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Targeted gene mutation in tetraploid potato through transient TALEN expression in protoplasts

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ABSTRACT

Potato is the third largest food crop in the world, however, the high degree of heterozygosity, the tetrasomic inheritance and severe inbreeding depression are major difficulties for conventional potato breeding. The rapid development of modern breeding methods offers new possibilities to enhance breeding efficiency and precise improvement of desirable traits. New site-directed mutagenesis techniques that can directly edit the target genes without any integration of recombinant DNA are especially favorable. Here we present a successful pipeline for site-directed mutagenesis in tetraploid potato through transient TALEN expression in protoplasts. The transfection efficiency of protoplasts was 38–39% and the site-directed mutation frequency was 7–8% with a few base deletions as the predominant type of mutation. Among the protoplast-derived calli, 11–13% showed mutations and a similar frequency (10%) was observed in the regenerated shoots. Our results indicate that the site-directed mutagenesis technology could be used as a new breeding method in potato as well as for functional analysis of important genes to promote sustainable potato production.

1. Introduction

Random mutagenesis techniques, based on physical or chemical mutagens, have been used in plant breeding for producing several thousands of new cultivars in the past 70 years (Shu et al., 2012). Such methods provide a cheap and simple way to induce new variability in germplasm which has already been adapted for cultivation (Ahloowalia et al., 2004; Waugh et al., 2006). However, these methods rely on an extensive phenotypic or genotypic screening to identify desirable mutants, and with many unwanted mutations in a single genotype as the common outcome (Shu et al., 2012). On the contrary, site-directed mutagenesis (SDM) allows predictable and precise editing of specific DNA sequences. This mutation technology has potential applications ranging from gene therapy to basic research and plant breeding (Fichtner et al., 2014; Gaj et al., 2013; Kim and Kim, 2014). In general, SDM can be achieved through introducing a double strand break (DSB) at a given DNA sequence in cells. Then the targeted cell's repair pathways, either based on homologous recombination (HR) or non-homologous end joining (NHEJ), is exploited for gene addition, gene correction or gene knock-out (Gaj et al., 2013). Zinc finger nuclease (ZFN), the first nuclease used in SDM approaches, along with homing endonucleases (HE or meganucleases) have been studied and used in plants (Curtin et al., 2012; Fichtner et al., 2014). However, not until the recent introduction of the Transcription Activator-Like Effector Nucleases (TALENs) (Christian et al., 2010) and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) system (Jinek et al., 2012), has the technology reached a level of simplification and efficiency sufficiently high to allow a rapid application among laboratories. The TALEN architecture is characterized by a nuclease domain fused to a DNA binding domain derived from the effector proteins produced by the plant pathogens in the genus *Xanthomonas*. The

Abbreviations: SDM, site-directed mutagenesis; DSB, double strand break; HR, homologous recombination; NHEJ, non-homologous end joining; TALEN, transcription activator-like effector nuclease; *ALS*, acetolactate synthase.

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DNA binding domain is a customizable modular structure featured by a repeat-variable diresidue (RVD) array assembled to achieve binding specificity. Dimerization of two nuclease domains, is generally required to obtain a DSB, thus at least a pair of TALEN has to be designed for each DNA target site. Both TALEN and CRISPR-Cas systems have recently been tested and proven to be functional in a broad range of organisms, including mammals, arthropods, and plants (Chen and Gao, 2013; Gaj et al., 2013).

In diploid crops, such as barley (Gurushidze et al., 2014; Wendt et al., 2013), rice (Li et al., 2012; Zhang et al., 2014; Zhou et al., 2014), soybean (Curtin et al., 2011; Haun et al., 2014), tomato (Brooks et al., 2014; Lor et al., 2014) and maize (Liang et al., 2013; Shukla et al., 2009), SDMs have been successfully employed for genome editing purposes. However, a number of important food crops are polyploid, such as wheat (*Triticum aestivum* L.) and potato (*Solanum tuberosum* L.). The major challenges in SDM of polyploid plants are the presence of multiple alleles of a certain gene, as well as frequent gene duplications resulting in a high number of possible targets in a single cell. The reports on successful applications of TALEN or CRISPR-Cas techniques in polyploid species are very limited. Recently, three homoalleles of the *MLO* gene in the allohexaploid wheat were successfully targeted employing TALEN and CRISPR-Cas (Wang et al., 2014), through a transgenic approach with stable integration. Moreover, Sawai et al. (2014) reported the mutation of all four alleles of the *St SSR2* gene involved in glycoalkaloid synthesis *via* stable transformation of a TALEN construct in potato.

Potato is the third most important staple crop with excellent yield potential and high nutritional value (Barrell et al., 2013). Potato cultivars are normally autotetraploid ($2n = 4x = 48$), but ploidy level can range from diploid ($2n = 2x = 24$) to pentaploid ($2n = 5x = 60$) (Spooner et al., 2005). Breeding efforts for improved cultivars are considerable, started more than 100 years ago; however, progress has been relatively slow and breeding is largely based on phenotypic selection among a large number of offspring. The high heterozygosity (Uitdewilligen et al., 2013) and tetrasomic inheritance in autotetraploid potato are the major drawbacks in potato breeding, because unfavorable alleles are continuously unmasked at each breeding cycle. Generally, it takes about 100,000 new genotypes to generate one new variety. Breeding programs based on ploidy reduction and selfing (to discard otherwise hidden unfavorable alleles) have been carried out, but the severe inbreeding depression and self-incompatibility of diploid germplasm have hampered these attempts (Lindhout et al., 2011).

