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Genetically Modified *Medicago truncatula* Lacking Calcium Oxalate has Increased Calcium Bioavailability and Partially Rescues Vitamin D Receptor Knockout Mice Phenotypes

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Abstract

Background: How the distribution and sequestered form of plant macro/micro-nutrients influence their bioavailability, and ultimately impact human health, are poorly understood. The legume *Medicago truncatula* has a portion of its tissue calcium (Ca) sequestered in the form of the Ca oxalate (CaOx) crystal which reduces its nutritional value in terms of Ca bioavailability. The *calcium oxalate deficient 5* (*cod5*) mutant has a total Ca content similar to wild-type (WT) plants, but sequesters less of its tissue Ca in the form of the CaOx crystal. Previous short-term mice feeding studies suggest that this difference is responsible for the improved Ca bioavailability of the *cod5* plants compared to WT plants.

Objectives: To perform long term feeding studies with Vitamin D Receptor Knockout (VDR-KO) mice and the nutritionally improved *cod5* line to assess the impact of increased Ca bioavailability on VDR-KO Ca deficiency phenotypes.

Methods: To assess the ability of diets containing *cod5* plant material as the sole Ca source to rescue the Ca deficiency phenotypes of the VDR-KO mice we conducted both short term and long term experiments. Specifically, Ca absorption and utilization were measured short term (24-hour) in the hind limb bones and duodenum tissue of VDR-KO mice that were fed either an intrinsically ^{45}Ca labeled *cod5* or WT Medicago diet. Long term (20-day) bodyweight gain and change in Bone Mineral Density (BMD) were also measured over a 20 day period in VDR-KO mice fed either a *cod5* or WT Medicago diet.

Results: In the 24-hour feeding study, ^{45}Ca incorporation was found to be 46.3% (male) or 53.9% (female) higher in hind limb bones (P<0.01); and 32.5% (male) or 38.5% (female) higher in duodenums (P<0.01) in VDR-KO mice fed *cod5* than those fed WT plants. In the 20-day feeding study, the VDR-KO mice (male) fed *cod5* gained 38.1% more bodyweight than those fed WT plants (P=0.06). The increase of BMD after 20 days in the VDR-KO mice (male) fed *cod5* diets was 22.5% higher than those fed WT diets (P=0.17).

Conclusions: Our study confirms and extends an earlier study by showing that *cod5* Medicago not only had higher Ca bioavailability but it can also rescue, in part, the VDR-KO Ca deficiency phenotypes. Thus, the removal of CaOx from a plant-based diet appears to be a viable long-term dietary option to boost bioavailable Ca levels and help combat Ca related disorders.

Keywords: Calcium bioavailability; *Medicago trunculata*; Vitamin D receptor knockout mice; Oxalate; Calcium Oxalate Deficient 5 (cod5)

Abbreviations: Ca: Calcium; CaOx: Calcium Oxalate; WT: Wild-type; BMD: Body Mineral Density

Introduction

Nutritional recommendations agree on the importance of a predominantly plant-based diet (http://www.choosemyplate.gov); however, sole reliance on plants for nutrition can limit the intake of the essential nutrients calcium (Ca), vitamins B12 and D [1]. Bioavailable Ca is critical to human health, which is needed specifically for bone growth and muscle control. Inadequate Ca uptake can cause imbalanced Ca levels in serum, resulting in hormone-regulated adaptation which promotes the mobilization of Ca from bone Ca phosphate and increases the risk of osteoporosis [2,3]. In recent years, concerns over the development of osteoporosis in an aging population has led to an increase in food fortification of Ca in the United States [4,5]. Several studies show that Ca-fortified vegetables can increase Ca uptake in mice and humans [6,7], but the long term efficacy of these fortification activities remains questionable [8].

Calcium in plants exist as complexes with oxalate, phytate, and fiber [9,10], all of which suppress Ca bioavailability. It is believed that the Ca oxalate (CaOx) content is the best predictor of Ca availability [11]. For example, spinach contains a high concentration of Ca but a significant amount of this Ca is complexed with oxalate which reduces its Ca bioavailability to about 5.0% [12]. However, in soybeans oxalate levels are similar to spinach but its Ca bioavailability is around 20%

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[13]. This variation among plants warrants further investigation of how CaOx impacts bioavailability.

