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Transgenic Alfalfa That Accumulates Piceid (Trans-Resveratrol-3-O–β-D-glucopyranoside) Requires the Presence of β -Glucosidase to Inhibit the Formation of Aberrant Crypt Foci in the Colon of CF-1 Mice

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Abbreviations: Aberrant crypt foci (ACF), *trans*-resveratrol-aglycone (Rag), Piceid = *trans*-resveratrol-3-0-β-D-glucoside (RG), *resveratrol-synthase* (RS), basal diet (BD), control, non-transgenic alfalfa (CA), transgenic resveratrol-glucoside accumulating alfalfa (TA).

Keywords: resveratrol, piceid, genetically-engineered crops, aberrant crypt foci, colon cancer

Abstract

Plants have been genetically enhanced to produce a number of products for agricultural, industrial and pharmaceutical purposes. This technology could potentially be applied to providing chemoprevention strategies to the general population. Resveratrol (3,5,4' – trihydroxystilbene) is a compound that has been shown to have protective activity against a number of cancers and could be an ideal candidate for such an application. Alfalfa that was genetically modified to express *resveratrol-synthase* was used as a model in applying biotechnological approaches to cancer prevention. The transgenic alfalfa, which accumulates resveratrol as a glucoside (piceid = *trans*-resveratrol-3-*O*-β-D-glucopyranoside) (152 ± 17.5 μg piceid/g dry weight), was incorporated into a standard mouse diet at 20% of the diet by weight and fed for 5 weeks to 6-week old, female CF-1 mice (N=17-30) that were injected with a single dose of azoxymethane (5 mg/kg body weight). While the addition of resveratrol-aglycone (20 mg/kg diet) to the basal diet reduced the number of aberrant crypt foci/mouse, the transgenic alfalfa did not inhibit the

number, size or multiplicity of aberrant crypt foci in the colon of the CF-1 mice relative to control alfalfa which does not accumulate resveratrol-glucoside. However, diets containing transgenic alfalfa with an exogenous β-glucosidase (860 U/kg diet) did significantly inhibit the number of aberrant crypt foci in the distal 2 cm of the colon of the mice relative to mice fed diets containing the transgenic alfalfa without the enzyme ($p<0.05$; Fisher's Combination of p-values). The β-glucosidase alone appeared to have no effect on the inhibition of aberrant crypt foci. These results suggest that piceid in transgenic piceid-accumulating alfalfa was not bioavailable.

Introduction

Colorectal cancer is the third most common cause of cancer-related illness in adults living in the United States (1). Data from case-control studies have suggested that diets rich in fruits and vegetables are protective against colon cancer (2), although findings from recent prospective studies reveal that this correlation may not indicate a causal relationship (3,4). Plants produce an array of phytochemicals as secondary metabolites for defense purposes (5). Several of these compounds have been shown to have cancer-preventing activity in laboratory studies (6). However, the concentration or bioavailability of these health-beneficial constituents is often very low in edible plants (7), which may partially explain the inconsistency between epidemiological and recent prospective studies. In recent years, there has been increased interest in developing strategies to grow crops for health-promoting purposes (8). Genetic-engineering approaches have been successfully employed to increase the yield or introduce polyphenolic compounds into plant crops (9). A successful chemoprevention strategy could involve genetically modifying crops to increase the availability of some of these bioactive constituents.

One of the most studied phytochemicals in recent years has been resveratrol (3,5,4'-trihydroxystilbene). Resveratrol is a phytoalexin synthesized in a variety of plant species in response to external stresses such as injury, UV irradiation and fungal infection (10). In the human diet, resveratrol was found in highest concentrations in red wine, grapes and peanuts (11,12). Epidemiological studies have shown an inverse correlation between the intake of red wine and the incidence of cardiovascular disease (13). It is proposed that resveratrol is partly responsible for the health benefit acquired from red wine.

Interest in resveratrol as a chemopreventive or therapeutic agent stems from an earlier report that showed that resveratrol inhibited cellular events associated with all stages of carcinogenesis - tumor initiation, promotion and progression (14). Since then, resveratrol has been shown to have growth inhibitory activity in a variety of human cancer cell lines and in animal models of carcinogenesis (15 and references therein).

