Plant Science 296 (2020) 110469

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

# Efficient CELI endonuclease production in *Nicotiana benthamiana* through transient expression and applications in detections of mutation and gene editing events

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ARTICLE INFO

*Keywords:*
Production of CELI endonuclease
Optimization of CELI digestion
Transient expression
Detections of mismatch and gene editing

ABSTRACT

Rapid and low-cost methods of detecting mutations and polymorphisms are crucial for genotyping applications including mutagenesis and gene editing. S1 family endonucleases such as T7E1, EndoV and CELI can potentially be used in enzymatic mismatch detection. Among them, CELI has been shown to be effective in detecting mutations in Targeting Induced Local Lesions IN Genomes (TILLING). However, current method of CELI purification from celery is laborious, and challenging for many non-biochemical laboratories, and the presence of post-translational modifications hinders efficient production of the enzyme in *E. coli*. Here, we report an efficient system for bulk production of enzymatically active CELI endonuclease through transient expression in a model plant *Nicotiana benthamiana*. We also optimized the reaction buffer, by additions of $Mn^{2+}$ and DTT, with enhanced mismatch cleavage activity. Using the new CELI production and reaction system, we were able to routinely detect mismatches in 1/32 mixed mutant and wildtype DNA samples. We believe the newly established system has many applications in characterization of mutations occurred in natural variations, mutagenized populations and gene editing.

## 1. Introduction

Modern genetic and genomic studies rely on the presence of natural variations or introduced mutations such as mutagenesis and CRISPR/Cas9-based gene editing in genomes. Establishment of efficient tools to detect such variations and mutations has received general attention [1,2]. In the past 15 years, researchers have developed different methods such as next-generation sequencing, microarrays, and enzyme-based detections [3–5]. Enzyme-based methods are relatively low in costs, and rely on single strand-specific endonucleases that recognize and cleave mismatch sites in heteroduplex DNA templates [5]. Single strand-specific S1 endonucleases such as CELI, EndoI, T7E1, and EndoV can potentially be used for such applications [6–11]. To date, only T7E1 and CELI endonucleases have been showed to be efficient and cost-effective for mismatch detections in animals and plants [2,12–16].

Although CELI endonuclease identified from celery (*Apium graveolens*) is effective in cleaving mismatches in neutral pH [17], the enzyme has to be extracted directly from celery and the extraction efficiency is extremely low (0.3 mg from 7 kg celery stalk) [17], which is challenging for many non-biochemical laboratories. *Nicotiana benthamiana* is a model plant widely used for studies in pathogen-plant interactions for many years [18]. Recently, agroinfiltration-based transient expressions using *N. benthamiana* have been proven to be effective for producing antibodies [18] and recombinant proteins [19], and for analyzing subcellular localizations and protein-protein interactions [20].

In this study, we report the expression and production of CELI endonuclease in *N. benthamiana* using the transient expression system. The CELI purified from *N. benthamiana* showed similar, even enhanced mismatch cleavage activity when compared with native CELI purified directly from celery. We also optimized the condition of CELI reaction, and showed that a combination of additional $Mn^{2+}$ and DTT in the reaction buffer significantly enhanced CELI cleavage activity and stability, leading the development of a cost-effective, highly sensitive

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https://doi.org/10.1016/j.plantsci.2020.110469
Received 24 December 2019; Received in revised form 12 March 2020; Accepted 14 March 2020
Available online 18 March 2020
0168-9452/ 

