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To cite this version:

Anouchka Guyon-Debast, Alessandro Alboresi, Zoé Terret, Florence Charlot, Floriane Berthier, et al.. A blueprint for gene function analysis through Base Editing in the model plant Physcomitrium (Physcomitrella) patens. New Phytologist, 2021, 230 (3), pp.1258-1272. 10.1111/nph.17171 . hal-03163698v2

HAL Id: hal-03163698

https://hal.science/hal-03163698v2

Submitted on 21 Sep 2022

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Methods

A blueprint for gene function analysis through Base Editing in the model plant *Physcomitrium (Physcomitrella) patens*

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Received: *5 October 2020*
Accepted: *21 December 2020*

New Phytologist (2021) **230:** 1258–1272
doi: 10.1111/nph.17171

Key words: adenine deaminase, APRT, base editing, Cas9, CRISPR, cytosine deaminase, *Physcomitrella patens*, *Physcomitrium patens*.

Summary

- CRISPR-Cas9 has proven to be highly valuable for genome editing in plants, including the model plant *Physcomitrium patens*. However, the fact that most of the editing events produced using the native Cas9 nuclease correspond to small insertions and deletions is a limitation.
- CRISPR-Cas9 base editors enable targeted mutation of single nucleotides in eukaryotic genomes and therefore overcome this limitation. Here, we report two programmable base-editing systems to induce precise cytosine or adenine conversions in *P. patens*.
- Using cytosine or adenine base editors, site-specific single-base mutations can be achieved with an efficiency up to 55%, without off-target mutations. Using the *APT* gene as a reporter of editing, we could show that both base editors can be used in simplex or multiplex, allowing for the production of protein variants with multiple amino-acid changes. Finally, we set up a co-editing selection system, named selecting modification of APRT to report gene targeting (SMART), allowing up to 90% efficiency site-specific base editing in *P. patens*.
- These two base editors will facilitate gene functional analysis in *P. patens*, allowing for site-specific editing of a given base through single sgRNA base editing or for *in planta* evolution of a given gene through the production of randomly mutagenised variants using multiple sgRNA base editing.

Introduction

Modification of the sequence of a protein is a powerful approach to decipher the protein's sequence–function relationship and understand its biological roles. In addition, production of different variants for a given gene and its corresponding protein also has practical applications and is a way to go beyond what evolution has shaped in terms of function for this protein. For a long time, intracellular protein engineering through substitution, insertion or deletion of nucleotides in its corresponding gene, was restricted to unicellular eukaryotes. One possible way to modify a gene *in planta* is the TILLING strategy, based on EMS-induced mutations in the genome (Jacob *et al.*, 2018). However, such *in vivo* mutagenesis is not targeted to a specific locus and, for this reason, needs first a thorough selection system to find the desired mutation in the target gene. In addition, TILLING will only very rarely allow the selection of simultaneous mutations in a given gene. Finally, such a strategy will necessitate the elimination of background mutations that could interfere with the functional analysis of the modified protein.

For a few years now, the CRISPR-Cas9 system has been efficiently applied to induce targeted mutagenesis in plant genes. Guided by an sgRNA, the Cas9 nuclease produces a targeted DNA double-stranded break (DSB) by site-specific cleavage, which typically results in insertion or deletion mutations after error-prone NHEJ DNA repair (Jiang & Doudna, 2017). In many cases these mutations correspond to loss of function due to out-of-frame mutations, premature terminations or aberrant splicing variants. By contrast, gene function analysis and the development of new traits of interest in crops need a more precise mutation system delivering more predictable mutations, including modification of one or more bases in a given gene. Such mutations that facilitate basic research can be achieved through different CRISPR-derived tools, as recently reviewed (Manghwar *et al.*, 2019; Veillet *et al.*, 2020a), but also accelerate plant breeding (Zhang *et al.*, 2019; Gaillochet *et al.*, 2020). While

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modification of one or more bases via CRISPR-mediated gene targeting constitutes an elegant strategy, its efficiency remains low in flowering plants (Huang & Puchta, 2019).

Recently, a strategy named Prime editing, has been developed in human cells (Anzalone *et al.*, 2019) and then adapted to wheat and rice (Lin *et al.*, 2020). Prime editing is based on the fusion of a Cas9 nickase to a reverse transcriptase that, guided by a Prime editing guide RNAs (pegRNAs), enables point mutations, insertions and deletions. This strategy is highly promising but still needs refinement to achieve editing that is efficient enough for routine use (Marzec & Hensel, 2020). As an alternative to gene targeting, and before the development of the promising Prime editing strategy, CRISPR-mediated base editors (BEs) have been developed, to induce targeted base modification, first in human and murine cell lines (Komor *et al.*, 2016) and then in rice (Li *et al.*, 2017; Lu & Zhu, 2017). Like Prime editing, base editing is a gene editing strategy free of donor DNA and double-stranded breaks. Two types of BEs exist, cytosine base editors (CBEs) and adenine base editors (ABEs) that correspond to a Cas9 nickase (nCas9) or a catalytically inactive Cas12a (dCas12a) (Komor *et al.*, 2016; Li *et al.*, 2018a) that is fused to either a cytosine- or an adenine-deaminase domain, respectively. BEs generate targeted nucleotide substitutions on ssDNA in a small editing window that is accessible during the CRISPR-mediated R-loop formation. The action of the cytidine deaminase of the CBE complex on a cytosine can generate transition (C-to-T) and transversion (C-to-A and C-to-G) substitutions, however addition of a uracil DNA glycosylase inhibitor (UGI) to the CBE will permit the production mostly of transition (C-to-T) substitutions (Evanoff & Komor, 2019). ABEs almost exclusively result in A-to-G substitutions (Evanoff & Komor, 2019).

BEs using nCas9 fusions have been used in different model and crop plants including rice, wheat, maize, potato and tomato (Lu & Zhu, 2017; Shimatani *et al.*, 2017; Zong *et al.*, 2017; Li *et al.*, 2018b; Bastet *et al.*, 2019; Veillet *et al.*, 2019a,b). These studies have shown that CBE or ABE activities can vary between plants, but also between target sites in term of editing efficiency of an effective deamination window and of occurrence of byproducts (insertions, deletions or unpredicted substitutions). In the model plant bryophyte *Physcomitrium* (*Physcomitrella*) *patens*, the use of CRISPR-Cas9 or CRISPR-Cas12a strategies has permitted efficient gene knock-out (Nomura *et al.*, 2016; Lopez-Obando *et al.*, 2016; Pu *et al.*, 2019; Mallett *et al.*, 2019) or gene knock-in (Collonnier *et al.*, 2017a; Yi & Goshima, 2020), but no base-editing strategies have been reported so far.

Physcomitrium patens is a well recognised model to study evolutionary developmental biology questions, stem cell reprogramming, and the biology of nonvascular plants (Rensing *et al.*, 2020). In order to expand the toolbox for gene function analysis in *P. patens*, we explored the possibility of editing specific bases of the genome through CBE and ABE in this model plant. For this purpose, we used the *APT* gene that we and other groups had previously used as a reporter of gene modification or modulation in *P. patens* (Trouiller *et al.*, 2006; Holá *et al.*, 2013; Orr *et al.*, 2020). We demonstrate here for the first time that the CRISPR-Cas9 deaminase systems CBE and ABE are very efficient tools for base editing, including multiplex editing, in *P. patens*. We characterised the respective efficiencies and deamination windows for CBE and ABE and demonstrated that they can be useful tools for gene function analysis. In addition, data gained from this study can be translated to drive directed *in planta* evolution of other targets. Finally, we propose here an original co-editing selection strategy, named selecting modification of APRT to report gene targeting (SMART), based on the restoration of the *APT* gene function, for efficient and easy-to-screen base editing of any gene of interest in *P. patens*.