In humans, oxalate cannot be metabolized so after ingestion and absorption it is usually excreted. In some instances; however, the oxalate can complex with Ca before excretion occurs and result in the formation of kidney stones, particularly in those with low Ca intake [14,15]. Patients suffering from hyperoxaluria, a condition characterized by over-production of oxalate in the body leading to the development of kidney stones, are advised to avoid high-oxalate foods and to supplement with Ca [16]. Given the importance of adequate Ca for human health, and the potential negative health effects of excessive oxalate consumption for those with oxalate sensitivities, manipulation of CaOx formation is an important target of biofortification efforts, which aims to enhance the bioavailability of Ca and reduce oxalate content in food crops [17].

The low oxalate mutant from the model forage legume *Medicago trunculata*, *calcium oxalate deficient 5* (*cod5*), has been previously identified [18]. The *cod5* plant is devoid of prismatic crystals of CaOx. Growth studies and total mineral composition measurements indicate no substantial difference between *cod5* and WT with the exception of oxalate crystal content [19]. The removal of crystalline oxalate in *M. truculata* results in increased Ca absorption in mice [6]. However, no long-term feeding studies have been carried out to evaluate the benefits of *cod5* diets due to the lack of easy-to-monitor physiological phenotypes in normal mice fed with low Ca diets.

The Vitamin D Receptor (VDR) is a member of the superfamily of nuclear receptors which regulates gene expression in a ligand activated manner [20]. The hormonal metabolite of vitamin D, 1, 25-dihydroxyvitamin D_3 (1, 25$(OH)_2D_3$) functions as the ligand for VDR [21-23]. VDR dependent transcellular Ca absorption is important in maintaining Ca homeostasis when Ca concentrations are low [22]. The VDR knockout (VDR-KO) mice exhibit decreased serum Ca levels, reduced duodenal Ca absorption, impaired bone formation and growth retardation on 0.5% Ca diet [22,24,25]. However, on an excessive 2% Ca diet, the serum Ca balance, duodenal Ca intake, bone growth and body weight gain in VDR-KO mice can be partially restored, likely through a VDR independent system [24-26]. Therefore the enhanced sensitivity to dietary Ca levels of VDR-KO mice makes them ideal models to test the bioequivalence and bioavailability of Ca in *cod5* lines during long term feeding regimes.

In our study, we used VDR-KO mice to test the bioavailability of Ca in *cod5* and WT Medicago leaf tissue to evaluate the long-term impacts of a *cod5* based diet. Our results demonstrate the longitudinal impacts of reduced CaOx content in plant diets in animals with defects in Ca metabolism.

Materials and Methods

Plant growth and preparations

Medicago truncatula WT (cv. Jemalong, ecotype A17) and *cod5* seeds were nicked with a razor blade and allowed to germinate on agar plates. The germinated plants were then grown hydroponically for 30 days before the leaves were cut back [27]. To intrinsically label WT and *cod5* Medicago with ^{45}Ca, the hydroponic solution was supplemented with 1 μCi ^{45}Ca/L after the removal of the leaves and grown for 30 additional days. Then the leaves were harvested and processed as described previously [6]. In brief, leaves were harvested and freeze dried [18,28], and then the material was frozen in liquid nitrogen and ground to a powder with a mortar and pestle [6].

Determination of bulk Ca and oxalate concentration

Calcium concentration measurements were carried out using inductively coupled plasma atomic emission absorption spectrometry on weighed leaf samples (Soil, Water and Forage Testing Laboratory, Texas A&M University). Each measurement was done in duplicate on three independently grown sets of plants. The results were averaged with standard error calculated [28].

Oxalate measurements were conducted as previously described [18]. In brief, weighed freeze-dried leaf samples were ground in water and centrifuged. The supernatant was decanted and the soluble oxalate measured using an oxalate diagnostic kit (Trinity Biotech 591-D). The amount of total oxalate was determined by simply omitting the centrifugation step and solubilizing the Ca oxalate crystals through the addition of H^+-Dowex in dilute acid. The mixture was heated at 60°C for 1 hour, and the pH of the mixture was adjusted to between 5 and 7. The mixture then was charcoal filtered and centrifuged. The supernatant was then analyzed for oxalate content using the oxalate diagnostic kit (Trinity Biotech). Measurements were done in triplicate on three independently grown sets of plants.