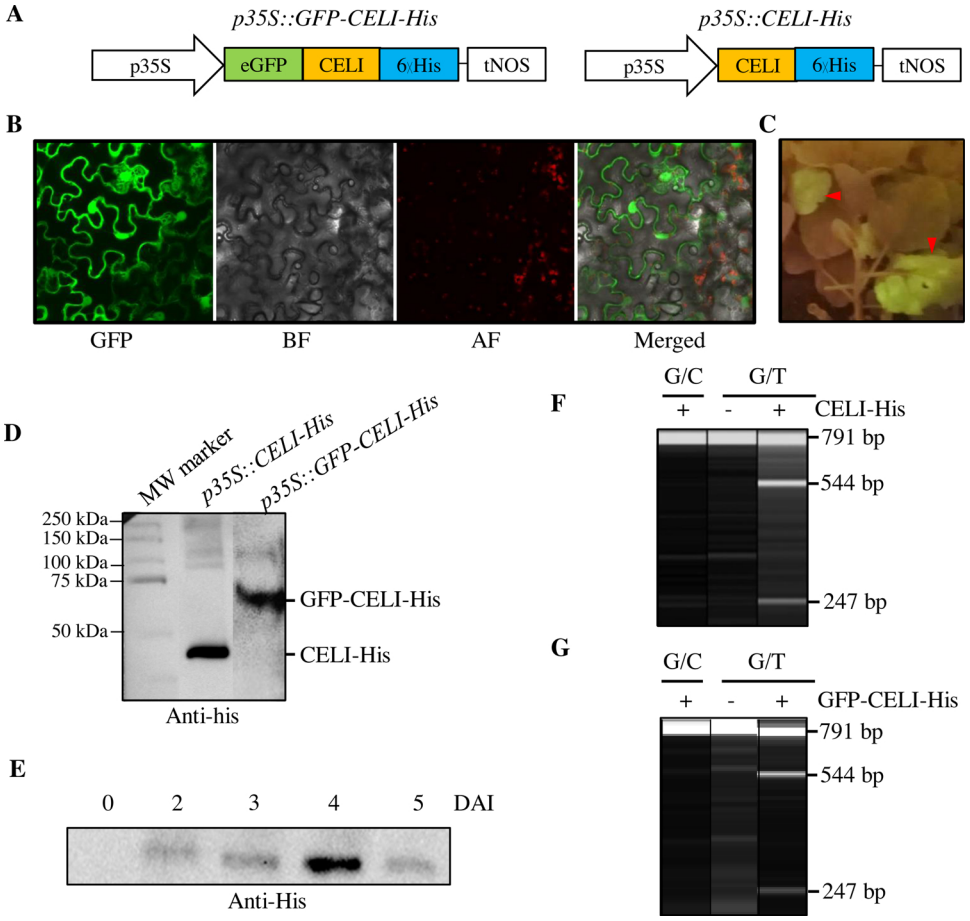


**Fig. 1. Purification of GFP-CELI-His and CELI-His. (A)** Schematic representation of the *p35S::GFP-CELI-His* (left) and *p35S::CELI-His* (right) constructs in binary vector *pCAMBIA2300*, to express *GFP-CELI-His* and *CELI-His*, respectively, driven by *CaMV 35S* promoter. **(B)** Confocal examination of *N. benthamiana* leaf at 4 DAI with *A. tumefaciens* EHA105 carrying *p35S::GFP-CELI-His*. From left to right, GFP siganl was collected with emission at 505–525 nm; bright field (BF), auto-fluorescence (AF), and merged images. **(C)** GFP signal (arrowheads) detected in *N. benthamiana* plants infiltrated with *p35S::GFP-CELI-His* at 4 DAI using a portable fluorescence lamp. **(D)** A western blot to show expressions of recombinant GFP-CELI-His and CELI-His fusion proteins at 4 DAI using anti-His antibody. **(E)** A western blot to show the expression of CELI-His, detected with anti-His antibody, in *N. benthamiana* leaves at different DAI. Note the most abundant CELI-His production was observed at 4 DAI. **(F)** and **(G)** Mismatch cleavage activities analyzed using CELI-His (**F**) and GFP-CELI-His (**G**), and PCR products of the *CHS* gene with mismatch (G/T) or without mismatch (G/C) were used as substrates. + and – indicate the presence and absence of endonucleases (CELI-His or GFP-CELI-His). Sizes of DNA fragments are indicated at the right side.

system for mutation detections.

## 2. Materials and methods

### 2.1. Construction of expression plasmids

The *CELI-His* coding sequence was amplified from *pAC::CELI-6×His* (kindly provided by Dr. A. Yeung from Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA) using primers with *Bam*HI and *Xba*I restriction sites (Table S1), and cloned into *Bam*HI and *Xba*I double digested *pCAMBIA2300-35S::eGFP(N)* and *pCAMBIA2300-35S::eGFP(C)* vectors, to produce *p35S::GFP-CELI-His* (GFP fused to the N-terminal of CELI-His, expressed under the control of *CaMV 35S* promoter) and *p35S::CELI-His* (CELI-His, expressed under the control of *CaMV 35S* promoter), respectively (Fig. 1A). These binary plasmids were extracted and transferred to *Agrobacterium tumefaciens* strain EHA105 by electroporation, and grown on LB plate with kanamycin (Kan, 50 μg/mL) and rifampicin (Rif, 25 μg/mL). Constructs in EHA105 were verified by sequencing, and stored in 25 % glycerol (v/v) at -80 °C.

### 2.2. N. benthamiana growth condition and transformation

Since CELI expressed in various prokaryotic hosts did not have activity in cleaving DNA mismatches [8], we employed the *N. benthamiana* transient expression system. Seeds of *N. benthamiana* were sowed individually in 5 cm by 5 cm by 10 cm square pots, and grown in 21 ± 2 °C growth room under for 14–21 days (16 h light/8 h dark), which can be used for transient expression [21]. EHA105 carrying *p35S::GFP-CELI-His* or *p35S::CELI-His* were first plated on a LB solid medium containing Kan and Rif, and cultured at 28 °C for 2 days before single colonies were picked and inoculated in 5 mL LB liquid medium supplemented with the same antibiotics, and grown at 28 °C on a shaker for 48 h. Afterwards, 1 mL culture was taken and diluted with the same LB medium to 200 mL, grew at 28 °C for another 2 days, collected and centrifuged at 5,000 g for 10 min. The pellet was then re-suspended in a solution with 10 mM 2-(N-morpholine)-ethanesulfonic acid (MES, pH 5.6), 100 μM acetosyringone and 10 mM $MgCl_2$, and adjusted to $OD_{595}$ = 0.8. Infiltration was conducted by slowly injecting the bacterial suspension to the lower surface of fully expanded leaves using 1 mL disposable syringe without needle, until the whole leaf showed water-soaked appearance. These infiltrated plants were kept under dark for 24 h, and then moved to the normal growth condition, and grew for 1–5 days.

### 2.3. Detections of GFP-CELI-His and CELI-His fusion proteins

To examine the expression of the GFP-CELI-His, infiltrated leaves at 4 days after infiltration (DAI) were excised and examined under a confocal laser scanning microscope (FV1000MPE, Olympus, Japan) using 488 nm excitation laser. GFP signal (emission at 505–525 nm), auto-fluorescence (emission at 655–755 nm) and bright-field images were captured. The GFP signal was also detected using a portable fluorescence lamp (LUYOR-3415, LUYOR, USA). For western blotting, 2 mg infiltrated *N. benthamiana* leaves, collected at 1–5 DAI, were ground to fine powder in liquid nitrogen, and recovered to 4 °C on ice. One mL extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA, 300 mM NaCl and 10 mM β-mercaptoethanol, pH 8.0) was added to the powder, vortexed, and centrifuged at 10,000 *g* at 4 °C for 5 min to collect the supernatant. Fifty μL supernatant was mixed with loading buffer containing 100 mM dithiothreitol (DTT), boiled for 3 min before loading onto SDS-PAGE with 12 % acrylamide, and then transferred to a polyvinylidene difluoride membrane (IPVH00010, Millipore, USA).