A transgenic alfalfa that accumulates resveratrol (152 ± 17.5 µg resveratrol-glucoside /g dry weight) was developed to protect alfalfa against root rot (16). Alfalfa normally

does not express *resveratrol-synthase* (RS) and, therefore, does not produce resveratrol. In the transgenic alfalfa, RS catalyzes the synthesis of resveratrol from the metabolic precursors p-coumaroyl CoA and malonyl CoA. Resveratrol in these plants is accumulated as *trans*-resveratrol-3-*O*-β-D-glucopyranoside (also known as piceid (RG)).

The aim of the present study was to investigate the potential effects of the alfalfa that was genetically-modified to express RS on colon carcinogenesis in the mouse model of azoxymethane (AOM)-induced carcinogenesis using aberrant crypt foci (ACF) as short-term markers. ACF are putative preneoplastic lesions that directly correlate to risk of colon cancer and tumor size in humans (17). Resveratrol was previously reported to inhibit the number and multiplicity (aberrant crypts per focus) of AOM-induced ACF in the colorectal mucosa of F344 rats (18). Mucin-depleted foci (MDF) and β-catenin accumulating crypts (BCAC) are subsets of ACF that have been identified on the bases of morphological and biochemical changes, respectively (19). Both MDF and BCAC are purported precancerous lesions but are currently not well characterized. Since ACF are widely accepted biomarkers for assessing the chemopreventive potential of agents in the colon, for our initial studies of the transgenic resveratrol-accumulating alfalfa, we elected to use classic ACF lesions to assay the potential potency of this crop against colon cancer.

We report here that transgenic alfalfa that accumulates RG in combination with an exogenous β-glucosidase inhibited the number of ACF in the distal colon of CF-1 mice. This apparent protective effect against AOM-induced ACF was not observed with the transgenic alfalfa or the β-glucosidase alone. These results suggest that RG is not bioavailable and that the modification of polyphenolic-glucosides by endogenous enzymes may be needed to realize the potential health benefits of this transgenic crop.

Materials and Methods

Reagents and Chemicals:

Trans-resveratrol-aglycone (Rag) was purchased from the Toronto Research Institute (Ontario, Canada). The purity of the resveratrol was estimated to be > 98% by the manufacturer. α-galactosidase from *Aspirgillus niger* was purchased from the National Enzyme Company (Forsyth, MO). High-performance liquid chromatography (HPLC) grade acetone and acetonitrile were purchased from Fisher Scientific (Liberty Lane Hampton, NH). All diet ingredients were purchased from Harland Tekland (Madison, WI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Transgenic Alfalfa:

A single alfalfa (*Medicago sativa*) genotype from the Regen SY germplasm (20) was transformed with *resveratrol-synthase* (*RS*) cDNA from peanut (*Arachis hypogaena*) and analyzed for the presence and expression of RS and the accumulation of the RG metabolite at the Samuel Roberts Noble Foundation in Oklahoma as previously described (16). Clones of the transformed plant as well as untransformed control clones of the same

genotype were grown at the Samuel Roberts Noble Foundation in Oklahoma and shipped to Iowa following drying and grinding as described below. They were planted in the field at Ames, IA in adjacent plots, last harvested in October 2004. When plants reached the late bud or early flower stage, they were harvested by clipping aboveground biomass at 5 cm. Biomass was subsequently washed with water and dried in a forage drying oven at 55°C. The dried plant material was coarsely ground in a Wiley mill and then re-ground to a powder in a UDY mill with a 1mm exit filter.

Mice and Diets:

CF-1 mice (6 weeks old) were obtained from the Charles Rivers laboratory (Wilmington, MA) and housed individually in stainless steel wire-mesh cages in a temperature controlled room with a 12-hour light:dark cycle. After one week of acclimatization, the mice received one intraperitoneal injection of AOM (5 mg/kg body weight) or saline. Three days after the injections, the mice were randomized and assigned to experimental diets, which they were fed ad-libitum, for 5 weeks (N=3-10/group in 3 replicates). Mice were assigned to one of four diets: 1). A basal diet (BD) based on the standard diet recommended by the American Society for Nutritional Sciences report for mature rats (AIN-93) (21), 2). BD with control alfalfa (CA), 3). BD with transgenic alfalfa (TA) and 4). BD with Rag.