Breeding to improve resistance to late blight (*Phytophthora infestans*) in potato using traditional methods has been very slow (Lindhout et al., 2011), although considerable progress has been achieved in introduction of resistance to nematodes, viruses and potato wart (Mackay, 2003). On the other hand, new plant varieties bred by modern breeding techniques, such as gene transformation, are tightly regulated in the European Union (EU) for commercial use. Genome editing may be an alternative, as mutated lines can be generated by a transgenic approach with stable integration of T-DNA carrying TALEN or CRISPR-Cas, which can subsequently be segregated out, and thus resulting in a mutant indistinguishable from classically bred varieties (Hartung and Schiemann, 2013). However from the legislative and the technical point of view, it would be more favorable to use a transient expression system (Podevin et al., 2013) in which no T-DNA is integrated into the plant genome and the generated mutants are of immediate interest as potential new varieties. This approach is particularly attractive for vegetatively propagated crops such as potato. In this study, we have successfully established a pipeline for tetraploid potato targeted gene mutation where TALEN was transiently expressed in protoplasts using acetolactate synthase gene (*ALS*) as the target. This gene encodes for a critical enzyme involved in the biosynthesis of branched-chain amino acids in plants (Tan et al., 2005) and has been used in experiments of targeted gene mutation in other species (Endo et al., 2006; Townsend et al., 2009; Zhang et al., 2013).

2. Materials and methods

2.1. TALEN design and construct preparation

About 50 million paired-end reads from *S. tuberosum* cv Desirée ($2n = 4x = 48$) obtained by RNA-seq were mapped against the two *ALS* gene sequences retrieved from *S. tuberosum* Group Phureja (clone DM 1–3) chromosome 7 (PGSC0003DMG400034102) and chromosome 10 (PGSC0003DMG400007078), using TopHat2 v. 0.8b (Kim et al., 2013). The read coverage was estimated using bedtools 2.20.1 (Quinlan and Hall, 2010) and the alignments imported in IGV (Robinson et al., 2011). The primer ALSFor (5′-GCCATCCCTCCACAATATGC-3′) and ALSRev (5′-GCAGCAGGTACGCCACAAG-3′) were designed in the two regions sharing 100% homology with the sequences of Desirée (Fig. 2a and b). The corresponding amplicon of 458 bp was further evaluated for putative TALEN targets with the TALEN targeter (https://tale-nt.cac.cornell.edu/node/add/talen) and a region of 55 bp, containing two 20 bp long conserved TALEN binding sites separated by a 15 bp spacer, was chosen (Supp. materials, 1).

The synthesis of the two repeat-variable diresidue (RVD) arrays for the chosen TALEN pair (Supp. material, 1) was performed with the GoldenGate TALEN kit 2.0 (AddGene, Cambridge, USA) as described by Cermak et al. (2011). The two RVD arrays were independently assembled in the vector pZHY500, carrying the NΔ152/CΔ63 truncated backbone. The released fragments by *Xba*I/*Bam*HI were subsequently cloned into the vector pZHY013 (Zhang et al., 2013) containing the EL/KK FokI obligated heterodimer and the T2A ribosomal skipping peptide (Halpin et al., 1999). The assembled coding sequences for the ALS-TALEN pair were then cloned into the Gateway (Life Technologies, Carlsbad, USA) compatible vector pK2GW7.0 (Karimi et al., 2002) under the 35S promoter, resulting in the plant expression vector p35S-ALS-TALEN with the final size of 16,010 bp (Supp. materials, 2). The plasmid extractions and purifications in this study were carried out using the Plasmid mini kit (Qiagen, Netherlands, EU).

2.2. Protoplast extraction and transfection

Leaves from *in vitro* propagated shoot cultures of *S. tuberosum* cv. Desirée were excised under sterile conditions for isolating protoplasts as described by Cardi et al. (1990) with some modifications (Supp. materials, 5a). The viability of the isolated protoplasts was assessed by fluorescine diacetate (FDA) staining as reported by Larkin (1976). The purified protoplasts were transfected as described by Craig et al. (2005) with modifications (Supp. materials, 5a). Briefly, for each transfection, 1.6×10^5 protoplasts (100 μl) were mixed with PEG4000 (12.5%) and 10 μg of the vector DNA, coding either for the green fluorescent protein (GPF) or the ALS-TALEN. The transfection reaction at room temperature (RT) was stopped after 3 min, followed by an incubation in the nutrient ½ Medium E (Supp. materials, 5b) for 24 h at RT. To determine transfection efficiency, two different vectors carrying the *GFP* gene were used: pGFP1 (pCW498-35S-GFiP-OcsT, 14,743 bp, Wood et al., 2009) and pGFP2 (pHBT-sGFP-NosT, 4230 bp, CD3-911, Chiu et al., 1996) (Supp. materials, 3). The GFP expression was evaluated after 24 h and the digital images of the transfected and non-transfected protoplasts were acquired using the stereoscope (LEICA M165 FC) and the microscope (LEICA DMLB100T) equipped with digital camera (LEICA DFC450C), GFP filter with excitation range 450–490 nm and fluorescent illumination system (PRIOR-Lumen 200). The