Materials and Methods

Molecular cloning

Guide RNA (sgRNA) sequences specific to the *APT* (Pp3c8_16590), Pp3c3_13220, Pp3c14_9040 and Pp3c17_3870 genes were chosen using the webtool CRISPOR 4.97 (Concordet & Haeussler, 2018). Expression cassettes sgRNA#5, sgRNA#7, sgRNA#21, sgRNA#23, sgRNA#24 and sgRNA#25, comprising the promoter of the *P. patens* U6 snRNA (Collonnier *et al.*, 2017a), the 5′-G-N(19)-3′ guide sequences targeting the *APT* gene and the tracrRNA scaffold were synthesised by Twist Bioscience (San Francisco, California, USA; Supporting Information Table S1). The sgRNA expression cassette sgRNAPp3c14, based on the same backbone but targeting the Pp3c14_9040 gene was synthesised by Twist Bioscience. sgRNA#5 and sgRNA#7 were subcloned into the pDONR207 vector by Gateway™ BP reaction (Invitrogen) to give psgRNA#5 and psgRNA#7. sgRNA#21, sgRNA#23, sgRNA#24, sgRNA#25 and sgRNAPp3c14 were cloned into the pDONR207-neomycin resistance (NeoR) vector using a Gateway™ BP reaction (Invitrogen) to give psgRNA#21, psgRNA#23, psgRNA#24, psgRNA#25 and psgRNAPp3c14. pDONR207-NeoR was obtained by cloning the 35S::neoR fragment (*Sma*I–*Apa*I, 1824 pb) from pBNRF (Schaefer *et al.*, 2010) into pDONR207. The sgRNAPp3c3, sgRNAPp3c17 and sgRNArestor expression cassettes, targeting the Pp3c3_13220 gene, the Pp3c17_3870 gene and a mutated version of the *APT* gene respectively, were synthesised and cloned in the pTwist Amp vector by Twist Bioscience to give psgRNAPp3c3 spgRNAPp3c17and psgRNArestor. psgRNA#2 containing the expression cassette sgRNA#2 has been described previously (Collonnier *et al.*, 2017a). All the expression cassettes used in this study are described in Table S1.

For the CBE system we used a CRISPR-nCas9 cytosine deaminase consisting of a fusion of nCas9 (D10A) to the *Petromyzon marinus* cytosine deaminase (PmCDA1). The pnCas9-CBE1 vector expressing this fusion enzyme is based on the pDicAID_nCas9-PmCDA_*Npt*II_Della vector (Shimatani *et al.*, 2017), from which the sgRNA expression cassette targeting the tomato *DELLA* gene was dropped out (*Aan*I digest). For The ABE system we used a CRISPR-nCas9-adenine deaminase consisting of a heterodimer of a wild-type bacterial tRNA adenosine deaminase (TadA) and a mutated version (TadA*), fused to nCas9 (Gaudelli *et al.*, 2017). For this purpose, using Invitrogen

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Platinum SuperFi DNA polymerase (Thermo Fisher Scientific), we PCR amplified the *ABE7.10-nCas9* gene fusion from pCMV-ABE7.10 (Addgene plasmid #102919) with AttB1 and AttB2 flanking sequences using the ABE7.10-AttB1 and ABE7.10-AttB2 primers. The PCR fragment was cloned into pDONR207 using the Gateway™ BP reaction and then subcloned into pBS-TPp-B (Thévenin *et al.*, 2012) using the Gateway™ LR reaction (Invitrogen) to give the pnCas9-ABE1 plasmid, in which *ABE7.10* is flanked by the rice Actin 1 promoter and CaMVter terminator. The pAct-Cas9 plasmid used in this study has been described previously (Collonnier *et al.*, 2017a). Sequences of the plasmids used in this study are listed in Table S2.

Moss culture and transformation

P. patens wild-type Gransden strain was propagated vegetatively as previously described (Cove *et al.*, 2009). Plants were grown on $PpNH_4$ medium ($PpNO_3$ medium supplemented with 2.7 mM NH_4-tartrate) in growth chambers set at 60% humidity with 16 h of light (quantum irradiance of 80 µmol m^{-2} s^{-1}) at 24°C and 8 h of dark at 22°C. Moss protoplast isolation and transfection were performed as previously described (Schaefer & Zryd, 1997). Protoplasts were transfected with a total of 20 µg of circular DNA divided as follow: 8 µg of the pAct-Cas9, pDIC-AID-APTgRNA#21, pnCas9-CBE1 or pnCas9-ABE1 plasmids and a mix of 12 µg of sgRNA plasmids. Regenerating protoplasts were spread on cellophane disks on $PpNH_4$ medium supplemented with 0.33 M mannitol for 1 wk. Plants on cellophane disks were then selected either on $PpNH_4$ supplemented with 50 mg l^{-1} G418 (Duchefa) to select clones that were transiently transfected (Lopez-Obando *et al.*, 2016) or directly on $PpNH_4$ supplemented with 10 µM 2-FA (Fluorochem) to select clones that were mutated at the *APT* locus (Collonnier *et al.*, 2017a). For the experiment consisting of the restoration of the APRT activity of the *apt* mutant ABEv#1, protoplasts isolated from this mutant were transfected with 10 µg of each of the pnCas9-CBE1 and psgRNArestor plasmids. Regenerating protoplasts were spread on cellophane disks on $PpNH_4$ medium supplemented with 0.33 M mannitol and 1.75 mM adenine (Sigma A8626) for 1 wk and then transferred onto $PpNH_4$ supplemented with 1.75 mM adenine for 3 wk for selection of clones in which APRT function was restored.

PCR and sequence analysis of the edited plants

For PCR analysis, genomic DNA was extracted from 50 mg of fresh tissue as previously described (Lopez-Obando *et al.*, 2016). The quality of the DNA samples was controlled using primers targeting the *P. patens RAD51-1* gene, PpRAD51-1#6 and PpRAD51-1#7. Molecular analysis was based on Sanger sequencing (Genoscreen, Lille, France) of PCR fragments using primers surrounding the targeted locus. For edited plants obtained using a single sgRNA, PpAPT#25 and PpAPT#5 were used for *APT*, Pp3c3#1 and Pp3c3#2 for the Pp3c3_13220 locus, Pp3c14#1 and Pp3c14#2 for the Pp3c14_9040 locus, Pp3c17#1 and Pp3c17#2 for the Pp3c1783870 locus. For edited plants obtained using multiple sgRNAs, molecular analysis was carried out using primers PpAPT#8 and PpAPT#10 primers for the sgRNA#7 locus, primers PpAPT#60 and PpAPT#61 for the sgRNA#5 and primers PpAPT#25 and PpAPT#5 for sgRNA#2 and sgRNA#21 loci. PCR primers used in this study are listed in Table S3.

Structural analysis of mutations in *P. patens* APRT

Nine APRT templates were selected with Modeller 9.18 (Webb & Sali, 2017), based on sequence identity (>20%) from: *Escherichia coli* (PDB:2DY0), *Saccharomyces cerevisiae* (PDB:1G2Q), *Giardia intestinalis* (PDB:1L1Q), *Thermoanaerobacter pseudethanolicus* (PDB:4LZA), *Rhodothermus marinus* (PDB:4M0K), *Yersinia pseudotuberculosis* (PDB:4MB6) and *Homo sapiens* (PDB:4X45). Multiple alignment was used to develop high quality models and the best model was chosen using the discrete optimised protein energy (DOPE) method (Shen & Sali, 2006) and/or the GA341 method (John & Sali, 2003; Melo *et al.*, 2009). Optimisation of the model was achieved using energy minimisation protocols available at YASARA software (Elmar *et al.*, 2010). The visualisation of the multiple alignment with the secondary structure was made using the ESPript3 server (Robert & Gouet, 2014).

In vivo measurements of chlorophyll fluorescence

Here, 10-d-old plants grown on $PpNO_3$ medium were probed for chlorophyll fluorescence with the modular version of the Dual PAM-100 fluorometer (Walz). Plants were dark adapted for 40 min and then induction curve analyses were performed using 850 µmol of photons m^{-2} s^{-1} red actinic light for 8 min followed by 8 min of dark recovery. Chlorophyll fluorescence was measured in plants dark adapted for 30 min that were then exposed to actinic light of 800 µmol of photons m^{-2} s^{-1} for 8 min. Fm and Fm′ are the fluorescence values after exposure to saturating pulses (6000 µmol of photons m^{-2} s^{-1}, duration 600 ms) in, respectively, dark adapted plants and plants exposed to actinic light. Non-photochemical quenching (NPQ) was calculated as Fm/Fm′ − 1 (Klughammer & Schreiber, 1994).