Diets were prepared by mixing all dry ingredients with water (25% by dry weight of diet). Diets were then rolled out, cut into thin strips and dried at room temperature. Alfalfa was added at 20% by dry weight into the diets in partial replacement of dextrin (60% of dextrin added to control diets) (**Table 1**). The TA that was added to the diets contained 152 ± 17.5 µg RG /g dry weight, thus these diets contained 30.5 ± 3.5 mg RG/kg diet. *Trans*-Resveratrol-aglycone (Rag) was added to the diets in equal molar concentration (20 mg/kg diet) to the RG by dry weight of the diet. In subsequent experiments, β-glucosidase was added to diets at 860 U/kg of dry diet. Since purified β-glucosidase was not commercially feasible at the time of this study, α-galactosidase, which was verified to have 12.5 ± 0.5 U β-glucosidase activity/mg, was used as a source of glucosidase activity. The amount of α-galactosidase (2600 α-galactosidase U/kg of diet) added to diets was determined by extrapolating the recommended human dose of Beano™ to mice on a daily energy basis (equal to 0.675 α-galactosidase U /Kcal in humans).

Resveratrol-glucoside identification in diets by HPLC analysis

To confirm the stability and uniform distribution of RG and Rag in diets, 2 g of crushed diet sample was extracted in 50 ml of a 90% methanol/10% water (v/v) solution at room temperature for 2 days under minimal light exposure. The samples were centrifuged at 1000 x g and the supernatants were evaporated and reconstituted in 1 ml methanol. The extracts were filtered and 10 µl of each extract was injected into a C18 column (2.1*150mm; Alltech Altima). Compounds were separated using a 45-minute gradient from 20-60% of acetontrile in water with a flow rate of 0.3 ml/min. Eluding

peaks were monitored at λ=308 and 280 nm with a UV diode array detector (Beckman Instruments, Fullerton, CA). The spectra of peaks at 308 nm were compared to those of a *trans*-resveratrol-glucoside standard (Apin Chemicals, UK).

Analysis of ß-glucosidase activity in diets

To estimate the β-glucosidase activity of α-galactosidase, the procedures described by King (22) were followed with some modifications. Briefly, various concentrations of α-galactosidase (250-2500 ng/ml) were incubated with 1 mM of 4-methylumbelliferyl-β-D-glucoside for 30 minutes at 37°C. The reaction was stopped using sodium citrate, and the florescence of the final product (4-methylumbeliferone) was measured using a Sequoia Turner Model 450 fluoromoter set at gain 1 with 360 nm excitation and 450 nm emission.

To measure β-glucosidase activity in the diets, 0.2 g of diet sample was crushed in a 0.1 M citric acid/0.2 M sodium phosphate buffer (pH=5.0). The suspension was vortexed and 50 µl was added to 450 µl of 1 mM 4-methylumbelliferyl-β-glucoside and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1.5 M sodium citrate. The β-glucosidase activity of assayed samples was quantified by comparing the fluorescent readings of the final reaction to the concentration vs. the fluorescence curve of a 4-methylumbeliferone standard.

Analysis of Aberrant Crypt Foci

After feeding the experimental diets for five weeks, mice were killed by decapitation. The colon and rectum were collected from each mouse and rinsed thoroughly with phosphate buffer saline (PBS). The colon and rectums were dissected longitudinally and fixed flat in 10% buffered formalin (pH 7.5) for 24 hours. The samples were stained with 0.2% methylene blue for 10 minutes and the ACF/colon were scored for each mouse at 10-fold magnification as described by Bird (23). ACF number, size, multiplicity (number of AC/focus) and distribution were recorded in 2 cm increments, starting at the rectum in a blinded fashion. The sizes of ACF were scored with an eyepiece graticule.