Anti-His antibody (D291-3, MBL, Japan) was used for the detection under the Chemiluminescent Imaging System (5200, Tanon, China).

### 2.4. CELI purification

Purification of native CELI from celery was performed as described [12,22]. For extractions of recombinant CELI-His or GFP-CELI-His proteins expressed in *N. benthamiana*, the protocol of Sparkes et al. [21] was followed with some modifications, and the whole procedure was performed at 4 °C, and all solutions were pre-cooled. In brief, 40 infiltrated *N. benthamiana* leaves (about 20 g) were harvested at 4 DAI, and homogenized in 100 mL extracted buffer containing 50 mM Tris−HCl, 0.2 M KCl, 0.1 % Triton X-100, 100 μM PMSF and 0.2 % Nonidet P-40 (pH 7.7), and centrifuged with 10,000 *g* for 20 min. The supernatant was then brought to 25 % $(NH_4)_2SO_4$ by adding $(NH_4)_2SO_4$ powder, mixed gently for 30 min at 4 °C. The supernatants were centrifuged at 16,000 g for 40 min before brought to 80 % $(NH_4)_2SO_4$ by adding $(NH_4)_2SO_4$ powder, and mixed gently for 30 min. The solution was centrifuged at 16,000 g for 1.5 h, and afterwards the supernatant was discarded carefully. The pellet was resuspended thoroughly in 20 mL binding buffer (50 mM Tris−HCl, 0.2 M KCl, 10 mM imidazole, 100 μM PMSF, pH 7.7) by inversions. The Econo-Pac chromatography column (7321010, BioRad, USA) with 5 mL high affinity Ni-charged IMAC resin (1560135, BioRad, USA) was first equilibrated with 50 mL binding buffer, and then the extraction solution was added to the column, to flow with gravity, and then eluted with 30 mL elution buffer containing 50 mM Tris−HCl, 0.2 M KCl, 100 μM PMSF and 100 mM imidazole, pH 7.7, and the elutes were collected with UV detection of 280 nm by Nanodrop 1000 (Thermo Fisher Scientific, USA). Collected enzyme sample about 20 mL was transferred to an ultra-filter tube (10 kDa Amicon, Millipore, USA), and centrifuged for about 15 min at 6,000 g until 300 μL solution remained. Activities of extracted recombinant CELI-His or GFP-CELI-His endonucleases were measured, and then diluted to 5 units/μL using the elution buffer with glycerol at the final concentration of 30 % (v/v), aliquoted and stored at -80 °C.

### 2.5. PCR amplification and heteroduplex formation

DNA templates for heteroduplex formation include: 1) the wildtype and a chalcone synthase (*CHS*) mutant of tobacco (*N. tabacum* L, Zhongyan 100 variety), with an G/T mismatch in a 791 bp amplicon; 2) the wildtype and a mutant of the *Os04g0650600* gene of rice (*Oryza sativa*, Zhonghua 11 variety), with an A/C mismatch in a 1,134 bp amplicon; 3) the wildtype and a mutant of the *Os03g0167600* gene of rice (*Oryza sativa*, Zhonghua 11 variety), with an T/G mismatch in a 800 bp amplicon; 4) the wildtype and a mutant of the *Os06g0341300* gene of rice (*Oryza sativa*, Zhonghua 11 variety), with an A/C mismatch in a 609 bp amplicon. DNAs from mutants were mixed with the wildtype DNA in different ratios (0:1; 2:1; 1:1; 1:4; 1:8; 1:16, 1:32 and 1:64) before amplified by PCR using primers listed in Table S1. PCR was performed in a 10 μL reaction containing 2 mM $MgSO_4$, 1 mM of each dNTP, 1 unit KOD FX DNA polymerase (KFX-101, TOYOBO, Japan), 10 ng genomic DNA and 400 nM primers as described [22], and the PCR program was set as: 2 min at 98 °C, followed by 40 cycles of 98 °C for 10 s, 60 °C for 30 s and 68 °C for 60 s, and then 68 °C for 10 min. Heteroduplexes were then formed by heating to 99 °C for 10 min, and 70 °C to 50 °C with a cooling rate of 0.3 °C/min, 20 s per cycle for 70 cycles, then held at 4 °C [12].

### 2.6. Endonuclease cleavage and optimizations

Cleavages with commercial endonucleases including T7E1 (M0302L, NEB, USA) and EndoV (EN0141, Thermo Fisher Scientific, USA) and CELII (706025, Transgenomics, USA) were performed with buffers provided by manufactories. Optimization of the mismatch cleavage was performed at 45 °C for 20 min in a 6 μL basic reaction buffer containing 0.2 μg/ml BSA, 0.002 % Triton X-100 (v/v), 10 mM KCl, 10 mM M HEPES, pH 7.5, 1 unit of endonucleases and 200 ng heteroduplexed PCR products, and optional 5 mM $CaCl_2$, 80 μM $MnCl_2$, 10 mM $MgCl_2$, 40 μM DTT, or combinations of these components. The optimal MD buffer established in this study contains 0.2 μg/mL BSA, 0.002 % Triton X-100 (v/v), 10 mM KCl, 10 mM HEPES, 80 μM $MnCl_2$, and 40 μM DTT, pH 7.5. The cleavages were terminated by adding 2 μL 0.225 M EDTA. Potential DNA degradations in CELI endonuclease were examined in the basic buffer with 5 mM $CaCl_2$, 80 μM $MnCl_2$, or 40 μM DTT, and 200 ng PCR products without mismatch, incubated at 45 °C for 0–50 min.

