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The Gln15Arg mutation in the transcriptional factor PALM1 produces multifoliate alfalfa --Manuscript Draft--

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Abstract:	The CRISPR/Cas9 system has proven to be highly valuable for genome editing in several important crops, including the allogamous tetraploid cultivated alfalfa (<i>Medicago sativa</i>). However, the fact that most of the beneficial mutations relevant to alfalfa breeding programs are recessive mutations identified in the autogamous diploid species <i>Medicago truncatula</i> is a limitation. Naturally, the identification of dominant mutations can overcome this constraint and maximize the benefits of genome editing. In this article, we present the identification of a novel dominant mutation producing multifoliate leaves. Specifically, we showed that the mutation of the conserved glutamine 15 of the transcriptional factor PALM1 to arginine produces heptafoliate leaves in alfalfa, improving its digestibility and nutritional quality. Finally, we discuss the potential use of this mutation to rapidly generate transgenic-free legume forages with higher nutritional value via adenine base editors.	

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The Gln15Arg mutation in the transcriptional factor PALM1 produces multifoliate alfalfa

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Key message We present the first non-synonymous nucleotide substitution causing multifoliate leaves in alfalfa. The Gln15Arg exchange in the transcriptional factor PALM1 produces heptafoliate leaves.

Abstract

The CRISPR/Cas9 system has proven to be highly valuable for genome editing in several important crops, including the allogamous tetraploid cultivated alfalfa (*Medicago sativa*). However, the fact that most of the beneficial mutations relevant to alfalfa breeding programs are recessive mutations identified in the autogamous diploid species *Medicago truncatula* is a limitation. Naturally, the identification of dominant mutations can overcome this constraint and maximize the benefits of genome editing. In this article, we present the identification of a novel dominant mutation producing multifoliate leaves. Specifically, we showed that the mutation of the conserved glutamine 15 of the transcriptional factor PALM1 to arginine produces heptafoliate leaves in alfalfa, improving its digestibility and nutritional quality. Finally, we discuss the potential use of this mutation to rapidly generate transgenic-free legume forages with higher nutritional value via adenine base editors.

Introduction

During the last decades, the extensive characterization of conserved genes from model plants has generated a wide variety of genetic engineering strategies applicable to improve crop species. Specifically, model plants with simple genetics such as *Arabidopsis* and rice have been helpful in identifying many conserved genes that contribute to plant growth in dicots and monocots, respectively. To improve the translatability of information from model to crop species, other plant species have been studied, including the model legume *Medicago truncatula*, an autogamous diploid species with a small genome.

Before the genome-editing era, the translatability of data from *M. truncatula* to legume crops, such as the allogamous tetraploid cultivated alfalfa (*Medicago sativa*), was relatively simple even in crops with complex genetics. This is because the classical genetic engineering approaches often use the production of transgenic crops overexpressing and silencing genes of interest, and the DNA insertions that generate the expression of the traits act as dominant alleles (Aung, et al. 2015; Gao, et al. 2018; Jozefkiewicz, et al. 2018; Yang, et al. 2008). Unfortunately, the deregulation of transgenic events involves long-term and expensive procedures, and consequently, most of these transgenic events are not commercially available.

Recently, the CRISPR/Cas9 system has begun to help to validate the strategies found in the model species *M. truncatula* in alfalfa. A notable example is the case of PALM1, a single-copy gene in the dicot genomes encoding a transcription factor that plays a critical function in compound leaf morphogenesis. In *M. truncatula*, null PALM1 mutants develop pentafoolate leaves rather than wild-type trifoliate leaves (Chen, et al. 2010). As expected, CRISPR/Cas9-mediated full-knockout of PALM1 in alfalfa generates pentafoolate leaves (Chen, et al. 2020). Since an alfalfa cultivar commonly comprises a large number of genetically and phenotypically heterozygous parent plants, alfalfa is recalcitrant to the introgression of recessive alleles into elite heterogeneous populations, limiting the potential applications of the beneficial traits obtained via the complete loss of the function of target genes. We here present the identification of a new dominant PALM1 allele characterized by a single nucleotide substitution that produces multifoliate leaves in a conventional alfalfa variety and discuss the potential applications of this mutation to produce legume forages with increased nutritional quality.

Material and methods

The occurrence of PALM1 mutant alleles in an alfalfa population with high multifoliate leaf expression generated by phenotypic recurrent selection (Odorizzi, et al. 2015) was analyzed by using next-generation sequencing (NGS). Genomic DNA of plants was isolated from leaf tissue by using the DNeasy plant mini kit (Cat.#69104, Qiagen) and diluted to a concentration of 10 ng/μl. The PALM1 gene was amplified with specific primers (5-TTCTCTACACACTGAAAAAGAGAGA-3 and 5-AATTTTCATCCCCCACCCTTATTA-3) using Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs) following the manufacturer's recommendations. Amplicons were analyzed next to a 1-kb plus DNA ladder (Thermo Fisher) by electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. The library was prepared with a Nextera™ DNA Flex Library Preparation Kit (Illumina) following the manufacturer's recommendations (Illumina, Nextera DNA Flex Library Prep Reference Guide) and the sequencing was done paired-end 2x250 on an Illumina MiSeq with a MiSeq Reagent Kit v2. This NGS technology allowed obtaining 5,863,671 paired-end reads, producing from 63,456 to 265,392 reads for each individual plant or bulk (10 plants) showing trifoliate or multifoliate phenotypes. DNA sequencing was performed at the Genomic Unit of the Instituto de Agrobiotecnología y Biología Molecular (IABIMO) of the Instituto Nacional de Tecnología Agropecuaria (INTA)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. The sequencing reads produced by NGS were mapped against wild-type alleles by using the Geneious assembler platform (www.geneious.com).

