previously described [37,48]. The binary plasmids described above were transferred into *Agrobacterium* strain C58pMP90 by heat shocking the competent cells at 42 °C for 90 s and then maintaining using standard microbiological techniques. For the classical editing approach that induces indels, plant tissues were grown on 50 mg/L kanamycin. For the PPE2 and PPE3 strategies, potato explants were grown on 50 mg/L kanamycin for 10 days. Then 2/3 of the explants were transferred to a medium containing 30 ng/mL chlorsulfuron, while the remaining explants were kept on a kanamycin selection pressure. After several weeks of growth, newly regenerated stems could be cut and individually transferred to a culture medium without kanamycin.

2.3. PCR and sequence analysis of the edited plants

All the PCR primers used in this study are listed in Table S3. Moss genomic DNA was extracted from 50 mg of fresh tissue as previously described [49]. The quality of the DNA samples was verified using primers targeting the *P. patens RAD51-1* gene, PpRAD51-1#6 and PpRAD51-1#7. Sequence analysis was based on Sanger sequencing (Genoscreen, Lille, France) of PCR fragments using primers amplifying the targeted loci. Primers PpAPT#25 and PpAPT#5 were used for the loci targeted by pegRNA#1, #3, #4, #5 and #6 and primers PpAPT#60 and PpAPT#61 for the loci targeted by pegRNA#2 and #7. Potato genomic DNA from regenerated plants and calli was extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. T-DNA was detected by PCR using the GoTaq G2 Flexi DNA polymerase (Promega, USA) with primers matching the *nptII* and the *Cas9* or *nCas9* sequence. HRM analysis was performed using the High-Resolution Melting Master (Roche Applied Science, Germany) on the LightCycler® 480 II system (Roche Applied Science, Germany), as previously described [37]. Plants that displayed a HRM mutated profile were then Sanger sequenced (Genoscreen, France) after amplification with the Superfi DNA polymerase (ThermoFisher Scientific, USA). Cloning of PCR fragments into individual plasmids was done using the TOPO TA Cloning Kit for Sequencing kit (ThermoFisher Scientific, USA), followed by isolation of plasmids and Sanger sequencing (Genoscreen, Lille, France).

3. Results

3.1. PE2 is functional in P. patens and its efficiency is pegRNA-dependent

PE2 constitutes the simplest technical form of *Prime Editing*, with the co-expression of the editing enzyme (PPE) and a single pegRNA (Fig. 1b). To test the feasibility of PE2 in *P. patens* we designed eight different pegRNAs targeting the ORF of the *PpAPT* gene (Fig. 1c–e, Table S1). The eight different modifications were predicted to generate mutations that conferred resistance to 2FA by creating deleterious SNPs or small indels (Fig. 1d). In addition, the length of the RT template 15–19 bps) and PBS (10–14 bps) were varied within the optimal range defined by Anzalone and collaborators [9]. Following protoplast transfection with the individual pegRNA vector and the pAct-PPE vector (see Fig. 1b for schematic representations and Material and Methods for details on vector assembly), regeneration and selection, six of the eight pegRNAs generated 2FA resistant plants with numbers spanning almost two orders of magnitude (Table 1). Of note, even the best observed mutation frequency at the *PpAPT* locus, which was observed with pegRNA#6 (0.06 % of the transformed protoplasts) was two orders of magnitude lower than the frequencies observed in either standard Cas-9 mediated mutagenesis [32,42] or using base editors [30]. To analyse the nature of the editions we PCR-amplified and sequenced the target loci from the

Table 1
Efficiency and predictability of 8 pegRNAs using PE2 and PE3 systems in moss.

Name	PE type	PE2 [a]			PE3 [a]			
		nb 2FA^R	mut. Freq. %	pred. PE % (n) [b]	dist. Nick	nb 2FA^R	mut. Freq. %	pred. PE % (n)
pegRNA#1	3 SNP	5	0,003	67% (3)	+41	3	0,002	33% (3)
pegRNA#2	ins4	16	0,009	93% (14)	−28	55	0,026	61% (38)
pegRNA#3	2 SNP	45	0,035	100% (45)	+41	104	0,061	100% (60)
pegRNA#4	2 SNP	2	0,0015	50% (2)	−52	4	0,002	100% (2)
pegRNA#5	del1	0	0	–	−52	1	0,0005	100% (1)
pegRNA#6	3 SNP	88	0,062	100% (71)	+151	57	0,026	98% (57)
pegRNA#7	3 SNP	2	0,0012	100% (2)	+272	9	0,004	100% (7)
pegRNA#8	del1 & 1 SNP	0	0	–	+83	0	0	–