Results

Efficient base editing in *P. patens* via RNA-guided cytosine or adenine deaminases

The APRT enzyme is a member of the Type I phosporibosyltransferase family and is involved in the nucleotide salvage pathway, by which organisms, including plants (Ashihara *et al.*, 2018), convert adenine to adenosine monophosphate (AMP). APRT enzyme, encoded by the *APT* gene, is also able to convert some adenine analogues into toxic compounds and this has been used for efficient selection of APRT-deficient mutants in many organisms (Taylor *et al.*, 1985). For this reason, we hypothesised that base modifications of the *P. patens APT* gene (Fig. 1a) could be easily monitored by selection on 2-FA in order to quickly assess the efficiency of base editing (Fig. S1). To determine whether programmable BEs could catalyse site-specific base editing in the genome of *P. patens*, we used both a CBE based on the cytosine

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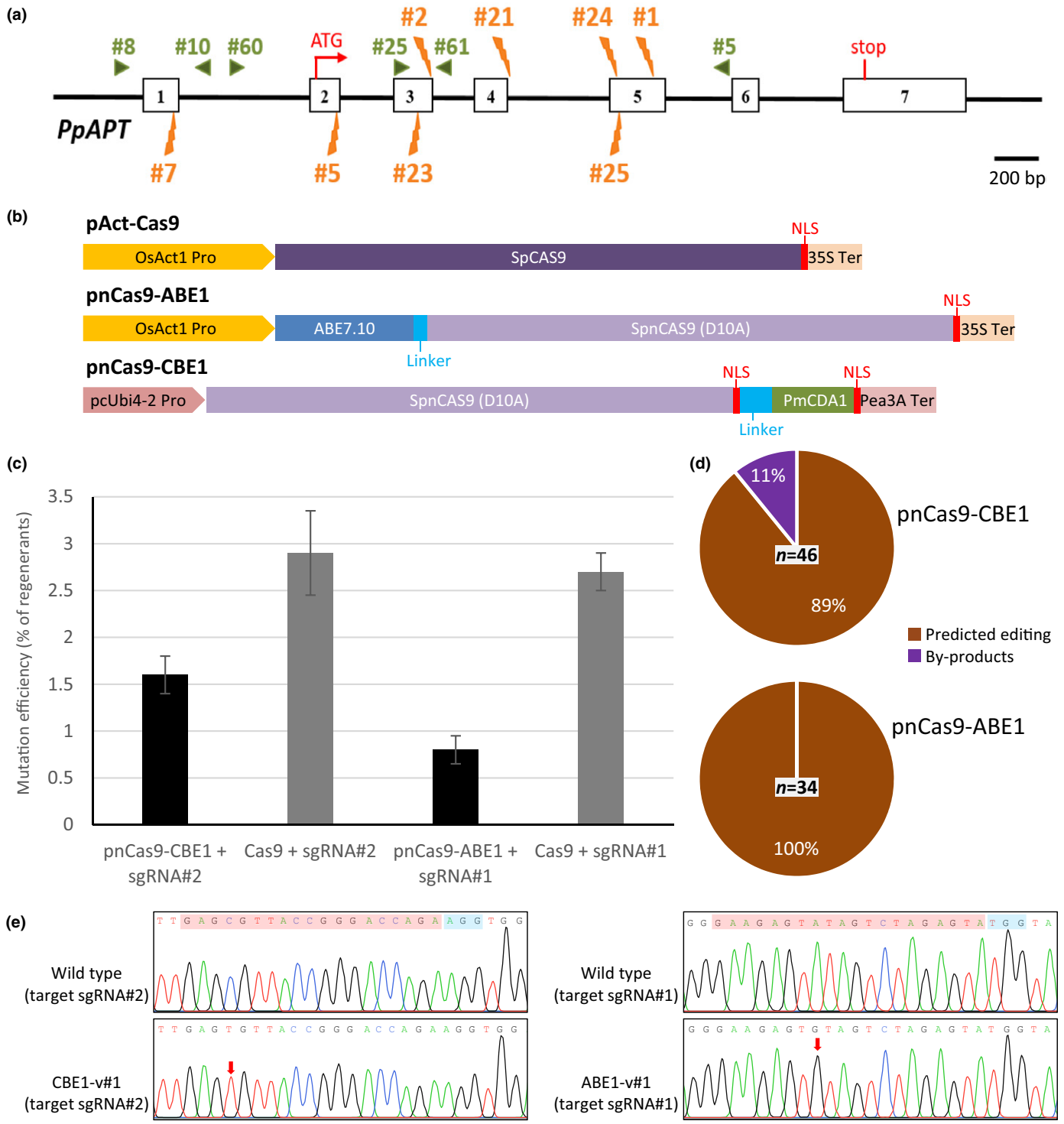

Fig. 1 Relative efficiency of mutagenesis and the nature of editing by CBE and ABE in *Physcomitrium patens*. (a) Structure of the *PpAPT* gene and sgRNAs positions. Boxes in white represent the exons and black lines represent the introns. The eight sgRNAs positions are indicated in orange, at the top for sgRNAs that target forward strand, and at the bottom for sgRNA that target reverse strand. Green arrows represent the primers used for PCR and sequencing. (b) Schematic representation of the native Cas9 and the two base editors. Linker sequences are in light blue, NLS sequences are in red. (c) Mutation rates using pnCas9-CBE1 or pnCas9-ABE1 (in black) vs active Cas9 (in grey). (d) Nature of the base editing was characterised by sequence analysis of 2FA-resistant plants. (e) Sequence chromatograms from wild-type and CBE or ABE edited clones. Target sequence (in red) and PAM (in blue) are highlighted in the wild-type (WT) sequence; red arrows point to the positions with edited base. Primers used for amplification and Sanger sequencing can be found in the Materials and Methods section. Number of analysed plants is indicated.

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deaminase from *P. marinus* (PmCDA1) and an ABE based on tRNA adenosine deaminase from *E. coli* (TadA) (Fig. 1b). We selected eight different sgRNAs targeting the *APT* reporter gene that contained cytosine or adenine residues in the predicted editing windows (Fig. 1a).

First, in order to validate the CBE and ABE constructs, we compared their respective editing efficiencies to that of the native Cas9 (pAct-Cas9). psgRNA#1 was transfected with pnCas9-ABE1 (for ABE) or pAct-Cas9 (for native Cas9) and psgRNA#2 was transfected with pnCas9-CBE1 (for CBE) or pAct-Cas9 (for native Cas9) respectively, in *P. patens* protoplasts. Regenerating protoplasts were transferred onto a medium containing 2-FA in order to detect plants that had been mutated at the *APT* locus. The relative efficiencies of *APT* mutagenesis, estimated by dividing the number of 2-FA-resistant plants by the number of initially regenerating plants, were 0.8% for ABE using sgRNA#1 and 1.6% for CBE using sgRNA#2 (Table S4; Fig. 1c). Using the same two guides, these values were, respectively, 2.7 and 2.9% when mutagenesis was performed with the native Cas9 system (Fig. 1c). Analysis of the type of mutations obtained with the two different BE strategies showed that a majority of mutant plants obtained with the CBE strategy corresponded to precise base-editing events (89%), and the remaining mutants corresponded to short insertions or deletions (hereafter called byproducts) (Fig. 1d,e). For the ABE strategy, 100% of the mutant plants corresponded to precise base editing (solely A-to-G substitution, Fig. 1d,e). This showed that CBE and ABE can be used to precisely modify cytosine or adenine bases in *P. patens*.

Multiplex base editing is efficient in *P. patens*

In order to evaluate the possibility of multiplex editing using the BE systems we co-transfected *P. patens* protoplasts with plasmids psgRNA#2, psgRNA#5, psgRNA#7, psgRNA#21 (Fig. 1) and pnCas9-CBE1 plasmids (for the CBE system), and with plasmids psgRNA#1, psgRNA#21, psgRNA#23 and pnCas9-ABE1 plasmids (for the ABE system) (Fig. 1). As before, regenerating protoplasts were grown on medium containing 2-FA, and plants that survived (*apt* mutants) were analysed. Sequence analysis of the *apt* mutants showed that, under these conditions, the relative efficiencies of mutagenesis (2.8% for CBE and 0.7% for ABE, Table S4) were comparable with those observed with the simplex strategy (1.6% for CBE and 0.8% for ABE, Table S4). For CBE, predictability of base editing was decreased, compared with the simplex strategy, as 47% (89% for the simplex) of the mutations corresponded to precise editing of a cytosine (Figs 2a, S2). Analysis of the mutations showed that the decrease observed was due to byproducts corresponding to small indels (Fig. S2), but also to the occurrence of deletions between the sgRNAs used for multiplexing. As expected, these deletions always involved sgRNAs that were in opposite orientation on the DNA (Figs 1a, S2), as nicking of both DNA strands by a pair of Cas9 nickases is known to lead to site-specific DSBs and NHEJ, and by contrast nicks on the same DNA strand were predominantly repaired through the high-fidelity base excision repair pathway (BER) (Ran *et al.*, 2013). Analysis of the mutations generated by the CBE system at the different targets in a given plant showed that precise multiplex base editing could be achieved, as 40% of the precisely edited plants (C substitutions with no byproducts and no guide-to-guide deletions, $n = 30$) were modified at the four targeted loci (Fig. 2b,c). For ABE multiplexing, the predictability of base editing was comparable with the simplex strategy, as 98% (100% for the simplex) of the mutations corresponded with the precise editing of an adenine (Figs 2a, S2). In addition, 14% of the precisely edited plants (A substitutions with no byproduct) ($n = 43$) showed concomitant mutations at the three targeted loci (Fig. 2b,d). These data showed that, when a cell is subjected to the CBE or ABE systems with multiple sgRNAs, concomitant editing of the targeted loci is possible, making multiplexing a powerful tool in *P. patens*.