### 2.7. Detections of mutations generated by CRISPR/Cas9-based gene editing

Transgenic rice (in Nipponbare background) carrying a CRISPR/Cas9 construct with a PAM site targeted to *Os06g0341300* were obtained from the lab, and genomic DNAs were extracted from each T0 plants, diluted and normalized to 10 ng/μL, mixed with (+WT) or without equal amount of wildtype DNA (-WT), before PCR amplification and mismatch detection were performed as described above.

### 2.8. Capillary electrophoresis

Capillary electrophoresis was performed at 9 kV for 30 s for a prerun, followed by sample injection and 40 min for sample separation in an AdvanCE FS96 capillary electrophoresis system (Advanced Analytical Technologies, USA) as described [22], and analyzed using the PROSize 2.0 software (Advanced Analytical Technologies, USA). Relative concentrations of each DNA fragments were quantified and determined from signal intensities and peak areas in comparison with molecular weight markers.

## 3. Results

### 3.1. Transient expressions of recombinant CELI endonucleases in N. Benthamiana leaves

To produce enzymatically active CELI endonuclease, two binary plasmids were constructed, one with CELI fused with a C-terminal 6×His tag (*p35S::CELI-His*), and another one with a N-terminal GFP and a C-terminal 6×His tag (*p35S::GFP-CELI-His*), expressed under the control of a *CaMV 35S* promoter (Fig. 1A and S1). *Agrobacterium* strains EHA105 carrying these plasmids were used to infiltrate well-developed *N. benthamiana* leaves as reported [21], and expressions of GFP-CELI-His and CELI-His fusion proteins (Fig. S2) were monitored at different DAI. Confocal examination of excised leaves infiltrated with *Agrobacterium* strain carrying *p35S::GFP-CELI-His* showed that the GFP-CELI-His produced was localized in the cytoplasm (Fig. 1B). Expression of the GFP-CELI-His in *N. benthamiana* leaves infiltrated with *p35S::GFP-CELI-His* could also be visualized non-invasively using a portable fluorescence lamp, and the maximum GFP signal was observed at 4 DAI (indicated by arrowheads, Fig. 1C). As a negative control, no GFP signal was observed when *p35S::CELI-His* was used (data not shown). Expressions of GFP-CELI-His and CELI-His in leaves infiltrated with *p35S::GFP-CELI-His* and *p35S::CELI-His*, respectively, were detected by western blot using anti-His antibody (Fig. 1D). Western blot analysis confirmed that the maximal CELI-His production was produced at 4 DAI, followed by a decline on 5 DAI (Fig. 1E), suggesting that the optimal time for extracting CELI fusion proteins is at 4 DAI.

### 3.2. Bulk productions and purifications of enzymatically active GFP-CELI-His and CELI-His fusion proteins

For bulk extractions of CELI fusion proteins, 20 g *N. benthamiana* leaves infiltrated with *p35S::GFP-CELI-His* or *p35S::CELI-His* were collected at 4 DAI, and GFP-CELI-His and CELI-His fusion proteins were

**Table 1**
Efficiencies of purifications of native CELI from celery and recombinant GFP-CELI-His from infiltrated *N. benthamiana* leaves.

| Species for CELI purifications | Starting materials used | Resin used for purification | Units of CELI purified | Units of CELI per g materials |
|---|---|---|---|---|
| Celery | 20 g | ConA-4B | 416 | 20.8 |
| Infiltrated *N. benthamiana* | 20 g | High affinity Ni-charged | 1000 | 50 |

purified using a Ni-column. In both cases, approximately 1,000 units of CELI was obtained, which is about 2.5 times more efficient than extraction from an equal amount of celery (Table 1). To determine whether the purified GFP-CELI-His and CELI-His fusion proteins had mismatch cleavage activity, a 791 bp *CHS* DNA fragment amplified from the wildtype and a mutant (A to G substitution) of *N.tabacum*var. Zhongyan 100, with a G/T mismatch, was used as the DNA substrate for cleavages. The heteroduplexed PCR product was digested with GFP-CELI-His or CELI-His, and analyzed by 96-well capillary electrophoresis. Results showed that GFP-CELI-His and CELI-His fusion proteins purified from *N. benthamiana* exhibited cleavage activities when samples with a mismatch (G/T) was used (Fig. 1F and G), producing two smaller fragments with 544 bp and 247 bp in lengths, while samples without adding the enzyme (-) or fragments without mismatch (G/C) did not show the specific cleavage (Fig. 1F and G), suggesting that both the GFP-CELI-His and CELI-His fusion proteins are functional endonucleases.

### 3.3. Optimization of the CELI reaction buffer for mismatch cleavages

We next optimized the reaction condition for endonucleases. The current reaction buffer used widely for CELI endonuclease contains 10 mM $Mg^{2+}$. However, results performed in this study showed that the addition of 10 mM $Mg^{2+}$ had no evident beneficial effect on mismatch cleavage, no matter T7E1, EndoV, native CELI from celery, commercial CELII, GFP-CELI-His or CELI-His were tested (Fig. 2A). In some cases, the presence of $Mg^{2+}$ compromised the mismatch cleavage activity (Fig. 2A), suggesting that additional $Mg^{2+}$ is not necessary for these enzymes. We also observed that T7E1 and EndoV endonucleases exhibited lower activities in mismatch cleavage, and higher in non-specific cleavages, producing more background bands when compared with CELI-related enzymes (Fig. 2A). This is consistent with the results reported [9].