[a] From 3 independent repetitions.
[b] (n) for number of sequenced mutant plants.

putative mutants. Overall the editing was very precise confirming the specificity of *Prime Editing* in the moss *P. patens* (Table 1, Fig. S5 for examples of successful edits). Notably pegRNAs #3 and pegRNA#6 systematically yielded perfect edits involving the edition of two and three nucleotides, respectively (Fig. 2a). Nevertheless, two different types of unintended event - namely partial editing and deletion - were detected for other guides. One mutant obtained with pegRNA#1 displayed partial editing with only two of the three targeted nucleotides. Also, short deletions were observed with pegRNA#2 (e.g. Fig. S6, plant#17). This later type of event is reminiscent of a classic Cas9-mediated mutation and may be due to a residual activity of the nCas9 associated with these specific pegRNAs.

3.2. PE3 does not improve significantly PE2 Prime Editing in P. patens

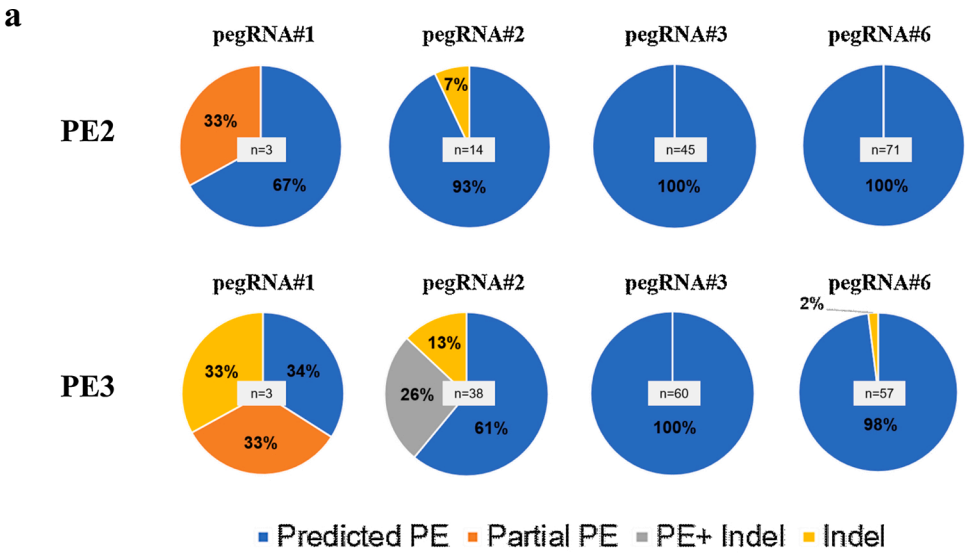
PE3 was designed to improve editing frequency by generating an extra nick in the vicinity of the editing locus to favor the fixation of the researched edition [9]. Nicking relied on the nickase activity of the PPE enzyme and was mediated by a standard sgRNA that targeted the opposite strand of the edit site. The distance of the nick from the edition site has been shown to be optimal at between 40 and 200 bps [9]. We used four sgRNAs positioned between 28–272 bps from their respective pegRNA (Fig. 1c, Tables 1, S2 for the targeting sequence). Following PE3 transformation the mutation frequencies were similar to those observed for PE2 (Table 1). Minor variations in frequency were observed in both directions depending on the pegRNA tested. The two most efficient pegRNAs, generating the most 2FA resistant plants, were pegRNA#3 and #6 for both PE2 and PE3. Two differences between PE2 and PE3 are worth noting. PegRNA#5, which did not yield any mutants in PE2, generated a single mutant in the PE3 setting indicating that this pegRNA can be functional, albeit at low rate. Also, pegRNA#2 generated more than three times more mutants in PE3 than in PE2. Sequence analysis provided an explanation for this increase: 39 % of the 2FA resistant plants generated by pegRNA#2 in combination with sgRNA-PE3#2 displayed a deletion either at the sgRNA-PE3#2 target site or between the two guides (Figs. 2a, S6). The distance between the two target sequences, 26 bps, is probably the cause of these deletions and reduces significantly the quality of the editing. The editing quality with the other pegRNAs remained almost perfect with the exception of pegRNA#1, which generated mutants that displayed either partial editing or a deletion at the pegRNA position (Fig. 2b, Plant #3). Overall, PE3 did not improve the quality and number of obtained edits. Worse when the second nick was too close to the edited sequence target it appeared to be mutagenic, generating small deletions at the target sites rather than correct editing of the targeted locus.

