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Original article

The *de novo* designed nutritive protein MB-1Trp does not resist proteolytic degradation in alfalfa leaves

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Abstract

We previously reported on a de novo designed protein "milk bundle-1Trp" (MB-1Trp) as a source of selected essential amino acids (EAA) for ruminant feeding. Here, we attempt to express this de novo designed protein in alfalfa. The microbial version of the gene encoding the protein was modified in order to achieve two expression strategies in transgenic alfalfa plants. Chimeric MB-1Trp genes alone or fused to a signal peptide and an endoplasmic reticulum retention sequence were introduced into alfalfa via *Agrobacterium*-mediated transformation. Polymerase chain reaction and reverse transcriptase polymerase chain reaction analysis performed on individual transgenic lines demonstrated that the MB-1Trp gene was correctly integrated and transcribed into mRNA. However, under our conditions, it was impossible to detect MB-1Trp protein expression in any of the transgenic plants analyzed. In order to assess MB-1Trp stability in alfalfa, *Escherichia coli*-derived MB-1Trp was incubated with proteins extracted from leaves of a non-transgenic plant. This study revealed a high susceptibility of mature MB-1Trp to alfalfa proteases, which may have contributed to its lack of accumulation.

Keywords: De novo designed protein; Protein stability; Transgenic alfalfa; Rumen digestion; Essential amino acids; Proteolysis

1. Introduction

The nutritional quality of forage crops is mainly determined by their content in selected essential amino acids (EAA), such as methionine and lysine [17]. Many crops used in animal diet formulations lack sufficient amounts of these EAA and require supplementation with pure methionine or EAA-rich proteins from diverse sources. This practice is costly and risky: the spread of mad cow disease was attributed to the use of animal protein meal contaminated with prions [3]. Thus, it becomes urgent to find new alternatives to support animal production.

Plant biotechnology has the potential to correct feed deficiency safely and economically [5]. Genes encoding heterologous EAA-rich proteins, or enzymes that enhance the synthesis of selected EAA, can be over-expressed in plants to correct their EAA balance [9,18]. Since the main focus of many laboratories was the improvement of the nutritive value of seeds, most of the transfected genes have been transferred into crops which encode for seed storage proteins such as albumins and zeins [12,15]. Less effort has been devoted to increasing EAA-rich proteins in vegetative tissue. The reported attempts dealing with this option used seed storage proteins under the control of the constitutive promoter 35S or the green tissue specific promoters Rbcs and Cab [2,18,21]. For instance, in order to promote protein accumulation, endoplasmic reticulum (ER)-targeting and retention signals were included in the constructs.

Among the most recent strategies for improving the nutritional value of crops is the creation and expression of de novo designed proteins with an enhanced content in EAA. Such proteins have a definite advantage over natural proteins or modi-

Abbreviations: E. coli, *Escherichia coli*; EAA, essential amino acid(s); EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycolbis(2-aminoethylether)-N,N,N′N′-tetraacetic acid; ER, endoplasmic reticulum; KDEL, lysine–aspartate–glutamate–leucine sequence; MB-1Trp, milk bundle-1Trp; MES, 2-morpholinoethanesulfonic acid; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcriptase from avian myeloblastosis virus, (E.C. 2.7.7.49); RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SFA8, sunflower 2S albumin 8 protein; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

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fied natural proteins in that they are conceived in order to harbor a controlled, tailored content in selected EAA. As a result, the desired EAA are not *added onto* a protein but *integrated into* a protein for which a target fold is selected. Ultimately, the novel protein is expressed in a simple organism, such as *Escherichia coli* (*E. coli*), and characterized in vitro prior to its expression in crops, in order to maximize its expression level and stability in such higher organisms. Keeler et al. [11] were the first to demonstrate the feasibility of this approach by designing and expressing a novel high-lysine α-helical coiled-coil protein, leading to a significant enhancement in lysine levels in the transgenic plants compared to the wild-type.

Alfalfa is the most widely used forage crop in the world [14]. However, alfalfa proteins alone are not ideal for ruminant nutrition due to its low content in methionine [19]. In our group, we designed the protein "milk bundle-1Trp" (MB1-Trp) which is characterized by a dominant content in selected EAA, especially methionine, lysine, threonine and leucine, which was designed to fold into a four-helical bundle structure [8]. This protein was intended for ruminant feeding, and progressively improved in order to maximize its resistance to ruminal degradation [16]. Using experiments modeling rumen conditions, the mutation of a tyrosine in position 62 to a tryptophan allowed stabilization of the original MB-1 to a level similar to some native plant proteins, such as sunflower albumin 8 (SFA8) [8]. An alfalfa plant harboring sufficient quantities of MB-1Trp could therefore be an efficient and economical source of critical EAA for ruminants. Here, we attempted to express the de novo designed protein MB-1Trp using a leaf specific promoter in order to improve the nutritive value of alfalfa forage.