The effects of $Ca^{2+}$ (5 mM $CaCl_2$), $Mn^{2+}$ (80 μM $MnCl_2$), and DTT (40 μM) on mismatch cleavages were also analyzed for different enzymes (Fig. 2A), and results showed that additions of either $Ca^{2+}$ or $Mn^{2+}$ enhanced the activities of CELI, CELII, and GFP-CELI-His and CELI-His, but not for those of T7E1 and EndoV (Fig. 2A). We noticed that in the presence of $Mn^{2+}$, compared with CELI-realted enzymes the disappearance of the full-length PCR product was faster when T7E1 and EndoV were used (Fig. 2A), suggesting that $Mn^{2+}$ somehow enhanced the non-specific degradation activities of these enzymes. And for this regard, CELI-related enzymes behave very differently from EndoV and T7E1. The addition of DTT did not improve, but partially compromised, the activities of these enzymes (Fig. 2A).

We then investigated whether combinations of these components further enhance the activities of CELI-His and GFP-CELI-His, and results showed that combinations of $Ca^{2+}$ and $Mn^{2+}$, $Ca^{2+}$ and DTT, or $Mn^{2+}$

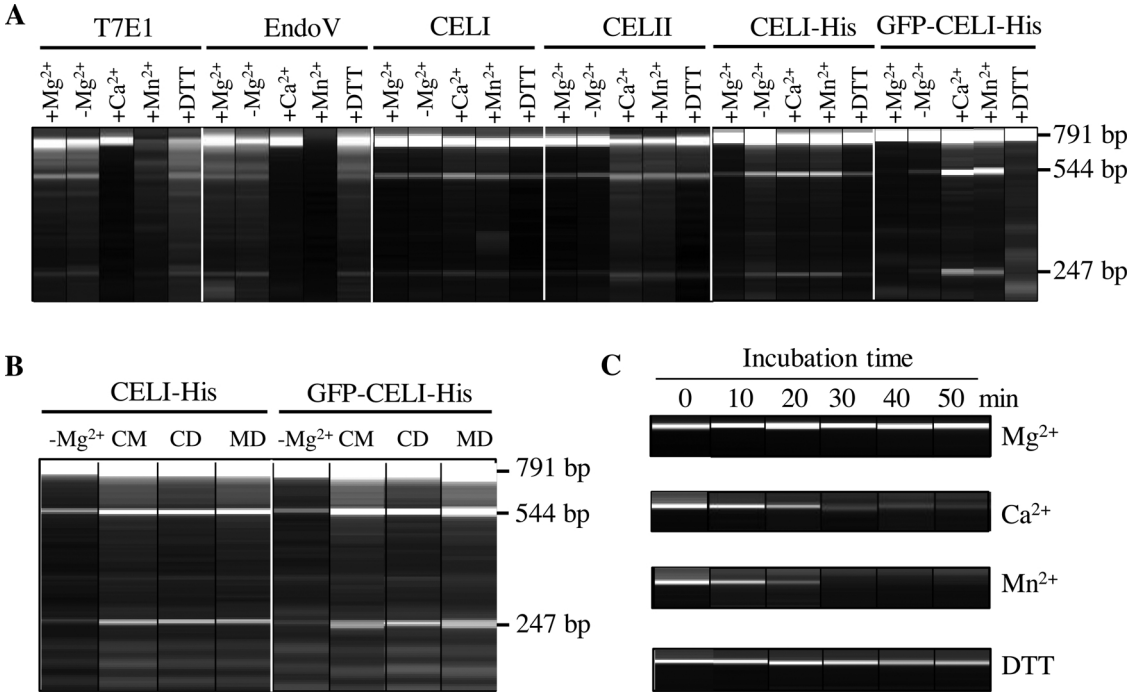


**Fig. 2. Optimization of the reaction buffer used for endonucleases in mismatch cleavages.** (**A**) Effects of 10 mM $MgCl_2$ ($Mg^{2+}$), 5 mM $CaCl_2$ ($Ca^{2+}$), 80 μM $MnCl_2$ ($Mn^{2+}$) or 40 μM DTT on commercial T7E1, EndoV and CELII, native CELI extracted from celery, and recombinant CELI-His and GFP-CELI-His endonucleases purified in this study in mismatch cleavages. Basic buffer without $Mg^{2+}$ (-$Mg^{2+}$) was used for all reactions, plus the additional components. Note that for CELI, CELII, CELI-His and GFP-CELI-His, optimal mismatch cleavage was observed when $Mn^{2+}$ or $Ca^{2+}$ were added, while additional $Mg^{2+}$ or DTT had negative effects. (**B**) Effects of combinations of $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$ and DTT on recombinant CELI-His and GFP-CELI-His endonucleases in mismatch cleavage; CM, with additional $Ca^{2+}$ and $Mg^{2+}$; CD, with additional $Ca^{2+}$ and DTT; MD, with additional $Mn^{2+}$ and DTT. Note that the combination of $Mn^{2+}$ and DTT together gave the optimal results for both enzymes. (**C**) Effects of $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$ or DTT on degradation of a 791 bp PCR product of the *CHS* gene from a wildtype plant (without mismatch) were examined by incubation at 45 ℃ with GFP-CELI-His, and the reaction time was set from 0–50 min. Size of DNA fragments is indicated at the right side of the panels.

and DTT improved mismatch cleavage activities of GFP-CELI-His and CELI-His fusion proteins, and prevented non-specific DNA degradation, as seen from the presence of higher amount of the 791 bp full-length DNA remained (Fig. 2B).