3.3. The guide targeting sequences and the pegRNA structure do not impede Cas9-mediated mutagenesis in P. patens

As the observed mutant frequencies obtained with the *Prime Editing* procedure were lower than those observed with standard Cas9 transfection approach, we set out to evaluate if either the structure of the pegRNA or the specific targeting sequence used in the pegRNA could be the cause for such a drop. We co-transfected protoplasts with pegRNA #2, #3, #6 and #8 in association with pAct-Cas9, an efficient CRISPR-Cas9 enzyme expresser in *P. patens* [29]. We then evaluated the mutation frequencies by selecting these protoplasts on 2FA containing medium. The results were unambiguous: the observed mutation frequencies (2.0%–4.6% of the regenerating protoplasts) were markedly higher than those observed with *Prime Editing* with the same pegRNA (Table 2) and of the same order of magnitude of those observed previously for other sgRNAs [32,42] indicating that the pegRNA structure does not hinder substantially its interaction with the Cas9 apoprotein and its DNA target. Subsequently, we performed standard CRISPR-Cas9 transfection using the sgRNAs sgRNA#C2, #C3, #C6, #C8, which have identical target sequences to those of the four pegRNAs tested above (see Table S2 for sequences). The mutation frequencies (4.8%–6.5% of the regenerating protoplasts) with these sgRNAs were in the same order of magnitude as those observed previously for other sgRNAs [32,42], an indication that the target sequences used for these pegRNAs were not causing the low *Prime Editing* frequency. Finally, as exemplified by pegRNA#8, which did not display any editing in the PE2 or PE3 settings but generated targeted mutants with standard Cas9, we observed that the efficiency of a given sgRNA in a standard CRISPR-Cas9 strategy appears not to be correlated with its capacity to mediate editing through *Prime Editing*.

3.4. Analysis of off-target editing events suggest that Prime Editing is highly specific in P. patens

The specificity of modifications to the targeted locus is a perennial question with the use of Cas9 technology. So much so that guide design algorithms such as CRISPOR [10] list potential off-target sites with up to four mismatches compared to the desired targeting sequence in the selected genome. We carried out an off-target analysis on the pegRNAs that generated the highest number of editing events, pegRNA#2, 3 and 6 (Fig. 2a). CRISPOR did not identified any off-target sites for pegRNA#3, but one and nine off-target sites were predicted for pegRNA#6 and pegRNA#2, respectively (Table S4). Two of the predicted off-target sites for pegRNA#2 correspond to a duplicated sequence in the *P. patens* genome. Using the CRISPOR-generated primer pair for each predicted