The three-dimensional structure of alfalfa PALM1 was predicted using its sequence obtained from the UniProtKB database (id:D7R6G4) (Bairoch, et al. 2005), including its Q15R mutant form, and the I-TASSER server based on the multiple threading alignments (with all parameters set as default) (Yang, et al. 2015). Dimer prediction was carried out using the multimer docking advanced option of the ClusPro 2.0 server (Kozakov, et al. 2017; Yueh, et al. 2017) and uploading the three-dimensional models of PALM1, wild-type and mutated, bound to a DNA molecule derived from the crystallographic data with pdbID:5V3J. The binding energies were obtained considering balanced coefficients in the energy equation. The PyMol software (PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC., Cambridge, MA, USA) was used to render the final complexes and to determine the interactions between partners.

Results and discussion

The bulked segregant analysis combined with the NGS of individual plants was used to identify the dominant mutation in PALM1 from an alfalfa population with high multifoliate leaf expression (Odorizzi, et al. 2015). Sequence analysis revealed that the mutant allele is similar to the wild-type allele, with the exception of a single nucleotide substitution within PALM1 (A44G), which changes the glutamine site to arginine (Gln15Arg) (Fig. 1a). In addition, we showed that the CAA codon of Gln-15 is highly conserved in diploid (*M. truncatula*) and polyploid (*Glycine max*) model legume plants and in allogamous forages (*M. sativa*, *Melilotus albus*, *Trifolium pratense*, *Lotus corniculatus* and *Vigna unguiculata*) (Fig. 1a).

A high leaf/stem ratio is a crucial agronomic trait for forage species, because it is positively correlated to the nutritional value of hay and pasture for animal and dairy productions (Etzet, et al. 1988; Sayed, et al. 2022). Consequently, the recently developed adenine base editors (Tan, et al. 2022), which convert an adenine (A) nucleotide to a guanine (G), probably offer a way to rapidly generate dominant traits in allogamous legume forages by changing the CAA codon that codes for glutamine to a CGA codon, which codes for arginine. Contrary to wild-type plants and null PALM1 mutants, which show trifoliate and pentafoliate leaves, respectively (Chen, et al. 2020), Gln15Arg mutants display mainly heptafoliate leaves (Fig. 1b). Then, the Gln15Arg dominant mutation on PALM1 causes a novel multifoliate phenotype. As expected, this mutation produces a higher leaf/stem ratio, higher crude protein and lower neutral detergent fiber under field conditions (Fig. S1).

Dominant mutations have been significantly associated with either increased stabilization or destabilization of homo-oligomers (Gerasimavicius, et al. 2022). In this context, we hypothesized that the Gln15Arg mutation might alter the stability of PALM1 dimers through intermolecule interactions. In this line, the dimer prediction of PALM1 proteins revealed significant differences in terms of complex energies (Fig. 1c). The dimer formed by PALM1 (Gln15Arg) mutant proteins showed a balanced energy of -2307.7 kcal/mol, lower than the energy predicted for the dimer of the wild-type proteins (-2284.5 kcal/mol) and for the dimer formed by a wild-type and a mutant protein (-1985.7 kcal/mol). This difference is most likely due to the presence of arginine, which can form a greater number of even stronger H-bonds (Fig. 1c). The increased stability of this transcription factor by the Gln15Arg change in the dimer formed by mutant proteins may thus rigidify them and

reduce their conformational flexibility, in turn causing a loss of function. In addition, the expression of the mutant protein Gln15Arg could interfere with the activity of the wild-type protein PALM1. The latter is most commonly observed for proteins that form homomeric complexes, where the mutant subunit can alter the assembly (Bergendahl, et al. 2019).

Independently of the action mechanism, the induction of the Gln15Arg mutation on PALM1 via base editing could be an ideal approach to rapidly improve the legume forage species because PALM1 is a single-copy intron-free nuclear gene without related genes. This contrasts with the case of the multifoliate alfalfa-associated gene NAC39, which belongs to a large group of plant-specific transcriptional factors with extremely high nucleotide identity (Jozefkiewicz, et al. 2021). Finally, the current low-cost production of different traits in legume crops by using base editing (Bottero, et al. 2022) and the rapid and cloning-free screening of edited plants via NGS (Stritzler, et al. 2022) suggest that the generation of Gln15Arg mutants and their introgression in elite germplasm of allogamous legume crops is a feasible option to improve forage quality in the next years.