Characteristics of BE and nature of the substitutions

First, in order to check that selection of the edited plants on 2-FA did not create a bias in the type of mutations that could be observed using BE, we aimed at analysing plants that were transfected with the CBE system without the *a priori* knowledge that editing would result in an alteration of APRT activity. For this purpose, protoplasts were transfected with the CBE system and sgRNA#2 or sgRNA#21. The regenerating protoplasts were transferred onto a medium containing the antibiotic G418 in order to select clones that expressed the transfected plasmids that contained a NeoR gene cassette (Table S5). We observed that 34.8% for sgRNA2 and 52.3% for sgRNA#21 of the G418 resistant transfected clones were mutated at the *APT* locus (Fig. 3). Of these mutated clones, 32.6% for sgRNA#2 and 20.9% for sgRNA#21 corresponded to precise base-edited plants, the other mutants corresponded to deletions (Fig. 3). Interestingly, a small fraction of the G418-resistant clones (2.2% for sgRNA#2 and 4.7% for sgRNA#21, Fig. 3) corresponded to chimeric clones composed of a mixture of wild-type and mutated cells (Fig. S3). Such chimeric clones were not observed using direct 2-FA selection. This suggests that, even if the BE system is preferentially active very early after protoplast transfection, it can still be active after multiple divisions of the originally transfected cell thanks to episomal replication of the vector (Muren *et al.*, 2009). Even if illegitimate integration of a nonhomologous supercoiled plasmid is low in *P. patens* (Schaefer & Zryd, 1997), episomal persistence of the vector in the clones in the presence of G418 could, in theory, lead to stable integration in the genome. In order to check for the level of unexpected integration of the ABE or CBE vectors in the selected plants, we tested the sensitivity of 288 ABE- or CBE-transfected clones to G418. We could observe that sensitivity to G418 was restored in most of the clones as only one clone out of 480 (0.21%) showed resistance to G418 (Fig. S4). All the edited clones presented in this study were G418 sensitive. Finally, in order to test whether the different mutations observed in the *APT* locus would have an effect on the activity of the APRT enzyme we sequenced the *APT* gene in the 132 G418-resistant (transfected) clones obtained with sgRNA#2 or sgRNA#21 and placed them on a medium containing 2-FA. Under these conditions, 100% of the clones containing a mutation (77 out of the

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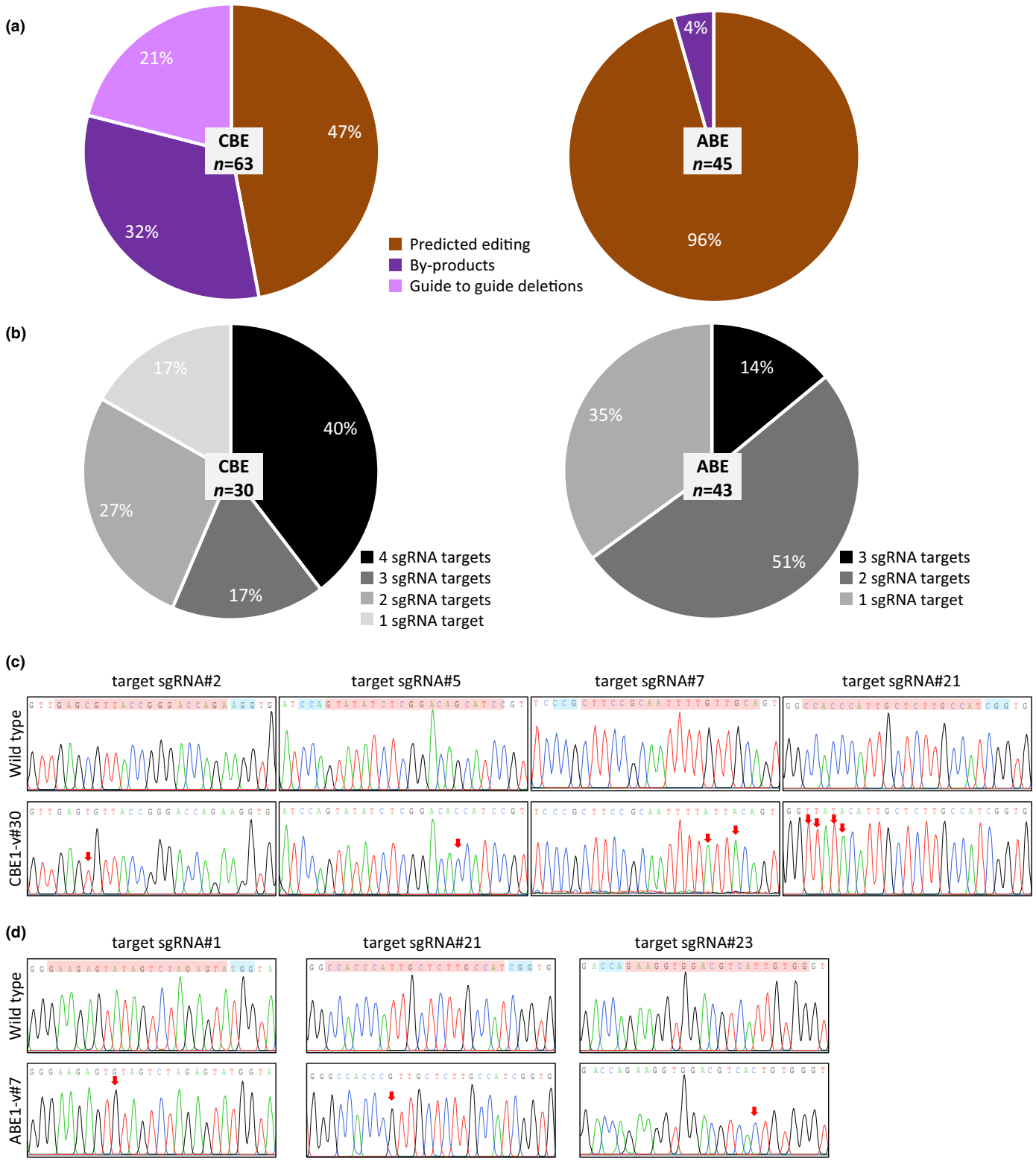

Fig. 2 Predictability and efficiency of multiplex editing by CBE and ABE in *Physcomitrium patens*. (a) Frequencies of predicted base editing, byproducts and guide-to-guide deletions using pnCas9-CBE1 with four sgRNAs or pnCas9-ABE1 with three sgRNAs. (b) Percentage of plants where the *APT* gene has been modified precisely (no byproducts or guide-to-guide deletions) at multiple sgRNA targets concomitantly. Primers used for amplification and Sanger sequencing can be found in the Materials and Methods section. Number of analysed plants is indicated. (c, d) Sequence chromatograms from wild-type and CBE or ABE edited clones. Target sequence (in red) and PAM (in blue) are highlighted in the WT sequence; red arrows point to the positions with edited base.

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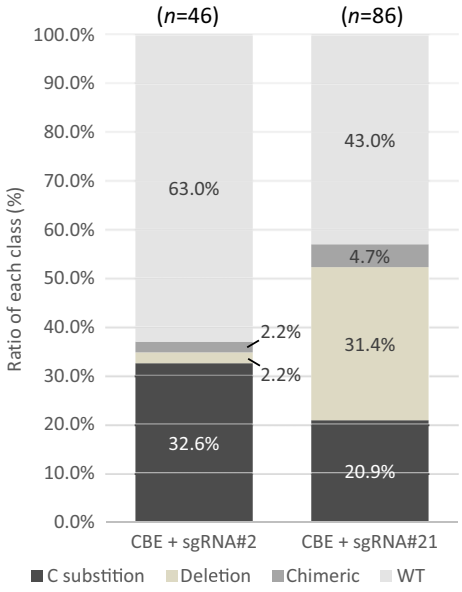

Fig. 3 Efficiency of mutagenesis and nature of editing by CBE in *Physcomitrium patens*. Efficiency of mutagenesis using pnCas9-CBE1 for sgRNA#2 and sgRNA#21 observed among transient transformants (G418 selection). Efficiency and nature of the mutagenesis were assessed by sequencing analysis using PpAPT#25/PpAPT#5 primers on G418-resistant plants. Number of analysed plants is indicated.

132 G418-resistant clones) were resistant to 2-FA (Table S6). This confirmed our hypothesis that the APRT enzyme is very constrained in terms of amino-acid changes and is therefore very sensitive to mutations. Therefore 2-FA screening is very effective in identifying any modification in the *APT* gene.