Statistical Analysis

The data presented in this paper were analyzed using SAS software (SAS Institute) and are expressed as means ± SE. The first experiment was conducted in three replicates and the significance of the differences between ACF means, changes in body weight (final weight-initial weight) and food consumption (g/food/day) were assessed by performing a two-way analysis of variance (ANOVA). Specific differences between groups were analyzed using a student's t-test as the post-hoc test. Because the availability of alfalfa at harvest times was limited due to the weak growth performance of both the non-transformed and transformed alfalfa lines, follow-up comparisons of diets with and without α-galactosidase were conducted in multiple studies following different harvests. In these follow-up studies, group means within experiments were analyzed using a one-way ANOVA followed by a student's t-test for specific comparisons. Treatment comparisons between follow-up experiments were made using Fisher's procedure for

combining p-values (24). All statistical tests performed on the data were two-sided, and a value of $P<0.05$ was considered statistically significant.

Results

B-glucosidase activity of α-galactosidase

The relationship between the amount of 4-methylumbelliferone (4-MU) formed vs. α-galactosidase concentration was linear in the concentration range of 125 - 2,000 ng enzyme/ml following incubation with the substrate 4-methylumbelliferone-β-D-glucoside (4-MUG) for 30 minutes at 37°C (Figure 1). The standard curve plot for the relationship between fluorescence vs. the amount of 4-MU was linear, in the range of 0.003-0.05 µmoles of 4-MU (Figure 1, insert). This plot was used to convert fluorescent readings to 4-MU produced in the β-glucosidase-catalyzed deglycosylation of 4-MUG. Based on this assessment, the β-glucosidase activity of α-galactosidase was estimated to be 12.5 ± 0.5 β-glucosidase U (µmoles 4-MU liberated from 4-MUG) mg enzyme^{-1} min^{-1}. To test the reliability of our assay, a β-glucosidase enzyme (G0395, Sigma Aldrich) with a reported activity of 2.1 β-glucosidase U mg enzyme^{-1} min^{-1} was measured as well. The measured fluorescence of the β-glucosidase enzyme was linear in the concentration range of 200-4000 ng/ml (figure 2) with an estimated activity of 1.9 ± 0.03 β-glucosidase U mg enzyme^{-1} min^{-1}.

Effect of diets on body weight and food consumption

The mice gradually gained weight throughout the duration of the experiment but, as **table 2** demonstrates, the change in body weight [(final weight (29.6 ± 2.4 g) – initial weight (26.4 ± 2.1 g)] between treatment groups was not significant (mean gain= 3.2 ± 1.6 g, p=0.5). Food intake (g/mouse/day) did not differ between treatments in the first experiment (4.0 ± 0.4 g/day/mouse, p-value=0.23). Also, there was no difference in change in body weight or in food intake in mice fed diets supplemented with α-galactosidase compared to mice that were fed diets that did not contain the enzyme in the follow-up experiments (data not shown).

Effect of treatments on formation of aberrant crypt foci in experiment 1

The number of aberrant crypts was scored for different regions of the colon as defined by 2 cm increments starting at the most distal end adjacent to the rectum. Aberrant crypts formed mainly in the distal 2 cm of the colon. The number of ACF in the most distal 2 cm of the colon was significantly lower in mice fed basal diets supplemented with Rag relative to the other diets (p-value < 0.05) (**table 2**). However, the number of ACF in the distal colon of mice fed TA was not significantly different from mice fed CA (p=0.5). Additionally, the number of ACF in the colon of mice fed CA with Rag (means= 2.5 ± 1.8 and 5.8 ± 1.5 in two replicates N= 5 and 9) was not significantly reduced compared to mice fed CA diets without Rag (means= 4.8 ± 2.5 and 8.8 ± 1.7 in two replicates N=10 each, p= 0.5 for first replicate comparison and p=0.2 for second replicate comparison).

There was no significant difference in the multiplicity (mean of all treatments= 1.4 ± 0.4 AC/focus, p=0.3) of ACF or number of ACF in the middle 2 cm (mean of all treatments=2.4 ± 2.5, p= 0.3) and proximal 1 cm (mean of all treatments=0.3 ± 0.7, p=0.1) of the colon in the CF-1 mice. There was also no significant difference in the size of ACF (mean=2.0 x 10^{-2} ± 0.7 x 10^{-2} mm^2, p=0.4), although this was assessed in only two of the three replicates. ACF were detected in 8% (4/50) of mice injected with saline and were not confined to any single treatment.