Figure 1. The mutation of the conserved glutamine 15 of the transcriptional factor PALM1 to arginine produces multifoliate alfalfa. (a) Emergence of a nonsense mutation (highlighted in orange) within the 5' region of PALM1 (A44G, Gln15Arg) responsible for the multifoliate alfalfa phenotype. (b) Gln15Arg mutants produce heptafoliate leaves rather than wild-type trifoliate leaves in alfalfa. (c) A dimer of PALM1 wild-type proteins (left), bounded with two DNA molecules and showed as green and blue in the “cartoon” representation, and a dimer of PALM1 mutant proteins right), in violet and yellow, are reported. In the boxes, are shown the amino acids of the interaction areas of the N-term domains that involve the mutation site Gln15Arg. Feasible H-bonds are reported as dotted black lines among amino acids represented in “ball and stick”.

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Conflict of interest. The authors declare that they have no conflict of interest.

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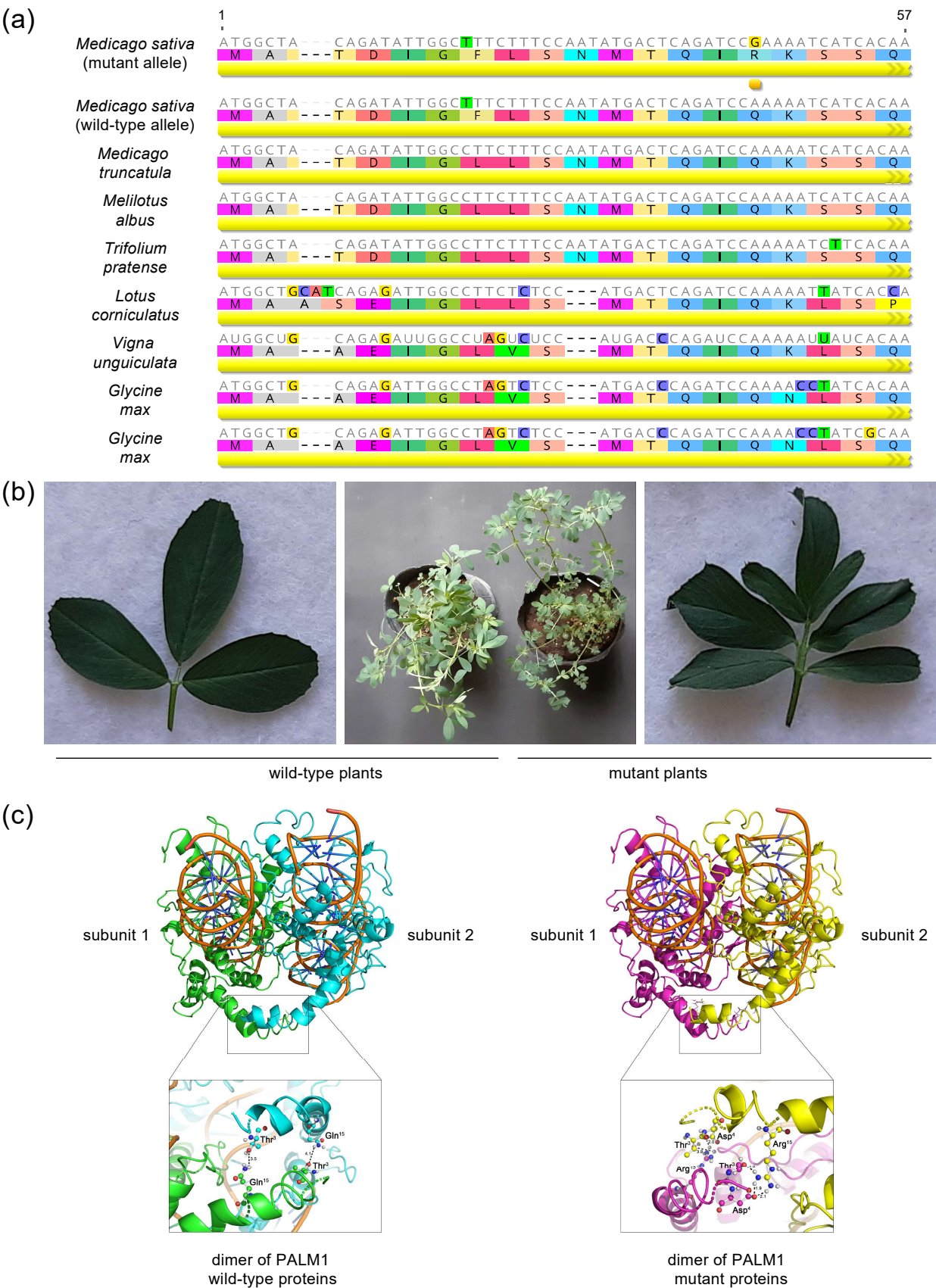
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Figure 1



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