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Calcium oxalate content affects the nutritional availability of calcium from *Medicago truncatula* leaves

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Abstract

It is known that oxalate, present in edible plants, can bind calcium in a crystalline form that reduces the availability of the bound calcium for nutritional absorption by humans. It is unknown, however, the degree to which the calcium oxalate content of a plant can be genetically altered and how much such alterations can impact the nutritional availability of the calcium present in plant foods. The recent identification of near isogenic *Medicago truncatula* mutants that contain a varying range in calcium oxalate content allows us to begin to address this gap in our knowledge. Here we assess, using an *in vitro* dialysis system that simulates the processes of digestion and absorption, the availability of calcium present in the leaves of *M. truncatula*. The results showed that calcium availability generally correlates inversely with the amount of calcium sequestered in the oxalate crystal. The plants with more calcium oxalate were found to have reduced calcium availability while the plant with less calcium oxalate was found to have enhanced calcium availability, compared to controls. Overall, this study supports genetically manipulating the form of calcium in edible plants as a viable strategy to improve the nutritional quality.

Keywords: Calcium; *Medicago*; Nutrition; Oxalate

1. Introduction

Efforts to understand the link between dietary calcium and health-related conditions such as osteoporosis [1], cancer [2], and renal stone formation [3] has brought attention to the importance of understanding calcium availability (the amount of calcium available for nutritional absorption) in foods. Calcium present in plant foods exists primarily as a complex in which it is bound to substances such as oxalate, phytate, fiber, fatty acid, protein, and/or other anions [4,5]. The substance to which calcium is bound can have a dramatic influence on its availability.

Most studies suggest that oxalate is an antinutrient that renders calcium and sometimes other minerals unavailable for nutritional absorption by human. A good example of such a study [6] compared calcium absorption from kale (low oxalate) and spinach (high oxalate). The findings from this and several other studies [6–9] have led to the general conclusion that the more calcium in the form of the oxalate salt, the less calcium available for nutritional absorption. A possible exception to this conclusion, however, was reported in a study using soybeans. Soybeans are rich in oxalate [10,11], yet soybeans were found to have a relatively high calcium availability [12]. In addition, in some instances calcium absorption from *in vitro*-generated calcium oxalate crystals gave higher than expected values [13]. Thus, studies using a single plant system that differs only in oxalate content would help in clarifying this apparent discrepancy.

As an initial step toward eliminating much of the heterogeneity associated with comparing different edible plants (e.g., spinach, soybeans, and kale) we recently assessed the calcium availability in the *Medicago truncatula* wild type and a genetically altered mutant, calcium oxalate defective (*cod*) 5 [9]. The *cod5* mutant was previously isolated from an EMS-mutagenized *M. truncatula* population and had near wild type amounts of calcium but little of it partitioned into the calcium oxalate crystal [14]. Calcium availability studies with these two plants showed that the *cod5* had an increase in calcium availability compared to control, and thus, indicated the feasibility of improving calcium availability in plant foods

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through genetic modification of its oxalate content [9]. In addition, biomass and mineral content comparisons between mutant and control showed no major differences when grown under controlled greenhouse conditions [15]. Thus, the removal of calcium oxalate does not appear to compromise growth or mineral content under controlled conditions. To ensure that the finding of improved calcium availability is a reflection of a reduction in calcium oxalate content, rather than some other secondary effect specific for the *cod5* mutant, the testing of additional plant lines is desirable before any firm conclusions are drawn.

We report here the analysis of additional EMS-mutants of *M. truncatula* that contain calcium oxalate contents above and below wild-type levels. One of the mutants, *cod6*, contains less calcium in the form of the oxalate crystal compared to wild type [14] while the mesophyll oxalate defective (*mod*) 2 and *mod4* contain more calcium bound as the oxalate salt compared to wild type [16]. Upon measuring calcium availability, using an *in vitro* dialysis method that simulates the process of digestion and absorption, a general trend emerged of increasing calcium availability with decreasing calcium oxalate content. Thus, the highest level of dialysable calcium was found in the modified plant with the lowest calcium oxalate content and lowest calcium availability was found in the plants modified to contain the higher oxalate content. The unmodified control which had intermediate calcium oxalate content also had an intermediate calcium availability compared to the other test plants.