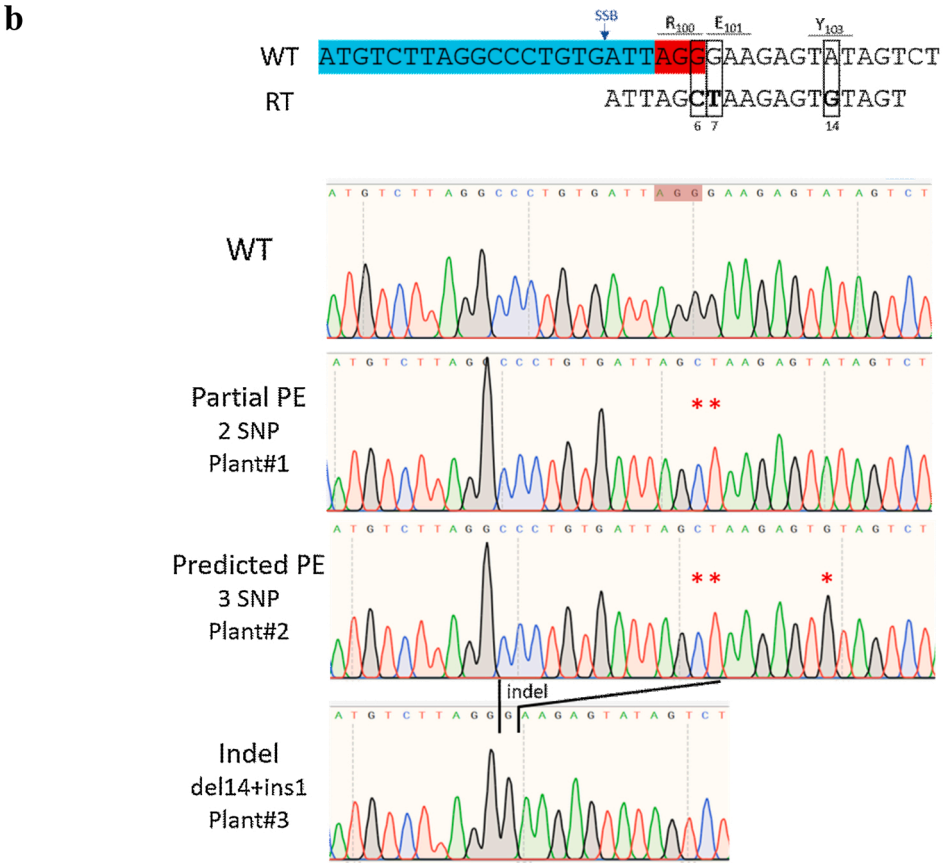


Fig. 2. Mutations induced using the PE2 and PE3 systems in moss.
(**a**) Mutations obtained with PE2 and PE3 for four pegRNAs (#1, #2, #3 and #6). The number of plants sequenced is indicated (n). (**b**) Examples of prime edited plants using PE3 with pegRNA#1. WT sequence, partial PE, predicted PE and deletion events, respectively. For plant#3, in addition to the short indel shown at the editing site, a second deletion occurred at the sgRNA position (41pb upstream of pegRNA#1).

off-target site, all loci were successfully PCR-amplified from 42 pegRNA#2 and 37 pegRNA#6 independently edited plants. Subsequent sequencing of each amplicon did not detect any modification at these sites (Tables S4 and S5). Therefore, *Prime Editing* in *P. patens* seems to be highly specific, at least concerning the predicted off-target sites (Table S4). The basis of this highly locus-specific edition, initially proposed by Anzalone and collaborators [9] and observed in rice [50], reposes potentially on the use of a nickase Cas9 in *Prime Editing*. As a result, even if the PPE enzyme recognizes and damages an off-target, this effects only one DNA strand leading to a single strand break that is generally resolved by the native DNA reparation machinery without the creation of mutation.

3.5. *PE2 is functional in potato but at low frequency*

To assess PE2 in potato, we used the same PPE enzyme as described above cloned behind the parsley Ubiquitin promoter into a modified pDe backbone [51], resulting in the pDePPE plasmid (Fig. 3a). pegRNA-StALS (Fig. 3b, Table S1) was designed to edit St*ALS* proline186 into a serine, an amino acid change that is known to confer resistance to chlorsulfuron in tobacco [52]. pegRNA-StALS -both targeting sequence and PBS- was designed in order to target both the *StALS1* and *StALS2*

Table 2
CRISPR-Cas9 mediated *APT* knock-out frequencies in moss.

Target	pegRNA efficiency of editing [a]		sgRNA efficiency of editing [a]
	via PE (pAct-PPE) [b]	via KO (pAct-Cas9)	via KO (pAct-Cas9)
APT#2	2,6. 10^{-2} (±0,7)	4,6 (±0,8)	5,6 (±0,9)
APT#3	6,1. 10^{-2} (±0,8)	2,0 (±0,3)	6,5 (±0,2)
APT#6	2,6. 10^{-2} (±0,7)	3,7 (±0,9)	4,9 (± 0,3)
APT#8	0	2,2 (±0,6)	4,8 (±0,6)

[a] From 3 independent repetitions.
[b] From Table 1 (PE3).