The amount of alfalfa added to the diets (20% by dry weight) was derived from previous studies in our lab showing that the number of AOM-induced ACF lesions in the colon of CF-1 mice fed diets containing resveratrol concentrations as low as 20 mg Rag/kg of diet (equivalent to the number of moles of piceid in diets containing 20% of the transgenic alfalfa) were significantly reduced (data not shown). Resveratrol at higher concentrations (40- 100 mg Rag/kg diet) significantly inhibited the number of ACF lesions formed, but the differences between these groups were not significant. Furthermore, we were concerned that supplementing alfalfa beyond 20% by dry weight would compromise the nutritional integrity of the diets.

Effect of treatments with β-glucosidase on formation of aberrant crypt foci in follow-up experiments.

As with the first experiment, the incidence of ACF was mainly limited to the distal 2 cm of the colon in all follow-up studies. The number of ACF in the entire colon and distal region of the colon was significantly reduced in the mice fed diets containing TA with α-galactosidase compared to the number of ACF in mice fed diets containing TA without exogenous α-galactosidase (**Table 3**). The difference in the number of ACF in the entire colon or distal 2 cm of the colon was not significant between the BD and the BD containing α-galactosidase nor was the number of ACF different between diets containing 20% CA and diets containing the CA with α-galactosidase. ACF number did not differ between treatments with or without α-galactosidase in the middle or proximal region of the colon in any of these studies (data not shown). Size of ACF was assessed in only one replicate for each treatment in follow-up studies. As with the first study, there was no significant difference in ACF size (mean= 2.7 x 10^{-2} ± 0.6 x 10^{-2} mm^2 , p=0.8) or multiplicity (mean= 1.4 ± 0.5; p=0.2) between treatments in the follow-up studies.

Discussion

The principal finding of this study was that TA that accumulates RG in combination with an exogenous β-glucosidase was effective in inhibiting the formation of ACF in the distal colon of CF-1 mice. Neither of these treatments alone was effective in reducing ACF. The number of ACF was reduced by an average of 32% and 87% in mice fed the TA with the exogenous glucosidase relative to mice fed the TA alone in two replicates where these groups were included together. ACF number was reduced by 52% in mice fed diets with purified Rag that was added in equal molar concentrations to that of the RG

in TA. The protective effect of the TA in combination with β-glucosidase was not due to the presence of other plant metabolites present in alfalfa since CA supplemented at 20% by dry weight into the diet did not inhibit ACF development in the colon of CF-1 mice regardless of whether or not β-glucosidase was added. These results imply that RG may not be bioavailable and modification of secondary metabolites by endogenous enzymes in the plant is an important consideration when assessing the potential health-benefits of a crop genetically-enhanced to produce high levels of these compounds. To our knowledge this is the first *in-vivo* demonstration of the potential health benefits of a genetically modified crop against a biomarker of cancer.

Resveratrol is found in low and variable amounts in relatively few sources in the human diet. Resveratrol content is very low in peanuts (< 1μg/g wt) and grapes (< 10 μg/g fresh wt), which are two major dietary sources of resveratrol (12,25). Furthermore, the majority of resveratrol found in grapes is in the form of *cis*- or *trans*-piceid with the aglycone comprising only a small fraction of the detectible levels of resveratrol (< 0.6 μg Rag/g fresh wt). In the human diet, resveratrol is found in highest concentrations in red wine. However, analysis of different red wines reveal that *trans*-resveratrol-aglycone content of wines can vary (0.1 – 14 mg/L) depending upon the cultivar of grape used, the climate in which the grapes were grown, and the maceration process followed (12, 26, 27, 28). *Trans*-piceid has also been identified at appreciable levels in red wine, in some cases as high as 50.8 mg/L. Genetic-engineering strategies could provide a means to increase the dietary availability of resveratrol to humans through additional food options.