Diet preparation and mice genotyping

Calcium limited AIN-93G diets were (Ca concentration 0.5%) obtained from Research Diets (New Brunswick, NJ). To obtain various diets with low (0.5%) or high Ca (2%) levels, Ca limited AIN-93G diets were mixed with freeze-dried WT, or *cod5* plants, or other Ca chemicals ($CaCl_2$, or CaC_2O_4 (CaOx)) (Table 1). 3.5 g of these diets were calorically and nutritionally equivalent (except for Ca) to the standard AIN-93G diet [26]. Animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee [29]. VDR-KO mice were generated by breeding heterozygous mice. At the age of ten days, tail clips were taken for genotyping. Genotyping Primers used were: Forward 5'-TTTGGATCCATGGAGGCAATGCAGCCAGC -3',

Reverse 5'-TTTGGTACCTCAGGAGATCTCATTGCCAAA CACCTC-3',

Insert 5'-TTTGGATCCATGAAGCGCAAGGCCCTGTTC -3' [21].

On day 21 postpartum, confirmed VDR-KO mice or WT mice were weaned and assigned to the experiments based on their genotype and gender. Throughout the experiments, mice were housed in cages with *ad libitum* access to water and food prior to the initiation of the experiment. Before the feeding experiments, mice were maintained on AIN-93G diet that contains 2% Ca [6].

Diet name	Ca Source	Weight of Ca Source (g)	Modified Low Ca^{2+} AIN diets (g)	Ca Content (%)	Kcal / kg
Low Ca	N/A	0	1000	0.5%	3670
2% Ca	$CaCl_2$	9.7	990.3	2%	3633
CaOx	CaC_2O_4	11.2	988.8	2%	3630
WT	WT Medicago	233	767	2%	3515
cod5	*cod5* Medicago	233	767	2%	3515

Table 1: The ingredients, calcium and calorie composition in five diets used in the 20-day feeding study (per total weight of 1000 g).

Measurement of Ca absorption in hind limbs and duodenum

The VDR-KO mice were stratified and deprived of food for 24 hours and separated into two treatment groups (n=12 per group, six males and six females) using a randomized block design. After the 24 hours fasting, 3.5 g of each diet (Table 1) was placed in a glass food jar and placed into the cage. The amount of diet fed to each mouse represented 14.0 kcal. The small serving size of diets also insured that the mice ate the majority of the test meal over the allotted 24 hours. All mice were euthanized in CO_2 chamber after 24 hours of feeding and placed at 4°C for 24 hours. The bones of the two hind limbs (femur, tibia, and fibula) were removed to measure bone Ca absorption. The soft tissue was surgically removed with a scalpel and the bones were then ashed to remove all the organic matter. The ashing was performed in a muffle furnace (Thermolyne Furnace, Barnsted International, Dubuque, IW) at 700°C for 30 hours. The duodenum was removed with a scalpel to measure duodenal Ca absorption. The tissue was collected in 1 ml PBS buffer and sonicated to homogenization. The amount of ^{45}Ca incorporated in the sample was determined by a scintillation counter.

Long term feeding study and measurement of bone mineral density (BMD)

In the pilot study, male VDR-KO mice demonstrated higher sensitivity to Ca intake (data not shown), so we chose male VDR-KO mice for long term feeding study. Ten or twenty (depending on the diet groups) male weaning VDR-KO mice were allotted into one of five different diets (Table 1) using a randomized block design [7]. 3.5 g of each diet was placed in a glass food dish and placed into the cage every day for 20 days. Water was allowed *ad libitum* throughout the experiment. Individual bodyweight was recorded every 24 hours. The BMD of the VDR-KO mice were measured by Dual Energy X-ray Absorptiometry (DEXA; PIXImus instrument, Lunar Corp., Madison, WI) on Day 1 and Day 20 for ten mice in each diet group. The precision was ± 1% coefficient of variation in vitro. DEXA test was taken on each mouse at the start and the end of the experiment to determine the bone mineral contents. The tests were carried out as previously described [30,31].

Data analysis

The statistical analysis was performed using SPSS Version 11. T-test among five groups was conducted and subsequent pairwise comparisons were performed with Sidak multiple comparison test.

Results

Previously, we showed that *cod5* diets display increased Ca bioavailability [6]. However, we have not demonstrated if this dietary change can impact animal growth and development. Here, we first tested ^{45}Ca incorporation into VDR-KO mice hind limbs and the duodenum. In a 24-hour feeding study, the VDR-KO mice fed ^{45}Ca-labeled *cod5* diets had 7.80 ± 0.51% (Male)/7.48 ± 0.91% (Female) hind limb bone Ca incorporation while VDR-KO mice fed ^{45}Ca labeled WT Medicago diets had 5.33 ± 0.46% (Male)/4.86 ± 0.77% (Female) hind limb bone Ca incorporation (Figure 1A). For duodenal Ca absorption, the VDR-KO mice fed ^{45}Ca labeled *cod5* Medicago had 0.57 ± 0.09% (Male)/0.54 ± 0.12% (Female) duodenal Ca incorporation while VDR-KO mice fed ^{45}Ca labeled WT diets had 0.43 ± 0.07% (Male)/0.39 ± 0.05% (Female) duodenal Ca incorporation (Figure 1B). The group fed the *cod5* diet displayed 46.3% (male)/53.9% (female) higher Ca absorption in hind limb bones (P<0.01) and 32.5% (male)/38.5% (female) higher Ca absorption in duodenums (P<0.01) than the group fed the WT diet.