genes, based on a recent release of a SNP map of the *Solanum tuberosum* cultivar Desiree [43]. Its RT template, which harbors the mutations to introduce, was designed to induce three base conversions. Two base substitutions (one C-to-T transition and one G-to-C transversion) aimed to generate the P186S amino acid shift to confer chlorsulfuron resistance, and one G-to-A transition aimed at modifying the PAM (synonymous mutation) in order to prevent nCas9 cleavage after edition of the locus (Figs. 3b, S7a). One week after pDePPE2-StALS (Fig. S4) *Agrobacterium*-mediated transformation, two-thirds of the explants were transferred to chlorsulfuron-containing medium in order to apply a selection pressure allowing only regeneration of edited cells (transgenic or T-DNA-free plants), while one third of the explants were maintained on kanamycin to select for stably transformed cells, as previously reported [37]. None of the 20 kanamycin-regenerated transgenic plants generated with pDePPE2-StALS were mutated at the target loci (*StALS1* and *StALS2*) based on high-resolution melting (HRM) analysis (Fig. S7b). However, while one of the two plants regenerated from the chlorsulfuron-containing medium harbored a melting curve profile similar to the control, indicating that this plant escaped the selection pressure, the other (Prime edited n°1) displayed mutated profiles at the targeted loci (Fig. S7c). Direct Sanger sequencing of PCR products from *StALS1* and *StALS2* (8 alleles) revealed a mixture of signals at the targeted nucleotides, corresponding to the expected base substitutions (Fig. S7a and c). To confirm that the mutations affected the same allele, we cloned the PCR products from the *StALS1* gene into individual plasmids. This analysis showed that this plant indeed harbored the 3 expected substitutions at the *StALS1* target locus (Fig. 3c). Based on the number of wild-type and mutated reads (Table S6), we postulate that the mutation may be stably present on a single allele and may also be somatic (i.e. a mosaic plant). Compared to our previous work on base editing of *StALS* genes [37], the number of resistant plants on the chlorsulfuron-containing medium appeared to be very low. We also sampled eight green, growing calli directly from the medium, but unfortunately no mutation was identified by HRM despite the presence of a T-DNA insert.

3.6. PE3 did not show editing in our ALS targeting strategy

PE3 used the same PPE enzyme and pegRNA as that employed in 3.5 supplemented with a standard sgRNA targeting *StALS* at 20 bps on the opposite strand of the edited sequence. Thus, we added the U6 driven sgRNA-StALS cassette to pDePPE2-StALS to build the transformation vector pDePPE3-StALS (Fig. S4b, Table S2). *Agrobacterium*-mediated transformation and transformant isolation was performed as with pDePPE2-StALS but none of the regenerated plants from kanamycin (6 transgenic plants) or chlorsulfuron-containing media (2 regenerated plants) were mutated according to a HRM analysis (Table S7). Additionally, we sampled 12 green, growing calli that harbored the T-DNA insert, but HRM analysis indicated that none of them displayed a mutated profile.

3.7. Low efficiency of Prime Editing in potato appears not to be due to the structure of the pegRNA

To evaluate if the very low efficiency of PE in potato was due to the structure of the pegRNA we performed a transformation associating the