In addition to alfalfa, several other plants have been genetically modified to express *resveratrol-synthase* for agronomical purposes, including tobacco (29), rice (30), barley (31), kiwi (32), and apple (33). Resveratrol was reported to accumulate as a glucoside in all of these plants when the presence of the resveratrol was assessed.

The present study suggested that RG was not as bioavailable or as bioactive as Rag for ACF prevention, since the addition of an exogenous β-glucosidase to TA was required to achieve a reduction in AOM-induced ACF in the colorectal mucosa of CF-1 mice. TA alone was not protective against AOM-induced ACF. We hypothesize that the supplemented glucosidase catalyzed the liberation of the more bioavailable aglycone moiety from RG in diets containing the TA. Diets which contained the exogenous glucosidase with CA did not reduce the number of ACF in the colon of CF-1 mice, suggesting that the protective effect of the glucosidase was associated with the RG in the transgenic alfalfa. This was a surprising observation as alfalfa contains numerous secondary metabolites (34,35). The total concentration of flavonoids in alfalfa has been shown to range from 0.24 to 0.78% in dry matter with the majority of identified compounds being glycosides of apigenin, luteolin and tricin (34). The TA used in our study contained, on average, 0.015% RG by dry weight. It is possible that most of the natural-occurring polyphenols in alfalfa are not bioactive or some of the more active compounds are not present in very high concentrations.

Few studies have reported on the bioactivity or bioavailability of RG. RG was shown to inhibit platelet aggregation (36) and inhibit thymine incorporation into DNA in Lewis lung carcinoma cells (37). However, the effective dose in the later study was 100 µM (IC > 1000 µM), bringing into question the potential bioactivity of RG. In the Kimura et al study (37), RG inhibited tumor growth in the paw of C57BL/6 mice that were transplanted with Lewis lung carcinoma cells, although his protective activity was achieved by the administration of very high doses of RG at 300 mg kg^{-1} daily for 32 days. Lower doses were not reported in this study. Assays conducted in Caco-2 monolayer systems reveal that the sodium-glucose co-transporter (38) and lactase phlorizin hydrolase (39) may play an important role in the absorption and deglycosylation of RG in the intestine. In the former study, the uptake of Rag in Caco-2 cells was more rapid than RG with the intercellular concentrations of Rag remaining at least 4 times higher than RG at all time points examined in Caco-2 cells that were treated with 150 µM of each compound (38). In a recent study, RG was detected in a variety of tissues, including the brain, heart, liver and lungs in Wistar rats within 20 minutes of the rats receiving a single, oral dose of piceid (50 mg/kg body weight) suspended in a 5% sodium carboxymethylcellulose solution (40). Plasma concentrations of RG reached a peak concentration of 0.9 ± 0.4 µM at 20 minutes post-dose administration. Unfortunately, side-by-side comparisons with Rag where not featured in this study. To our knowledge, the colon bioavailability of piceid has not yet been examined.

Rag was shown to inhibit chemically induced ACF in rodents in at least two other studies. Rag at daily doses of 200 µg kg^{-1} body weight via 10% ethanol in drinking water reduced the number, multiplicity and size of AOM-induced ACF in the colorectal mucosa of F344 rats (18). In a more recent study, Sengottuvelan et al showed that Rag (8 mg kg^{-1} body weight p.o., daily) reduced the number, multiplicity and size of ACF in rats injected weekly with 1,2-dimethylhydrazine (DMH) (20 mg kg^{-1} body weight for 15 weeks) (41). This group has also reported that Rag reduced the number of DMH-induced colonic tumors in rats regardless of whether Rag was administered before or following DMH injections (41, 42). In contrast, reports of resveratrol treatment on tumor incidence in C57BL/6J APC^{Min} mice with genetic colon cancers caused by mutant adenomotosis polyposis coli (APC) have been inconsistent (43, 44). Studies with Rag have been conducted in Min mice between the ages of 4-5 weeks, which may represent a more advanced stage of cancer since preneoplastic lesions begin to develop in the small intestine of these mice *in utero* (45) and may indicate that resveratrol may be more value as a preventive rather than a therapeutic agent in the colon. A number of biological activities have been ascribed to resveratrol which may, at least partially, explain any anti-cancer properties that the chemical may possess. Resveratrol has been demonstrated to prevent free-radical formation and have anti-mutagenic activity; inhibit activity of cytochrome P450 enzymes; inhibit cyclooxygenase-2 catalyzed reactions; induce phase-2 drug metabolism; induce cell cycle arrest and apoptosis (46 and references therein).