Hind limb bone and duodenal Ca incorporation are important parameters reflecting body Ca uptake levels [25]. Therefore, these results demonstrated that the *cod5* diet has higher Ca bioavailability in VDR-KO mice. However, the long-term physiological impact of Ca in *cod5* diets was unknown. Previous studies show that VDR-KO mice have bone and growth defects that are associated with low Ca absorption. These pathological phenotypes in VDR-KO mice can be partially rescued by high Ca intake [25]. Here we tested whether the removal of oxalate crystals in *cod5* could be a sufficient dietary alteration to rescue VDR-KO mice phenotypes. Male VDR-KO mice were divided into five dietary groups (n=10 for 0.5% Ca, 2% Ca and CaOx groups; n=20 for WT and *cod5* groups), and the diets were prepared as shown in table 1. A low Ca diet (0.5% Ca) was used as a negative control. A diet that contains 2% $CaCl_2$ was used as positive control. The CaOx diet which contains 2% total Ca but only 0.5% unbound Ca was used as a control to demonstrate that oxalate crystals act as antinutrients for Ca. Both WT and *cod5* diets contain 2% total Ca. After 20 days on each diet, the mice fed the 0.5% Ca diet gained an average of 1.75 ± 0.47 g of weight while those fed the 2% Ca diet gained an average of 3.91 ± 0.60 g of weight, showing the effect of increased Ca to rescue growth in these mutant mice. The CaOx diet group gained only 2.14 ± 0.36 g of weight supporting the antinutrient effect of oxalate crystals. The VDR-KO mice fed *cod5* diets for 20 days gained 3.37 ± 0.45 g body weight, while the mice in the group fed WT diets gained 2.44 ± 0.54 g of weight. In comparison, the *cod5* diets supported 38.1% more body weight gain than the WT diets (P=0.06) (Figure 2).

The Bone Mineral Density (BMD) is an important indicator for bone health [30]. The VDR-KO mice have lower BMD than C57BL/5 WT mice due to impaired Ca uptake and metabolism [32,33]. BMD was measured at day 1 and day 20 of the dietary regime and the increase of BMD was calculated for mice given each of the five diets (Table 1) groups (n=10). The percentage increase of BMD at day 20 compared

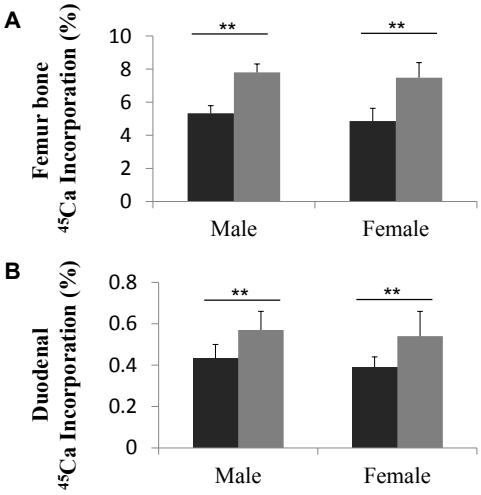

Figure 1: Hind limb bone and duodenal ^{45}Ca incorporation in VDR-KO mice fed wild-type (■) or *cod5* (■) Medicago diet.
A, Incorporation rate of ^{45}Ca per 1 gram of ash in the femur bones in both male and female VDR-KO mice. B, DuodenalCa^{45} incorporation rate in both male and female VDR-KO mice. For both panel A and B: n = 6; The error bars represent standard deviation. **: P value for T-test between wild-type and *cod5* group is less than 0.01.

to day 1 was calculated for each mice. The 0.5% Ca diet group showed an average of 13.65 ± 1.78% BMD increase while the 2% Ca diet group showed an average of 27.82 ± 1.55% BMD increase. The CaOx diet group had an average of 16.03 ± 1.81% BMD increase. The *cod5* diet group showed an average of 20.50 ± 2.98% increase while the WT diet group showed an average of 17.56 ± 2.21% increase in BMD. The *cod5* diet supported 16.7% higher BMD increase in VDR-KO mice than WT Medicago diet (P=0.17) (Figure 3).