We next addressed why a combination of $Mn^{2+}$ and DTT improved the sensitivity of mismatch cleavage by looking into potential DNA degradations. GFP-CELI-His was incubated at 45 °C with PCR products without mismatch for different times, and results showed, in the presence of DTT or $Mg^{2+}$, the full-length DNA band was stable for more than 45 min, while in buffers with only $Ca^{2+}$ or $Mn^{2+}$, the PCR product was degraded after incubation for 20 min or longer (Fig. 2C). Although $Mg^{2+}$ also inhibited the degradation of the PCR product efficiently (Fig. 2C), it reduced the activity of endonucleases as well (as showed in Fig. 2A). Thus, the basic buffer with additional $Mn^{2+}$ and DTT is optimal for CELI, and we named it as 'MD buffer', used for all following experiments.

To define the optimal time needed for completely digesting the heteroduplexd DNA in a sample, we used 50 ng denatured and re-annealed *CHS* DNA amplified from 1:1 mixed wildtype and homozygous mutant DNA, with a G/T mismatch, as the substrate for evaluating mismatch cleavage efficiency at different incubation time. It is expected that, if the denaturation and re-annealing is complete, 1/3 of DNA in the mixture will be homozygous wildtype DNA, 1/3 homozygous mutant DNA, and remaining 1/3 heteroduplexed DNA that could be digested by endonuclease. In a 6 μL reaction, 0.2 μL GFP-CELI-His was added, and incubated in the MD buffer at 45 °C for different times. The maximal cleaved heteroduplexed DNA fragments (320 bp and 289 bp) were observed 20 min after incubation (Fig. S3). No increase of the cleaved products was observed afterwards, suggesting that the heteroduplexed DNA has been completely digested. A reduction of both the full-length (609 bp) and cleaved DNA fragments were observed at 30 and 40 min after incubation (Fig. S3), indicating that non-specific DNA degradation had occurred. We thus defined one unit of CELI as the amount of enzyme needed to completely digest heteroduplexed DNA in 50 ng total DNA at 45 °C in 20 min.

Comparison of commercial endonucleases including T7E1, EndoV and CELII, using buffers provided by their manufactories, with native CELI from celery and recombinant CELI purified from infiltrated *N. benthamiana* leaves using the MD buffer showed that affinity purified GFP-CELI-His and CELI-His endonucleases in combination with the MD buffer gave an greatly improved mismatch cleavage, and the optimal result was observed when GFP-CELI-His was used (Fig. 3A and B). It is plausible that the GFP tag in the recombinant enzyme has somehow enhanced the activity or stability of the endonuclease. Thus, GFP-CELI-His was used for most experiments followed.

### 3.4. Applications of GFP-CELI-His in detecting mutation and gene editing events

To determine the mismatch detection sensitivity of GFP-CELI-His under the optimal condition identified above, two PCR products total length of 1,134 bp and 800 bp were amplified from DNA of mutants with single base point mutation G to A or C to T mixed with the wildtype DNA with a ratio of 0:1, 2:1, 1:1, 1:4, 1:8, 1 :16, 1:32 or 1:64, and denatured and re-annealed to form heteroduplexes. The heteroduplexed DNAs were digested with GFP-CELI-His using the MD buffer, followed by capillary electrophoresis, and results showed that mismatch cleavages were effectively detected by recombinant GFP-CELI-His endonuclease even with a dilution factor of 1:32 (Fig. 4A and B). Considering the presence of heterozygous mutations in mutagenized populations, in all TILLING analyses followed in lab, a dilution factor of 1:16 was routinely used (data not shown).

For detecting gene editing events, genomic DNAs were extracted from CRISPR/Cas9-edited rice plants, with a PAM site targeted to *Os06g0341300*, amplified with (+WT) or without the presence of the wildtype DNA (-WT) (with 1:1 ratio), and denatured and re-annealed

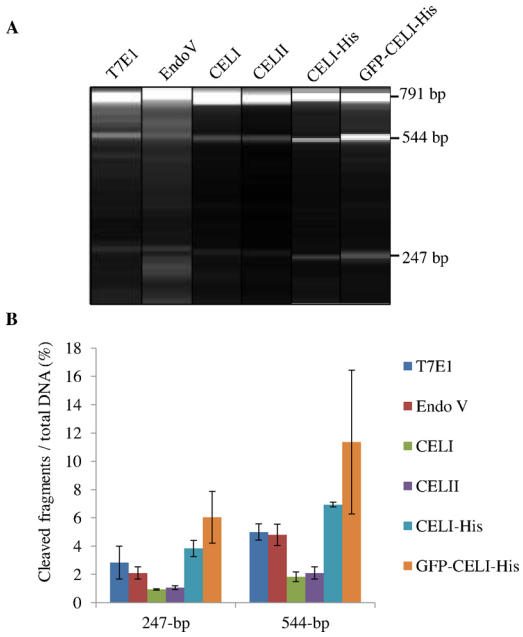


**Fig. 3. Comparison of mismatch cleavage activities of different endonucleases. (A)** A heteroduplex DNA sample of a 791-bp PCR product of the *CHS* gene with a mismatch (G/T) were digested with T7E1, EndoV and CELII, using buffers provided by their manufactories, native CELI from celery, and recombinant CELI purified from infiltrated *N. benthamiana* leaves, using the MD buffer. Note that the optimal cleavage was observed when GFP-CELI-His was used. **(B)** Calculated relative amounts of each cleaved fragments in total DNA products in (**A**) using PROSize 2.0 software ($n \geq 3$).