Based on the fact that a majority, if not all, of the mutations induced by CBE or ABE lead to a nonfunctional APRT, we decided to pool the different events of editing, obtained through selection on G418 or directly on 2-FA, in order to analyse the nature of the CBE or ABE editing products. For CBE, the analysis of the nature of the cytosines modifications ($n = 693$ modified cytosines of 2005 analysed) showed that a majority corresponded to C-to-T substitutions (75.8%), but some C-to-G (18.6%) or C-to-A (5.8%) substitutions were also observed (Fig. 4a). Analysis of the positions of the cytosines that could be substituted in eight different sgRNAs targets showed that cytosines present in the 5-bp editing window (positions −19 to −15 from the PAM) previously described (Nishida *et al.*, 2016), are efficiently edited also in *P. patens*. However, a significant number of C substitutions (17%) could also be observed outside this editing window, including a cytosine in position −21 from the PAM that was outside the 20-bp target sequence of sgRNA (Fig. 4c; Table S7). For target sequences presenting more than one C in the editing window, as is the case for sgRNA#21, at least two Cs and up to five Cs could be modified simultaneously (Fig. S3). In addition, important variation of the efficiency and nature of the substitutions could be observed for a cytosine in a given position from one targeted locus to another (Figs 4b, S5a, S5), confirming the influence of the environment of the cytosine on the efficiency and nature of its modification. Finally, we observed that the existence of multiple Cs in the editing window, as is the case for sgRNA#21, favoured the occurrence of deletion byproducts at the targeted locus, that in this case corresponded to 31.4% of the mutations (Fig. 3a). For ABE, analysis of the nature of the adenine modifications ($n = 156$ modified adenines) showed that, as observed in other organisms, all the modifications corresponded to A-to-G substitutions (Figs S5b, S7). It has been shown recently in animal cells that, in addition to converting adenine to guanine, ABEs could also convert cytosines that are in a narrow editing window (positions −16 to −14) and in a confined 'TCN' nucleic-acid sequence context, into guanine or thymine (Kim *et al.*, 2019). In order to check whether this unpredicted activity was present in *P. patens*, we designed two sgRNAs, sgRNA#24 and sgRNA#25 containing a 'TCN' sequence context with the C present in the narrow editing window (Fig. S7). Analysis of 75 plants that were modified by the ABE editor at the sgRNA#24 or #25 targets showed efficient substitution of the A in the editing window with no co-substitution of the C in the 'TCN' sequence context (Fig. S7), suggesting that the phenomenon observed in animal cells could not be generalised. Concerning the positions of the adenines that could be substituted, analysis in five different sgRNAs targets showed that adenines present in the 4-bp editing window (positions −17 to −14) previously reported (Eid *et al.*, 2018), were efficiently edited also in *P. patens*. Interestingly, exclusive substitution of an A in position −13 could also be observed (sgRNA#1 and sgRNA#24, Figs S5b,S7). Finally, for target sequences presenting more than one A in the editing window, as was the case for sgRNA#23 (Fig. S5b), at least two As could be modified simultaneously.

Predicted potential off-target sites are not affected by base-editing activity in *P. patens*

The sgRNAs used in this study were designed to minimise potential off-target cleavage in the *P. patens* genome (Phytozome 3.1) using the CRISPOR software package (Concordet & Haeussler, 2018). We focused our analysis on two sgRNAs (sgRNA#1 and sgRNA#2) for which no perfect 20-bp matches were found, but potential off-target sequences presenting three to six mismatches were identified: nine for sgRNA#1 and four for sgRNA#2 (Table S8). All these potential off-target loci were amplified with surrounding primers and sequenced in 39 clones transformed with pnCas9-ABE1 and psgRNA#1, and 39 clones transformed with pnCas9-CBE1 and psgRNA#2 that were all previously identified as mutated at the *APT* locus. No mutation could be detected in the potential off-target sequences for any of the tested clones, suggesting that Cas9-dependent predicted off-target activity may be low in *P. patens*.

BE allows the generation of multiple variants of the APRT enzyme

Altogether, use of CBE and ABE strategies with multiple sgRNAs allowed the selection of 38 plants containing different variants of the APRT enzyme and showing combinations of one to four amino-acid modifications (Table S9; Fig. S8). To understand

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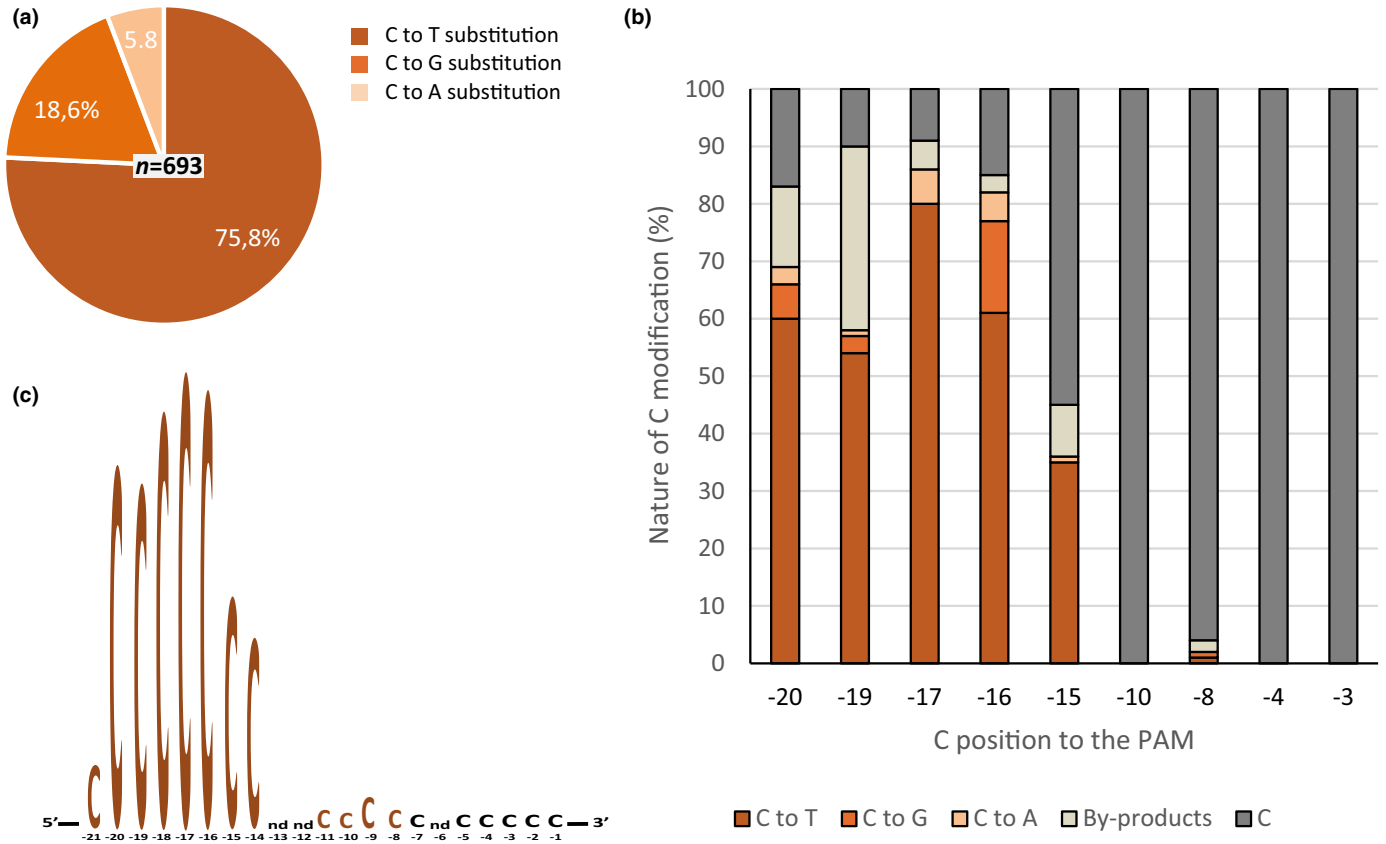

Fig. 4 Type of cytosine substitutions and importance of the position of the cytosine. (a) Nature of cytosine editing using pnCas9-CBE1 with the different sgRNAs used in this study was characterised (693 cytosine substitutions analysed). (b) Nature of cytosine editing using CBE with gRNA#21 for each cytosines present in this 20-bp target (n = 100 *Physcomitrium patens* plants). (c) Frequency of substitution for cytosines at each position of the eight sgRNAs used in this study (2005 cytosines analysed, 693 substituted), only cytosines in positions −13, −12 and −6 were not present (for number of cytosines at each position see Supporting Information Table S7). Primers used for amplification and Sanger sequencing can be found in the Materials and Methods section.

how CBE- and ABE-induced substitutions were associated with APRT loss of function, we focused our analysis on *apt* mutants showing one amino-acid change, and investigated the role of these single mutations on the APRT 3D structure. Because of the high level of identity of the *P. patens* APRT to the APRT proteins from different kingdoms (45% and 42% to *E. coli* and human APRT proteins, respectively) and the availability of several APRT 3D structures, we used homology modelling to build a *P. patens* APRT 3D model. All the sequences were aligned using Modeller 9.18 (Fig. S8). Based on multiple alignments of the APRT sequences, a model was created for the *P. patens* APRT (Fig. 5). The *P. patens* APRT monomer model is a single-domain structure composed of eight α-helices and nine β-strands that can be divided further into the 'hood' (residues 1–39) responsible primarily for base recognition and the definition of substrate specificity, the 'flexible loop' (residues 98–115) and the core (residues 40–184) (Fig. S8) that corresponds to the conserved type I PRTase fold (Phillips *et al.*, 1999; Shi *et al.*, 2001).