Discussion

While multiple avenues are available to achieve adequate dietary Ca intake, adequate Ca nutrition remains an unresolved nutritional issue [8]. Since the bioavailability of Ca varies greatly among various types of foods, information merely listing the amounts of Ca in foods is misleading [34]. Calcium absorption from plants appears to be inversely proportional to the crystalline oxalic acid content in food [2,14,15,35]. The ability to rapidly identify and characterize gene function and then utilize these genes to engineer plant metabolism has been a driving force in biofortification efforts [36]. The *cod5* mutation in Medicago causes removal of crystalline CaOx complexes but unchanged total Ca levels and presents an attractive target for efforts to increase Ca bioavailability.

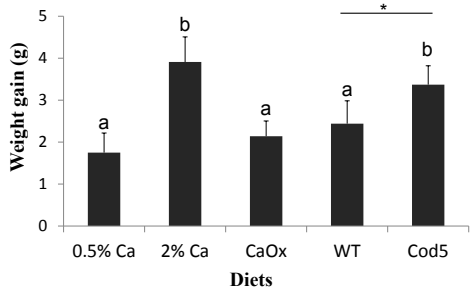

Figure 2: Weight gain of VDR-KO mice fed with five isocaloric diets containing different calcium sources over 20 days.
0.5% Ca: diet in which the Ca source is the AIN-93G diet base; 2% Ca: diet in which additional $CaCl_2$ was added to AIN-93G to achieve final Ca concentration of 2%; CaOx: diet in which additional CaC_2O_4 was added to AIN-93G to achieve final Ca concentration of 2%; WT and *cod5*: diets in which either processed WT or *cod5* Medicago was added to AIN-93G to achieve final Ca concentration of 2%. n = 10 for 0.5%, 2% Ca and CaOx group; n = 20 for WT and *cod5* group. The error bars represent standard error. a and b represent statistically significantly different groups. P value for T-test between a and b group is less than 0.02 except for WT vs. *cod5* group (*: P = 0.06).

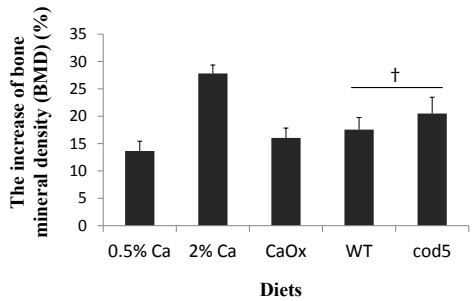

Figure 3: The percentage increase of bone mineral density (BMD) in VDR-KO mice fed five isocaloric diets containing different calcium sources over 20 days.
0.5% Ca: diet in which the Ca source is the AIN-93G diet base; 2% Ca: diet in which additional $CaCl_2$ was added to AIN-93G to achieve final Ca concentration of 2%; CaOx: diet in which additional CaC_2O_4 was added to AIN-93G to achieve final Ca concentration of 2%; WT and cod5: diets in which either processed WT or cod5 Medicago was added to AIN-93G to achieve final Ca concentration of 2%. n = 10 for all diet groups. The error bars represent standard error. †: P = 0.17.

In our previous work [6], we fed equal number of male and female mice four ^{45}Ca-containing diets: WT *M. truncatula* extrinsically or intrinsically labeled with ^{45}Ca, and *cod5* extrinsically or intrinsically labeled with ^{45}Ca. Extrinsic labeling adds the tracer to the food before ingestion, whereas for intrinsic labeling plants are grown in the presence of the label to incorporate the tracer into the plant matrix. Using both diets, absorption of the tracer was determined one day after consumption. Only in the intrinsically labeled diets, did we note a difference among the diets? We postulate these findings during the formation of crystals in Medicago, much like oxalate crystals in spinach, Ca is bound in a non-bioavailable form. In this study we used only intrinsically labeled foods.