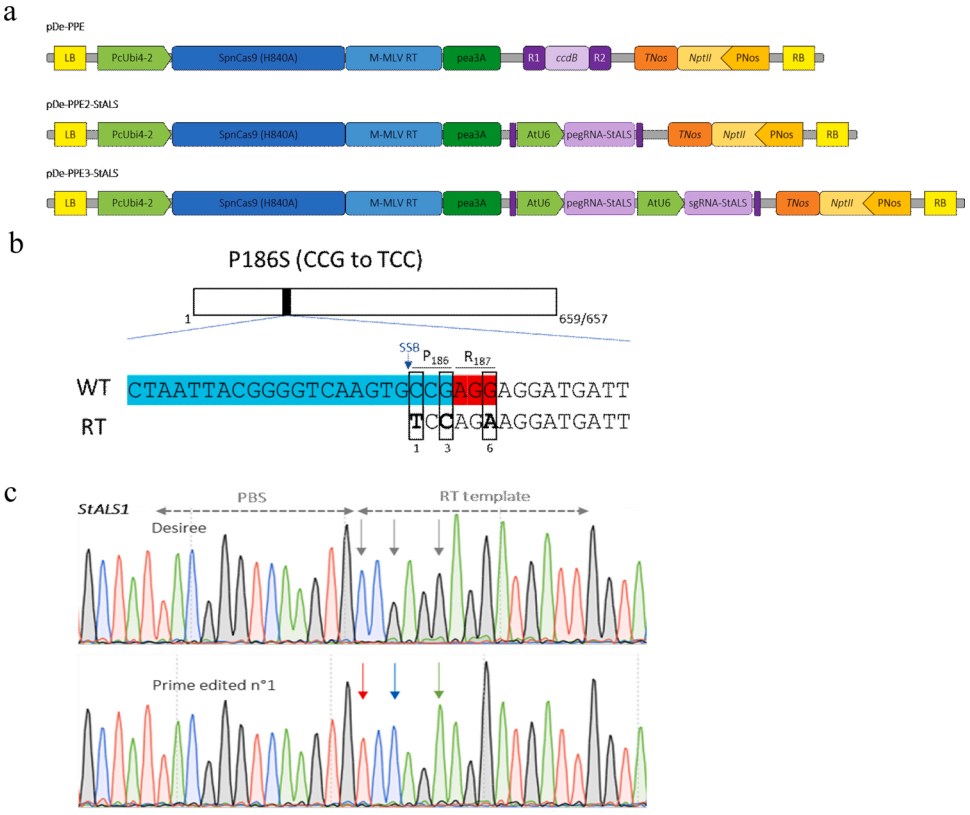


Fig. 3. Prime editing targeting of *StALS* loci in potato.
(**a**) Schematic representation of the prime editor pDe-PPE used for expression in dicot species through *Agrobacterium*-mediated transformation. The pDe backbone was used and the Cas9 cassette was replaced by the SpnCas9 (H840A)/M-MLV RT fusion. The pegRNA and sgRNA were swapped with the ccdB cassette using an LR gateway reaction to give pDe-PPE2-StALS and pDe-PPE3-StALS. (**b**) Schematic representation of the ALS protein with the wild-type genomic sequence (WT) and expected RT product (RT) using StALS-pegRNA (3 SNPs), leading to the modification $Pro_{186}Ser$. The target sequence is highlighted in blue and the PAM in red, the blue arrow represents the SSB site and the relative position of mutations are indicated below the expected RT product sequence. (**c**) Examples of sequencing chromatograms obtained from Desiree (WT) and prime edited n°1 plant after *StALS1* allele cloning into individual plasmids. Targeted nucleotides are indicated with grey or color arrows on WT or mutated chromatograms, respectively. A: green; T: red; C: blue; G: black.

pegRNA-StALS construct described above with a standard Cas9 that has been shown to be functional in potato [53]. We cloned pegRNA-StALS into a modified pDeCas9 backbone [51] resulting in the *Agrobacterium*-mediated transformation vector pDeCas9-StALS (Fig. S4c). Following potato explant transformation, regeneration and kanamycin selection, twelve resistant plants were processed further. Because the ALS enzyme is essential for the plant cell, it is likely that only cells that were not affected by frameshift mutations in all the alleles could have grown and regenerated to a whole plant. Genomic DNA was extracted from regenerated potato plants and a HRM analysis targeting both *StALS1* and *StALS2* (8 alleles) was performed in order to identify mutations at the target loci. Eleven out of twelve regenerated transgenic plants harbored mutations at the target sites (92 % efficiency). Intriguingly, after Sanger sequencing of PCR products from both *StALS1* and *StALS2*, we observed a clearly identifiable mutated trace in the chromatograms of only 6 of the 11 HRM positive plants, which may be due to higher sensitivity of the HRM analysis, especially for the detection of mosaic plants that harbor a small proportion of mutated cells. Overall, our results indicated that the pegRNA structure did not substantially impair Cas9 activity and that the pegRNA was functional for Cas9-mediated mutagenesis suggesting that low PE efficiency is probably not the result of poor targeting of the editing complex to the targeted locus.

4. Discussion

In this study, we have demonstrated that *Prime Editing* is functional in *P. patens* and potato. It allowed precise single or multiple base editing in these species that could not be obtained through base editing (BE). In *P. patens Prime Editing* also allowed insertions of one or more nucleotides at the targeted locus using either the PE2 or the PE3 approach. In contrast to the BE technique [30,54], *Prime Editing* allowed both guanine and thymine editing as well as more predictive cytosine and adenine editing. As observed with BE in *P. patens* [30], no predicted pegRNA-dependent off-target activity was detected (Table S5). Concerning possible unpredictable off-target activity of PE, it should be noted that no such activity was detected in rice [15,19] and maize [22]. The results presented here for *P. patens* tend to confirm that the PE3 approach does not increase *Prime Editing* in plants compared to PE2 [19, 26,28], despite the fact that an increase has been observed with animal cells [9]. For PE3, we show that, if the sgRNA target is too close of the pegRNA target (26 bps in the case of the couple pegRNA#2/sgRNA-PE#2), the quality of editing can drop drastically. With the couple pegRNA#2/sgRNA-PE#2, 45 % of the detected $2FA^R$ mutants corresponded to small indels located either at each guide site or between the two sites (Fig. S6). This observation suggests that either two DNA nicks close to each other or the presence of two PPE enzyme associated with their guides at a short distance on the DNA could generate DSBs and inhibit proper editing, as has been observed in other plant [19] and animal cells [9,18].