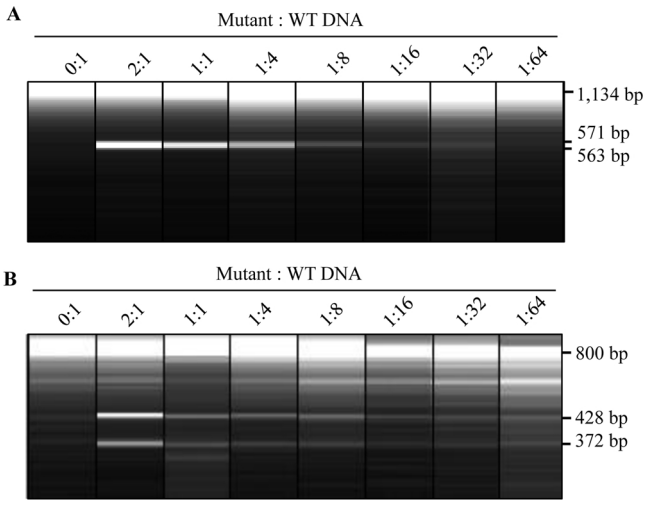


**Fig. 4. The sensitivity of GFP-CELI-His in combination with the MD buffer in detecting mismatches**. Genomic DNAs isolated from two different rice mutants with mutations in *Os04g0650600* **(A)** and *Os03g0167600* **(B)** were mixed with the DNA from the wildtype in different ratios (0:1, 2:1, 1:1, 1:4, 1:8, 1:16, 1:32, 1:64), used for PCR amplifications and mismatch detection using GFP-CELI-His in optimized MD buffer. Note that the mutation could be detected with a high sensitivity in 1:32 mixed templates. Sizes of DNA fragments are indicated at the side.

before digested with GFP-CELI-His in the MD buffer and detected by capillary electrophoresis. Results showed effective mismatch cleavages were observed when compared with the DNAs from the wildtype, and the zygosities (heterozygous in samples 5A,5B, and 5C, and homozygous in sample 5D, Fig. 5, left). Sequencing analyses of these plants showed that samples5A, 5B, and 5C were biallelic gene editing, while sample in Fig. 5D was a homozygous gene editing. Detailed sequencing

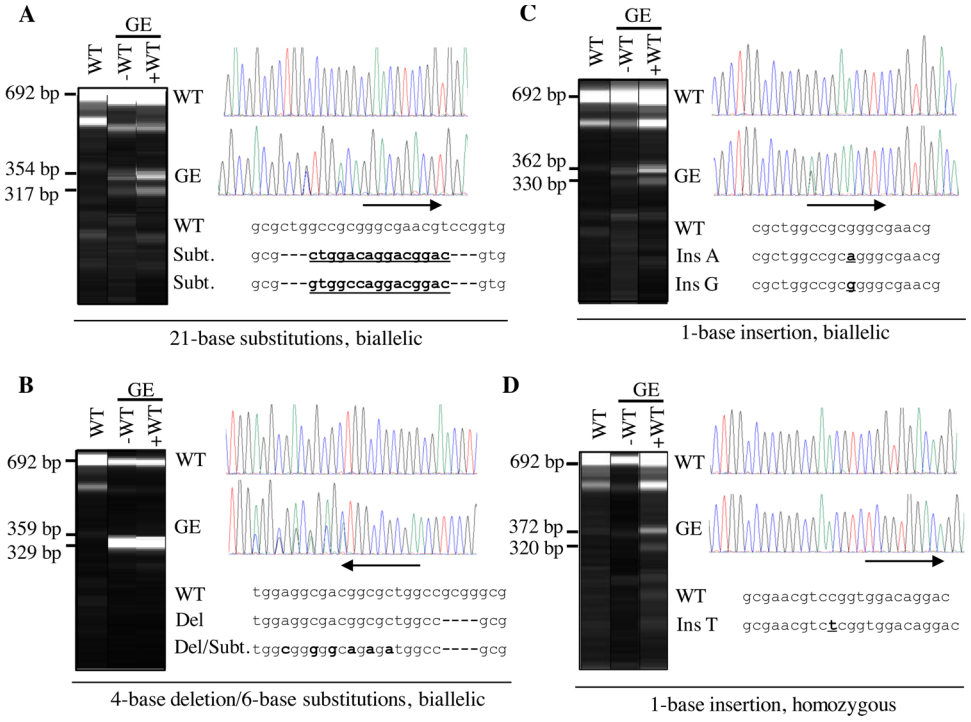


**Fig. 5. Detections of gene editing events using GFP-CELI-His endonuclease in the MD buffer.** Genomic DNAs extracted from gene edited rice plants (GE), with a NGG PAM targeted to *Os06g0341300*, were mixed (+WT) or not mixed (-WT) with the DNA from the wildtype (WT) in 1:1 ratio, and used as the templates for PCR amplification, and mismatch detection with GFP-CELI-His in the MD buffer (left). Note that homo- and hetero-zygosity status of each individual lines could deciphered by comparison of reactions with or without wildtype DNA, but heterozygous lines identified in gel may represent biallelic gene editing events: **(A)** biallelic gene editing with two different alleles with 21 base substitution, **(B)** biallelic gene editing one 4 base deletion and another one with 6 base substitution, **(C)** biallelic gene editing with two different single base substitutions, and **(D)** homozygous single base pair insertion, as showed by sequencing analyses. Positions of mutation are marked by black arrows, and sizes of cleaved DNA fragments were estimated.

analyses showed that sample in Fig. 5A had 21 base substitutions in two homozygous chromosomes, sample in Fig. 5B had a 4 base deletion and a 6 base substitutions in two homozygous chromosomes, sample Fig. 5C had two different 1 base insertions, while sample Fig. 5D was homozygous for one base insertion (Fig. 5, right). These results suggest that mismatch detection by GFP-CELI-His is very sensitive, and heterozygous editing plants identified on gel may represent biallelic gene editing events, which could be separated in their progenies.