The primarily limitations of using C57BL/6 WT mice is that there is no phenotypes to "rescue" with the modified diets. The level of Ca in *cod5* diets, though low, is sufficient to support C57BL/6 mice growth and development so that no obvious phenotypes can be detected. VDR-KO mice have lost the ability to absorb Vitamin D3 -dependent Ca [37]. The VDR-KO mice serum/bone Ca levels are 40% lower than WT mice when fed with 0.5% Ca diets (equivalent to Ca in AIN-76A diets) [37]. Due to this decreased Ca absorption, VDR-KO mice have impaired body growth and bone formation [21]. However, VDR-KO mice have the same serum/bone Ca levels as the WT when fed high Ca diets [37]. Continuous feeding with Ca enriched diets partially rescues the bodyweight and bone formation phenotypes [21]. Utilizing VDR-KO mice, here we simultaneously tested Ca bioavailability in *cod5* in the hind limbs and duodenum. The phenotypes associated with VDR-KO mice disruption are most pronounced in the intestine as this is a key tissue that regulates Ca uptake [30,32]. Our results indicate that the higher levels of bioavailable Ca in *cod5* plants lead to increased Ca absorption in duodenum and incorporation in hind limbs in VDR-KO mice. Moreover, in the 20-day feeding study, our results showed that *cod5* was able to provide more bioavailable Ca to VDR-KO mice than WT Medicago and support bone mass and body weight gain in a similar manner to diets supplemented with exogenous Ca. Overall, our results further support the hypothesis that single gene alterations in CaOx formation in plants can boost Ca bioavailability.

Previous work with C57BL/6 mice has noted sex differences in bone formation. This may indicate a sex-based difference in preserving structural properties of the skeleton [38]. In our feeding study, we also detected male VDR-KO mice were more sensitive to Ca uptake and this might be a result of the male mice's need for more Ca to support its higher bodyweight than females (Data not shown). However, both male and female VDR-KO mice fed *cod5* demonstrated better Ca uptake and bone health and the benefits of the cod5 diets in VDR-KO mice is not gender specific (Figure 1). Our test groups were relatively small and we noted substantial individual variability in feeding behaviors that likely resulted in P value being higher than 0.05. The P value for the long term bodyweight and BMD tests were P=0.06 and P=0.17 respectively. These values would have been smaller than 0.01. However, one VDR-KO mouse fed the cod5 diet did not consume the food during the test period and the animal's bodyweight and BMD didn't increase (data not shown). Thus, while are results are not statistically significant, the trend is apparent. Large quantities of plant material for the long-term feedings were required and plant rearing space was an issue. In order to provide the diets for 20 days we compromised on the number

of animals analyzed. Nonetheless, our data demonstrate a difference between *cod5* and WT Medicago lines (Figure 2).

Disruption of one allele of the VDR-KO gene has been shown to impact bone development and impart metabolic consequences [39]. VDR heterozgyous mutant (VDR HET) male mice have normal skeletal development until 16 weeks of age but show significantly less gain in fat mass than WT mice. In contrast, female VDR HET mice show decreased total-body BMD at 8 weeks. In the future, studies using *cod5* diets in the VDR HET mice may be informative.

Using foods to attenuate genetic disorders is not a novel concept. Several genetic diseases have been linked to altered mineral nutrition and can be attenuated by dietary therapies. For example, Mitral valve prolapse syndrome (MVP) is a frequent disorder characterized by a number of complaints that lessen the quality of life. Many patients with MVP have low serum magnesium, and supplementation of this ion leads to improvement in most symptoms [40]. However, using modified foods to suppress genetic disorders is an exciting prospect that will require further testing.

If Ca levels are low, Ca supplements may be prescribed to ameliorate phenotypes. Sometimes, Ca based phosphorus binders are prescribed to treat both low Ca and high phosphorus levels. Our work here suggests that foods high in Ca but low in CaOx may also be used as a dietary regime to help patients with chronically low Ca levels.

The low Ca bioavailability in common plant food sources makes it difficult for most vegetarians to meet their Ca intake demands. The work performed with Medicago can serve as a guide for biofortification effort in food plants. Based on our findings, we can predict the benefits in removing oxalate in other common vegetables such as spinach. Currently, work is undergoing to determine the genetic lesion in the *cod5* lines. Once the COD5 gene is cloned and characterized, work can be undertaken to modify the COD5 homologs in spinach and other vegetables [17].

Conclusions

Our results further confirm that *cod5* Medicago has increased Ca bioavailability compared to WT Medicago. In a long term feeding regime, removal of oxalate from the plant matrix increased absorbed Ca and deposited bone Ca in the consuming animal, thus conveying the idea that genetic changes in food crops can help attenuate effects in genetically susceptible individuals. Our study supports the concept of decreasing antinutrients in crops as an exciting strategy for long-term biofortification strategies to improve nutrient intake.

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