The four mutated residues (single mutations) associated with APRT loss of function were reported on the APRT 3D model, where they were all in important structural or functional domains (Figs 5,S9). Arginine 54 (Arg_{54}) at the end of the helix H3, isoleucine 63 (Ile_{63}) in β-strand S3, proline 75 (Pro_{75}) in the helix H4, are all located in the core subdomain, and tyrosine 103 (Tyr_{103}) is at the end of β-strand S5. Arg_{54} is surrounded by residues implicated in intraside-chain polar contact (Tyr_{55}–Glu_{154}–Gln_{58}/Asp_{6}–Tyr_{176}) and the $R_{54}C$ mutation could impair the formation of this interaction, and therefore the correct folding of the protein. It is highly likely that the mutation at position 75 could disturb the helix H4 and lead to abnormal protein folding. Tyr_{103} is highly conserved in the eight APRT enzymes of the different kingdoms, and is thought to be involved in the conformation of the flexible loop that is proposed to close the active site during catalysis (Shi *et al.*, 2002).

Efficient base editing of a gene of interest in *P. patens* using CBE and ABE

We have shown before that *APT*-base-edited plants could be isolated after selection on G418 of CBE-transfected protoplasts. In order to test the portability of this BE strategy based on selection on G418, for genes for which the mutations cannot be positively selected we decided to target three genes located on three different chromosomes in the *P. patens* genome (Fig. S10) and encoding respectively, violaxanthin de-epoxidase (VDE) protein (Pp3c3_13220), a C2H2 zinc finger transcription factor

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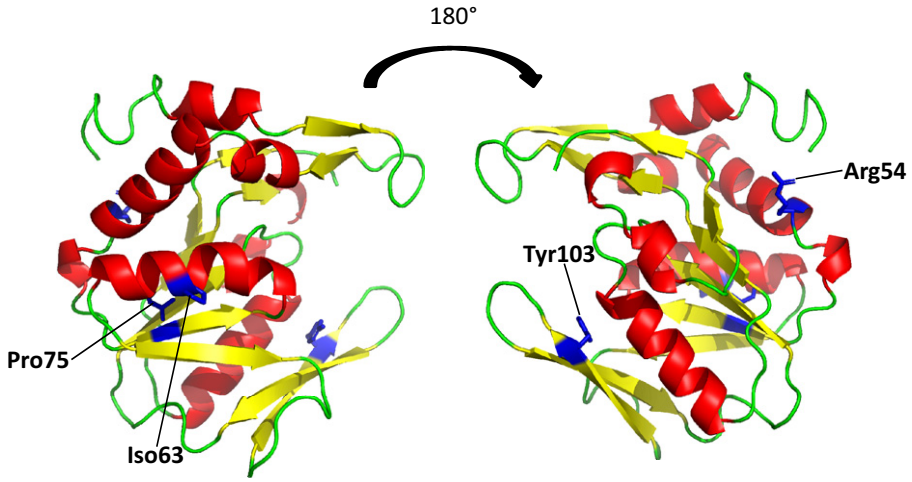

Fig. 5 Single amino-acid modifications by CBE or ABE in the *Physcomitrium patens* APRT. View of the *Physcomitrium patens* APRT 3D model. The APRT protein consists of eight α-helices (in red) and nine β-strands (in yellow) which can be divided further into the hood (residues 1–39), the flexible loop (residues 98–115; β5 and β6) and the core (residues 40–184). Amino acids that could be modified as single substitutions using CBE or ABE are indicated.

(Pp3c14_9040), and the tetratricopeptide-repeat protein 39C (TTC39C) protein (Pp3c17_3870). Protoplasts were transfected with the pnCas9-CBE1 plasmid and either of the sgRNAs expression vectors targeting the three genes. The regenerating protoplasts were transferred onto a medium containing the antibiotic G418 in order to select clones expressing the transfected plasmids. Sequence analysis of the targeted genes showed that 55% for sgRNAPp3c3, 23% for sgRNAPp3c14 and 51% for sgRNAPp3c17 of the transfected (G418 resistant) clones showed a cytosine edited at the targeted locus (Fig. 6a). As observed for the sgRNAs targeting the *APT* gene, the ratio of C-to-T, C-to-A and C-to-G varied from one sgRNA to the other, but with a majority of C-to-T substitutions (Figs 6b, S6). Analysis of the mutations induced at the three different loci showed that editing of the cytosines present in the editing window led to the production of different amino-acid changes and premature stop codons for the three different proteins.

The genomic region targeted in the *P. patens VDE* gene has a key role in regulation of photosynthesis. When plants are exposed to excess light, VDE catalyses the conversion of the carotenoid violaxanthin into another one, zeaxanthin (Baroli *et al.*, 2000). The VDE-dependent zeaxanthin synthesis contributes to the dissipation of excess energy through a mechanism called NPQ. The assessment of NPQ levels, calculated from the measurement of chlorophyll *a* fluorescence, therefore allows inferring VDE activity *in vivo* (Pinnola *et al.*, 2013). The availability of structural data on VDE allows the identification of key residues for its activity (Arnoux *et al.*, 2009) such as amino acids involved in violaxanthin binding that have been shown to be essential for protein activity in Arabidopsis VDE thanks to *in vitro* assays (Saga *et al.*, 2010) (Fig. S11). Base editing of the three cytosines present in this catalytic region led to the production of six variants. Variant *vde#20* contains a premature stop codon leading to a truncated VDE, the other variants are modified on one or two of amino acids D_{177}, W_{178} and Y_{179} (Table S10; Fig. S11). All *vde*-edited plants showed an impaired NPQ response, with a phenotype similar to *vde* KO plants (Fig. 6c,d). These results confirmed on one side the essential *in vivo* role of the targeted amino acids in VDE activity in *P. patens*, and more importantly in this context, provided an *in vivo* demonstration of the specific alteration of protein activity using gene editing of selected amino acids (Fig. 6).

These data showed that selection of transfected clones on G418 can be used to detect base-editing events in the three targeted genes of this study leading to efficiencies ranging from 23% to 55%. However, it must be noted that 12–16% of the edited clones were chimeric (Fig. 6a).

The SMART strategy for efficient base co-editing of a gene of interest in *P. patens*

In order to increase the efficiency of selection for base-edited clones and reduce the risk of chimerism we used the *APT* gene as a reporter of editing efficiency. Because the *apt* mutants show a developmental phenotype (decreased number of gametophores, see Fig. S8b) that could potentially interfere with phenotyping of a mutant in a given gene of interest, we could not use a strategy based on co-editing of the wild-type *APT* gene and the gene of interest. For this reason, we based our strategy on the reversion of an existing *apt* mutation toward the wild-type allele of the *APT* gene. Co-editing of this *apt* mutation and of a gene of interest should permit the selection of individuals mutated in the gene of interest but wild-type for the rest of their genome. For this purpose, we took advantage of the hypersensitivity of the *apt* mutants, compared with the wild-type, to the substrate of APRT, the adenine (Figs S1, S8b). We selected the *apt* mutant ABEv#1 obtained in this study (Fig. S8b) and tried to suppress the sensitivity to adenine in this mutant by reverting the initial mutation. In ABEv#1, the $Y_{103}C$ substitution that confers resistance to 2-FA and hypersensitivity to adenine, can be restored to the wild-type sequence via a $C_{103}Y$ substitution. We designed an sgRNA, sgRNArestor, that through a C-to-T base editing with the CBE