The overall *Prime Editing* editing rate in *P. patens* and potato compared to standard Cas9 or BE mutagenesis techniques at the same target was low. We showed that this low rate was neither due to the targeted loci nor to the structure of the pegRNA, as higher mutagenic rates were obtained using the same pegRNAs against the same target loci using standard Cas9-mediated targeted mutagenesis (Table 1). This observation is consistent with the results of similar experiments on other plants with alternative target loci. In land plants the efficiency of target mutagenesis using stable transformation of standard Cas9 varies can attain 100 % and routinely reaches 80 % (see [55] for review). The best edited plant / transformed plant ratio obtained for *Prime Editing* so far is 51 % reported for maize [26] but, with rare exceptions, values for rice vary between 1% and10 % [19,22,24]. Additionally, several pegRNAs appear to never generate editing events [19,22,28,56] as was the case for pegRNA#8 in the present study. Differences between the expression systems could partly explain these variations. Promoters already validated in land plants are generally used for robust expression of both the enzyme coding gene and the guide RNAs. But, promoter strength can vary strongly from one experimental system to another. For example, the *At*U3 promoter was shown recently to greatly outperform *At*U6 promoter, used successfully in different angiosperms, in driving RNA guide expression in poplar [57] and tomato [58]. Therefore, improvements are probably possible, notably in the potato system where we have used a parsley promoter to express the PPE gene and an *At*U6 promoter to express the pegRNA. Nevertheless, the present breath of tested plants is enough to indicate that the PE system as its stands generates modifications less efficiently, even if with more precision, than do other Cas9-based systems.

The present study and most of other laboratories currently rely on the functional units established by Anzalone and collaborators [9]. Improvement in overall enzyme efficiency and particularly in the coordination between the nCas9 and the RT activity associated with optimization of the pegRNA in its PE-RT couple interaction domain could improve *Prime Editing*. Use of species-specific codon optimization (e.g. [19,59]) or of *Streptococcus aureus* Cas9 instead of *S. pyogenes* Cas9 [22] did not improve *Prime Editing* rates in rice and tomato, respectively. However, many other Cas9 and RT genes are available and these could be a means to increase editing frequency. As far as improving pegRNA design for *Prime Editing* is concerned, a careful PBS temperature optimization study showed that the optimum temperature for the PBS in rice is 30 °C [15]), an indication that hybridization temperature more than length is a factor in the design of guide RNAs. Recently, it has been shown that degradation by exonucleases of the 3′ extension of the pegRNA could impede PE efficiency [60]. To circumvent this phenomenon, structured RNA motifs have been incorporated at the 3'end of pegRNAs to prevent RNA degradation, resulting in engineered pegRNAs (epegRNAs) that broadly improve PE efficiency in different cell lines [60]. This strategy constitutes an interesting approach for the development of prime editing in plant species.

In conclusion, our results indicate that the CRISPR-Cas9-mediated *Prime Editing* is achievable in *P. patens* and in the tetraploid potato, albeit with a lower efficiency compared to the CRISPR-Cas9-mediated technique. Nevertheless, the quality of the edits obtained at the target locus and absence of off-target mutagenic events at predicted off-target loci are very positive points even if, clearly, the *Prime Editing* system needs further improvement if it is to be broadly used for basic research and precision crop breeding. This need for improvement is especially true for vegetatively propagated and polyploid species where editing efficiency needs to be high in the first generation, as no selfing can be performed to obtain multiallelic edited plants. In this perspective, use of a model system such as *P. patens,* in which *Prime Editing* innovations can be rapidly deployed and evaluated, represents an efficient way forward to prepare for the transfer of this technology to crop plants.

Author contributions

FN, P-FP, AG-D and FV designed the research; P-FP, AG-D and FV performed the research with the help of MP K and J-LG; P-FP, FN, AG-D and FV wrote the manuscript with contributions from all the authors.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2021.111162.

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