2. Results

2.1. MB-1Trp gene transfection and transcription

The MB-1Trp gene was first optimized for alfalfa production and transfected into the host plant as described in Section 4. As shown in Fig. 1A, for 22 out of the 23 transgenic lines analyzed, both MB-1Trp and *npt*II (encoding kanamycin resistance) genes were detected. Amplification products of the expected size were obtained from RNA extracts only when the reverse transcriptase (RT) was present (Fig. 1B) confirming that the MB-1Trp gene sequence was efficiently transcribed into mRNA for 17 out of 23 plants.

2.2. MB-1Trp protein expression

Calibration of immunodetection showed that 5 ng of purified bacterial MB-1Trp protein mixed with 30 μg of total soluble proteins from a non-transgenic plant could be routinely detected. Immunoprecipitation was then applied to larger amounts (up to 1 mg) of total soluble proteins using similar conditions (with or without protease inhibitors). No immunoreactive material at the expected molecular weight of the control

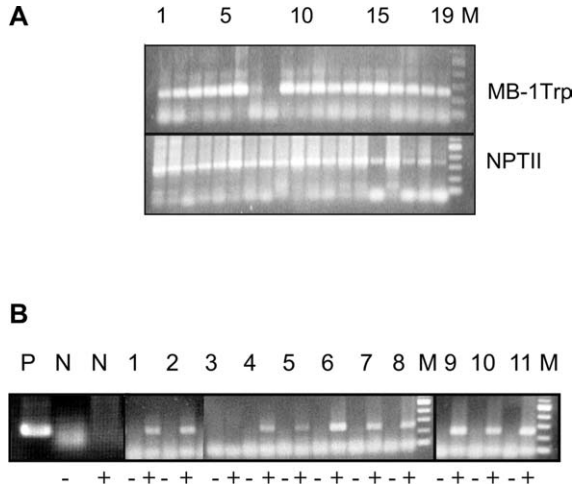

Fig. 1. Molecular analysis of transgenic alfalfa plants. **Panel A.** PCR amplification of genomic DNA from transgenic plants using MB-1Trp and *npt*II specific primers. 1–8: MB-1Trp-C transgenic plants; 9–19: MB-1Trp-ER transgenic plants. Plant no. 8 did not harbor MB-1Trp gene. **Panel B.** RT-PCR detection of MB-1Trp mRNA in leaves of selected transgenic alfalfa plants. Lanes 1–5: total RNA from MB-1Trp-C transgenic plants; 6–11: total RNA from MB-1Trp-ER transgenic plants. N: negative control, total RNA from non-transgenic plants; P: positive control, amplification from a plasmid DNA containing the MB-1Trp gene sequence. Below the gels: + indicate the use of RT. Transgenic line no. 3 did not show any RNA for MB-1Trp gene.

MB-1Trp was detected, regardless of the presence of protease inhibitors (results not shown).

To determine whether MB-1Trp was expressed despite the fact that it was undetectable on Western blots, several investigations were carried out: 1- reverse transcriptase polymerase chain reaction (RT-PCR) products obtained from transgenic lines encoding both MB-1Trp-C (cytoplasmic expression) and MB-1Trp-ER (ER targeted accumulation) were sequenced. No sequence abnormalities such as stop codons or deletions were detected; all the RT-PCR sequences were identical to the original sequence of MB-1Trp cloned into the transfection vectors; 2- a study was conducted to monitor bacterial MB-1Trp stability in alfalfa extracts. As seen in Fig. 2, the MB-1Trp protein

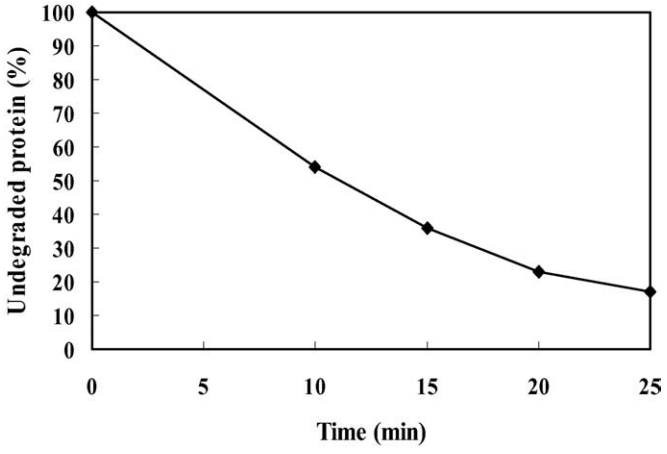

Fig. 2. In vitro stability of MB-1Trp produced in *E. coli* and incubated with alfalfa leaf extract. Percentages are obtained using the quantity of MB-1Trp detected on Western blot before incubating with alfalfa protein extract at room temperature.