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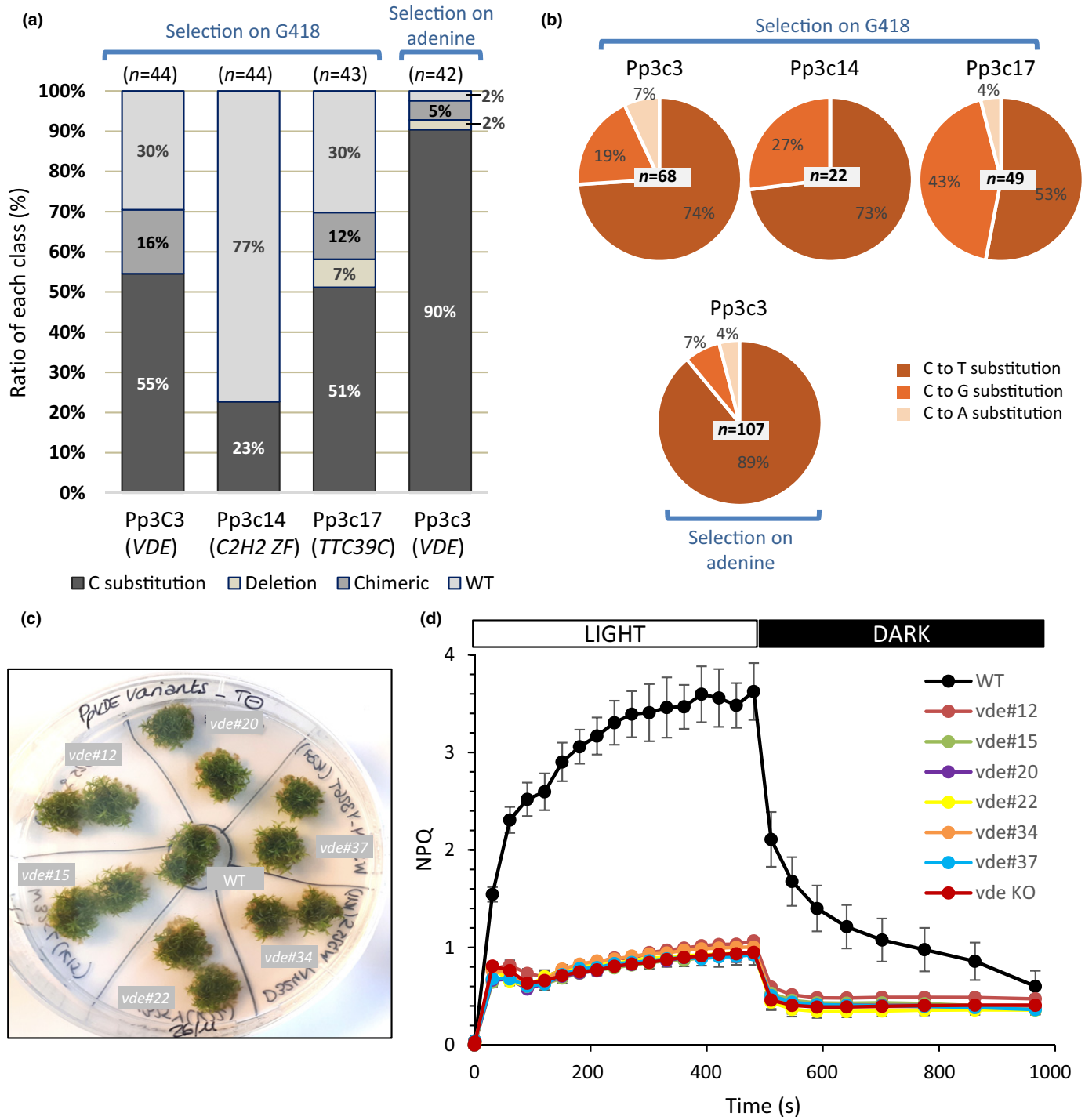

Fig. 6 Efficiency and nature of editing by cytidine deaminase on *Physcomitrium patens* genes of interest and consequences on NPQ activity for the *VDE* (Pp3c3_13220) variants. (a) Efficiency of CBE editing of Pp3c3_13220 (*VDE*), Pp3c14_9040 (*C2H2 ZF*) and Pp3c17_3870 (*TTC39C*) via transfection of wild-type (WT) protoplasts and selection of transient transformants on G418 and, for Pp3c3_13220 via transfection of ABEv#1 protoplasts and co-selection on adenine. Editing efficiency was assessed by sequence analysis performed on G418-resistant plants and also on adenine-resistant plants for Pp3c3_13220. Primers used for amplification and Sanger sequencing can be found in the Materials and Methods section. Number of analysed plants is indicated. (b) Nature of editing for Pp3c3_13220, Pp3c17_3870 and Pp3c14_9040 loci was assessed on 68, 22 and 49 C substitutions for selection on G418, respectively, and 107 for selection on adenine for locus Pp3c3_13220. (c) Phenotype of 20-d-old wild-type and *VDE* variant plants. (d) NPQ induction and relaxation kinetics of the wild-type and *VDE* base-edited variants. The wild-type is compared with clones mutated for the *VDE* gene by cytosine base editing (Supporting Information Table S10). A null mutant of *VDE* (*vde* KO; Pinnola *et al*, 2013) was used as a control for VDE inactivation. Data are mean values of plants grown on three independent plates ± SD.

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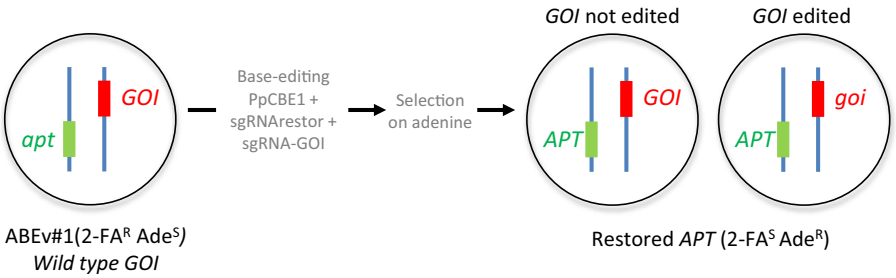

Fig. 7 The SMART strategy for efficient base co-editing of a gene of interest in *Physcomitrium patens*. Principle of selection of base-edited events using adenine selection. The ABEv#1 mutant is co-transfected with the CBE system and two different sgRNAs, sgRNArestor and a sgRNA targeting a gene of interest (GOI). Base editing at the *apt* locus with sgRNArestor can restore APRT function and confer resistance to adenine. Adenine-resistant clones can be co-edited or not at the GOI locus.

system could cause this mutation. This reversion should lead to clones that are sensitive to 2-FA but resistant to adenine (Figs 7, S12).

Based on this, protoplasts from the ABEv#1 mutant were co-transfected with the CBE system and two different sgRNAs, sgRNArestor and sgRNAPp3c3 used previously to target the *VDE* gene. The regenerating protoplasts were transferred onto medium containing adenine in order to select clones of the ABEv#1 mutant that were edited and in which function of the APRT enzyme was restored. Sequence analysis of the *APT* gene in these clones (Table S11) showed that sgRNArestor can efficiently restore a wild-type APRT activity by reverting the $Y_{103}C$ mutation to $C_{103}Y$. This reversion was sufficient to restore a perfect wild-type APRT sequence (38% of the clones), or it could be accompanied by the replacement of the glutamic acid in position 102 by an aspartic acid, a lysine or a glutamine. Interestingly, restoration of a wild-type APRT activity could also be obtained by replacement of the cysteine in position 103 by a phenylalanine ($C_{103}F$) accompanied or not by the replacement of the glutamic acid in position 102 by an aspartic acid. Sequence analysis of the *VDE* gene show that its concomitant editing with the *APT* gene was very efficient (Table S11; Fig. 6a). Using this strategy of selection on adenine for co-editing of the gene of interest and of the *APT* reporter gene, the efficiency of base editing of the *VDE* gene reached 90%, compared with the 55% efficiency observed using selection on G418, and furthermore increased the number of VDE variants. In addition, the number of chimeric clones was decreased more than three-fold (Fig. 6a), making this system of selection, that we named SMART, a very powerful tool for base editing of genes of interest in *P. patens*.

Discussion

A wide range of gene function analysis tools is available for the model plant *P. patens*, such as gene replacement through homologous recombination, gene knock-down through RNA silencing or gene knock-out through CRISPR-Cas (Rensing *et al.*, 2020). Here, we adapted the plant CBE and ABE systems to efficiently and specifically achieve targeted modification of single or multiple cytosines and adenines in *P. patens*. We showed that CBE and ABE strategies are efficient in *P. patens* and deciphered their different characteristics and outputs.

The results presented here show that, in *P. patens*, the PmCDA1-based CBE strategy allows substitution of one or more cytosines in an editing window slightly larger than the one previously described (Nishida *et al.*, 2016) including nucleotides −20 to −14 from the PAM. The majority of the modifications corresponded to C-to-T substitutions but, depending on the sgRNA used, the ratio of edited plants showing indel mutations at the targeted site could reach almost 50%. This is in line with what can be observed in rice, for which the different CBE strategies gave rise to 22% to 71% of plants showing indel mutations (Li *et al.*, 2017; Ren *et al.*, 2018). The observed byproducts occurred in the editing window, and were more frequent when the editing window contained more than one cytosine. These are likely to be due to the unfaithful BER of the uracil through nonhomologous end joining. As previously observed in other plants or animal cells, the TadA-based ABE strategy allows efficient substitution of one or more adenines present in the predicted editing window by guanines with very few byproducts, which were observed only when multiple nearby sgRNAs were used at the same time. These byproducts are close to the predicted cutting site of the nickase, and are probably due to Cas9 nickase activity. For ABE, only very few byproducts could be found with more than 98% of the modifications corresponding to A-to-G substitutions. In animal cells, ABE has been shown to be able to convert cytosines that are in a confined 'TCN' nucleic-acid sequence context into guanine or thymine (Lee *et al.*, 2018; Kim *et al.*, 2019). We could not detect this additional activity in *P. patens*, reinforcing the high level of precision of the ABE strategy in this organism.