## 4. Discussion

Sensitive and high-throughput technologies to detect natural variations, and introduced mutations by mutagenesis or gene editing technology, in genomes is increasingly important for genetic and genomic studies [23]. In addition to next-generation sequencing that requires extensive sample preparations and data analyses, detections of such genomic variations can also be achieved efficiently by mismatch-specific cleavage using endonucleases such as CELI, followed by electrophoresis. Currently, CELI endonuclease needs to be prepared from celery by extraction, precipitation and column fractionation [5,24–25]. Heterologous production of CELI has been tried in various prokaryotic hosts and baculovirus, but so far enzymatically active CELI has only been produced in baculovirus-infected insect cells [8,26], or silkworm [27]. Since post-translational modifications are crucial for the activity of CELI endonuclease, lacking or incorrect modifications could explain why functional CELI cannot be expressed in other organisms. For this reason, the use of endonuclease in genotyping so far is limited. Here, we report a simple and efficient technology for bulk production of enzymatically active CELI through transient expressions of CELI-His and GFP-CELI-His fusion proteins in *N. benthamiana* leaves. We also showed that, compared with the traditional crude extracted CELI from celery, the affinity purified recombinant CELI endonucleases, especially the GFP-CELI-His from *N. benthamiana* is more efficient.

Constructs of recombinant CELI endonucleases, one with His-tag and another one with both GFP- and His-tag, were introduced to *N. benthamiana* leaves by *Agrobacterium* infiltration, and maximal productions of these fusion proteins were observed at four DAI. This is similar to transient expressions of other proteins in *N. benthamiana* reported [28]. Compared with His-tagged CELI-His that can only be monitored using anti-His antibody, the expression of GFP-CELI-His in infiltrated leaves could not only be monitored by anti-His antibody, but also by confocal examinations and non-invasively by a hand-hold fluorescence lamp. Most likely due to the higher purities of CELI-His and GFP-CELI-His endonucleases obtained from the affinity purification using Ni-charged column, both of them showed elevated activities and less background bands in mismatch cleavage when compared with the native CELI purified from celery, and commercial endonucleases such as T7E1, EndoV and CELII. It is interesting to note that the activity of GFP-CELI-His is better than that of CELI-His. It is plausible that the presence of GFP in the fusion protein somehow enhanced the activity, or protected the stability of the enzyme, which shall be elucidated in the future.

To achieve the optimal activity of the CELI endonucleases, we optimized the buffer solution used for mismatch cleavage. The traditional buffer used for CELI endonuclease contains $Mg^{2+}$ [5–8,17]. We showed that supplements of $Ca^{2+}$ and $Mn^{2+}$ enhanced the mismatch cleavage activities in all CELI-related endonucleases such as the native CELI, CELII, CELI-His and GFP-CELI-His, while the presence of $Mg^{2+}$ did not enhance, or sometimes inhibited, the activities of these enzymes. Although the addition of DTT also compromised slightly their cleavage activities, DTT in combination with $Mn^{2+}$ in the buffer improved sensitivity of the mismatch detection, especially when GFP-CELI-His was used. We also showed that the presence of DTT inhibited effectively the degradation of PCR products, most likely due to the inhibition of exonuclease activities of the CELI, or other DNases contaminated from the extraction. Thus, the MD buffer that contains both $Mn^{2+}$ and DTT developed in this study gave stable and optimal mismatch cleavage for GFP-CELI-His. Consequently, using GFP-CELI-His produced in *N. benthamiana* and the optimized MD buffer, we were able to perform TILLING routinely in 1:32 mixed mutant and wildtype DNA samples, which is more efficient than previously published TILLING protocols [24,25].

CRISPR/Cas9-based gene editing creates point mutations including small deletions and insertions in regions targeted by guide RNAs [29,30], which is very useful in functional genomics and molecular breeding [31]. In this study, we demonstrated that the combination of GFP-CELI-His endonuclease produced in *N. benthamiana* and the

improved reaction buffer system creates a possibility to analyze gene editing events efficiently. We showed that GFP-CELI-His can effectively cleave different types of gene editing events without bias. By mixing with or without the wildtype DNA samples, it is also quite easy to discriminate the zygosity (homozygous or heterozygous) of edited plants. We also showed by sequencing that those heterozygous lines may represent biallelic gene editing events, which may allow to segregate different editing lines from their progenies. Together with the sensitive detection system developed in 96-well capillary electrophoresis [12,32–34], we strongly believe that the combined CELI production and reaction system developed in this study has great potentials in different genotyping applications.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We thank Dr. Anthony T. Yeung (Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA) and Dr. Meiping Zhao (Peking University, Beijing, China) for critical discussions of this work.

## Funding

This work was supported by grants from National Transgenic Research Projects of China (2019ZX08010003-002-009; 2016ZX08009-001-006-002; 2016ZX08009-003-005).

## 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.2020.110469.

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