2. Materials and methods

2.1. Plant growth

M. truncatula cv jemalong ecotype A17 seeds (wild type and mutant) were scarified with a razor blade and allowed to germinate on agar plates. The plantlets then were grown in hydroponics as previously described [9]. Leaves were harvested from 4- to 6-week-old plants and freeze dried.

2.2. Calcium analysis

Calcium content was determined by ICP analysis (Nutrient Analysis Laboratory, Cornell University) on the freeze-dried tissue samples. Each measurement was done in duplicate on five independently grown plant sets. The results were averaged and standard error calculated. Statistical analysis was done according to Minitab statistical software package (Minitab, State College, PA).

2.3. Oxalate measurements

Oxalate content was measured as described previously [14]. In brief, the freeze-dried samples were weighed and ground in water. Total oxalate levels were determined using an oxalate diagnostic kit (Trinity Biotech, St. Louis, MO). Crystals were solubilized by the addition of H^+-Dowex in dilute acid. The mixture was heated at 60 °C for 1 h to dissolve the oxalate crystals. The pH of the mixture was then adjusted (pH 5–7), followed by charcoal filtration and centrifugation. The supernatant then was analyzed for oxalate content according to the manufacturer's instructions (Trinity Biotech). Standards were prepared from oxalic acid dihydrate (Sigma, St. Louis, MO) and used for total oxalate measurements as recommended by the manufacturer. Measurements were done in duplicate on five independently grown sets of plants, the results averaged, and standard error calculated.

2.4. Dialysable calcium

Tissue digestion and assessment of calcium bioavailability was conducted as previously described [9]. In brief, freeze-dried leaves were ground and HCl added until the homogenate reached a pH 2.0. Pepsin was added to simulate digestion and the mixture was shaken in a beaker for 2 h at 37 °C. A sodium bicarbonate (0.075 M) loaded dialysis bag, simulating the intestine, was placed in each beaker containing the digested mixture followed by the addition of a pancreatin/bile solution. After incubation the contents of each dialysis bag was recovered and the calcium content determined. Analysis was done in duplicate on five independently grown sets of plants.

3. Results and discussion

A number of studies have shown, using various plant foods, that oxalate can bind calcium in a crystalline form and reduces the amount of calcium available for nutritional absorption. This issue of calcium availability is important considering the reliance of different populations around the world on plant foods as their main source of calcium and other nutrients. The question remains, however, as to the extent calcium availability can be altered through genetic modification of the calcium oxalate content present in edible plants. The recent identification of *mod2*, *mod4* [16], and *cod6* mutants [14] that hyper- and hypo-accumulate crystals of calcium oxalate, respectively, has allowed us to address this question.

Visual inspection of the foliage from control and mutant plants showed a similar appearance (Fig. 1A–D). Microscopic inspection of a cleared leaf from each plant, however, revealed differences in calcium oxalate crystal accumulation (Fig. 1E–H). Prismatic crystals were observed accumulating along the vascular strand of control leaves, but were reduced in the leaves of the hypo-accumulating *cod6* mutant (Fig. 1E and F). The *mod2* and *mod4* mutants accumulated prismatic crystals along the vascular strand like wild type, but in addition, formed a second type called druse (spherical aggregate of crystals), within the mesophyll tissues (Fig. 1G and H).

Calcium (Table 1) and oxalate (Fig. 2) measurements conducted on leaves from each plant were done to determine if the level of each crystal component correlated with the corresponding calcium oxalate phenotype. The amount of oxalate (Fig. 2) was found to correlate with the crystal phenotypes (Fig. 1E–H) with *mod2* and *mod4* showing the highest levels, wild type showing an intermediate level, and *cod6* showing the lowest level of oxalate. The amount of calcium, however, did not correlate with the crystal phenotypes