Because BE strategies are based on dead or nickase Cas9, and as such do not produce double-stranded breaks, a theoretical major advantage of BE over classical editing via Cas9 is the recovery of a more precise edited product with few or no off-targets. Low levels of gRNA-dependent off-target DNA base-editing activity for CBE or ABE editors have been shown in animals (for a review see Molla & Yang, 2019) and in plants such as rice or tomato (Shimatani *et al.*, 2017; Hua *et al.*, 2018). However, unbiased whole genome analyses (WGA) in rice, oilseed rape and mouse embryos (Jin *et al.*, 2019; Zuo *et al.*, 2019; Cheng *et al.*, 2021), have shown that CBEs derived from the rat APOBEC1 deaminase can induce substantial genome-wide Cas9-independent off-target mutations. Interestingly, this gRNA-independent off-target DNA base-editing activity was not observed for the

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ABE editor in rice (Jin *et al.*, 2019). Such unpredicted off-target activity of CBEs was also demonstrated for alternate cytosine deaminase domains in *E. coli*, and motivated the production of engineered CBEs with low levels of Cas9-independent off-target activity (Doman *et al.*, 2020). We have shown in this study that the PmCDA1-based CBE and TadA-based ABE editors used in this study have a low or null predicted gRNA-dependent off-target activity. Concerning a possible unpredicted off-target activity of these BEs, it must be noticed that no such activity could be detected in rice for the ABE used in our study (Jin *et al.*, 2019). For CBE, if the PmCDA1-based CBE showed only low levels of Cas9-independent deamination in *E. coli* compared with other cytosine deaminases, this activity was significantly higher in mammalian cells (Doman *et al.*, 2020). Only an unbiased WGS analysis would address the possible unpredicted off-target activity of these BEs in *P. patens* and permit a conclusion on the precision of these BEs in this plant.

Using selection on G418 for CBE- or ABE-transfected protoplasts we could demonstrate efficient editing of genes of interest. This allowed us to produce multiple variants of the APRT enzyme. In humans, alterations of APRT activity can lead to kidney stone disease (nephrolithiasis), and more than 40 mutations have been described to date leading to this metabolic defect (Rumsby, 2016). Some of these mutations are present in domains that were affected in our *P. patens* APRT variants. For instance, the common D65V mutation found in British, Icelandic and Spanish patients affected by nephrolithiasis modifies APRT helix H4, as do our variants P75I, P75L and P75R. Mimicking in the *P. patens* the APRT mutations causing human nephrolithiasis could be potentially informative in terms of conservation between kingdoms of APRT functions. Taking into consideration the potential sgRNAs (using an NGG PAM) present in the *APT* gene, 40% of the APRT amino acids could theoretically be modified using a combination of the two ABE and CBE editors used in this study. Recently, we have shown that the SpCas9 variant, SpCas9-NG, is active in *P. patens* for CRISPR-mediated gene knock-out applications (Veillet *et al.*, 2020b). Because this variant recognises NGN PAMs, setting up a BE strategy based on a SpnCas9-NG variant should theoretically permit the modification of 100% of the amino acids encoded by the *APT* gene, reinforcing the usefulness of these ABE and CBE strategies. The great potential of these BE strategies for functional analysis was confirmed for three other genes, including the *VDE* gene. The latter allowed us to demonstrate *in vivo* the essential role of the targeted amino acids in VDE activity in *P. patens*. Nevertheless, analysis of the edited clones obtained after selection on G418 showed that significant numbers of edited clones were chimeric. This should be taken into consideration when *P. patens* protoplasts clones were selected for transient transfection of BE editors using antibiotics. The possibility of the existence of such chimeric clones in other protoplast-based BE editor transfection systems (e.g. potato or tomato) would probably deserve attention.

Finally, by using *APT* as a reporter gene, we propose the SMART approach to efficiently select base editing in target genes. Using the SMART selection system, based on co-editing of a gene of interest and of a mutated version of the *APT*, and selection on the APRT substrate adenine, we could both diminish the proportion of chimeric clones and increase the efficiency of precise cytosine base editing significantly, reaching an efficiency of editing of 90%. This strategy is also possible for ABE starting from an *apt* mutant obtained via the CBE strategy. Furthermore, as multiplex base editing has been reported in dicot and monocot plants (Shimatani *et al.*, 2017, 2018; Hua *et al.*, 2018) and because the APRT function is a very conserved enzymatic function in all kingdoms, the SMART strategy presented here should be applicable to different flowering plants. It would potentially be an additional tool for transgene free editing in crops, already obtained using the *ALS* gene for example (Veillet *et al.*, 2019b), but would present the advantage of not including a selection step on herbicides or antibiotics.

In conclusion, we demonstrated here that CBE and ABE editors can be very useful tools for in-depth gene function analysis in *P. patens*. We provide information on the nature of the edited products, windows of editing, simplex versus multiplex systems and selection strategies, which should facilitate their use in this model plant. BE editors extend the already imposing tool box for precise genome editing in *P. patens*, such as gene replacement through homologous recombination (Schaefer, 2001), that could be made more efficient and precise using a CRISPR-Cas9 strategy (Collonnier *et al.*, 2017b), or via an elegant recent strategy also based on CRISPR-Cas9 but using oligonucleotide templates (Yi & Goshima, 2020). In theory, all these strategies could benefit from the SMART co-editing selection system described here. Finally, in addition to being an easy-to-implement alternative to these base modification strategies, the BE system described here makes possible the *in vivo* random mutagenesis of a given gene, a powerful new tool for gene function analysis in *P. patens* that should reinforce the status of *P. patens* as a powerful platform for functional analysis of plant genes.

Acknowledgements

We are grateful to Akihiko Kondo who provided the Target-AID plasmid. We thank Mark Tepfer and Pierre-François Perroud for their critical revision of the manuscript. The work, including study design, data collection, analysis and interpretation and manuscript writing, was supported by the French National Research Agency (ANR11-BTBR-0001-GENIUS). The IJPB benefits from the support of the LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS). The authors declare no conflict of interest.

Author contributions

FN and AG-D designed the research; AG-D performed the research with the help of AA, ZT, FC, FV, FB, PV-M, JMC, TM and J-LG; FN and AG-D wrote the manuscript with contributions from all the authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Schematic description of the *APT* reporter gene and APRT function.

Fig. S2 Examples of deletions observed during BE multiplexing.

Fig. S3 Examples of multiple cytosines editing or chimerism observed in some clones.

Fig. S4 G418 sensitivity of ABE and CBE single and multiplex edited clones after relaxing of the antibiotic selection pressure.

Fig. S5 Nature of editing using CBE or ABE for each cytosine or adenine present in the target locus.

Fig. S6 Nature of editing using CBE on genes of interest for each cytosine in the target locus.

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Fig. S7 Sequence of two sgRNAs containing cytosines potentially target of ABE activity and nature of ABE editing using these sgRNAs.

Fig. S8 Alignment of APRT sequences from different species and phenotype of the *apt P. patens* mutants.

Fig. S9 View of the *P. patens* APRT 3D model with amino acids (in blue) that could be modified as single substitutions using CBE or ABE.

Fig. S10 Structure of the *Pp3c3_13220*, *Pp3c14_9040* and *Pp3c17_3870* targeted genes.

Fig. S11 Sequence alignment of VDE from Arabidopsis and *Physcomitrella*.

Fig. S12 Use of the *APT* gene as a marker of base-editing efficiency.

Table S1 List of sgRNAs expression cassettes used in this study.

Table S2 Sequences of plasmids used in this study.

Table S3 List of PCR primers used in this study.

Table S4 Mutation rates of the CBE and ABE systems tested (2-FA direct selection).

Table S5 Transfection efficiency of the CBE and ABE systems.

Table S6 Mutation rates of the CBE system after preselection on G418.

Table S7 Frequency of substitution for cytosines at each position of the eight sgRNAs used in this study.

Table S8 Sequences and positions of possible off-target sites for sgRNA1 and sgRNA2.

Table S9 List of amino acids modified in the APT gene using the CBE or ABE strategy.

Table S10 Consequence of CBE editing in the different edited clones for the three genes of interest.

Table S11 Sequence analysis of the APT and Pp3c3_13220 locus in adenine-resistant clones obtained after co-transfection of the ABEv#1 mutant with the CBE system and the two sgRNAs, sgRNArestor and sgRNAPp3c3.

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