Additional studies are warranted to determine resveratrol's mechanism of action at the site of the colon.

In conclusion, we have demonstrated that transgenic alfalfa that accumulates resveratrol-glucoside did not inhibit AOM-induced ACF in the colorectal mucosa of CF-1 mice, but the addition of an exogenous glucosidase enzyme to diet preparations containing the transgenic alfalfa seemed to be protective against AOM induction of ACF in the distal colon of CF-1 mice. Our data suggests that future studies on the bioavailability and bioactivity of RG are essential in determining the therapeutic value of resveratrol as a nutraceutical. In addition, we have offered a strategy whereby other transgenic crops may be analyzed for their heath-benefiting value.

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Table 1: Comparison of Diets

	Basal	Diets with alfalfa
Casein (g/kg)	200	200
Dextrin (g/kg)	499.5	299.5
Alfalfa (g/kg)	-	200
Dextrose (g/kg)	150	150
Fiber (g/kg)	50	50
Mineral mix (g/kg)	35	35
Choline (g/kg)	2.5	2.5
Methionine (g/kg)	3.0	3.0
Vitamin mix (g/kg)	10	10
Corn oil (g/kg)	50	50

Trans-resveratrol-aglycone was added to some basal and control alfalfa diets at 0.002% by dry weight which was equivalent to the number of moles of piceid present in diets containing the transgenic alfalfa which accumulates 170 µg piceid/g wt of plant. In follow-up studies, α-galactosidase was added at 860 β-glucosidase U (2600 α-galactosidase U/kg) to basal, control alfalfa and transgenic alfalfa diets.

Table 2: Number of azoxymethane-induced Aberrant Crypt Foci and multiplicity (Average number of aberrant crypts per focus) in the colon of CF- mice.

Treatment	N	Gain in Body Weight (Initial Body weight) (g) (ANOVA p-value=0.5)	Total ACF/mouse (ANOVA p-value =0.09)	Region of colon (ACF/mouse)			Multiplicity (AC/focus) (ANOVA p-value=0.3)
				D (ANOVA p-value=.0023)	M (ANOVA p-value =0.3)	P (ANOVA p-value =.11)	
Basal (BD)	25	2.8 ± 0.3 (26.9 ± 0.4)	8.4 ± 1.1	5.4 ± 0.7	2.8 ± 0.5	0.2 ± 0.2	1.5 ± 0.1
Control Alfalfa (CA)	17	3.3 ± 0.5 (26.3 ± 0.5)	7.9 ± 1.3	4.3 ± 0.9	3.1 ± 0.6	0.5 ± 0.2	1.5 ± 0.1
Transgenic Alfalfa (TA)	21	3.1 ± 0.4 (27.1 ± 0.5)	7.2 ± 1.2	5.1 ± 0.8	1.9 ± 0.6	0.4 ± 0.2	1.5 ± 0.1
BD + resveratrol-aglycone	30	3.4 ± 0.3 (25.8 ± 0.4)	4.4 ± 1.0[1]	1.9 ± 0.6[2]	2.2 ± 0.5	0.3 ± 0.1	1.3 ± 0.1

Values represent mean ± SE (2-way ANOVA with student's t-test post-hoc analysis; SAS software). This experiment was conducted in three replicates with between 3 and 10 mice per group. The regions of the colon are defined as follows: D= distal 2 cm, M= second 2cm from distal end, P=proximal 1 cm. [1] $p<0.05$ compared to BD and CA groups for total ACF. [2]$p<0.05$ compared to BD, CA and TA treatments in rectal region of colon. ACF size was also assessed in some of the studies but no significant difference between treatments was observed in all of the studies where this parameter was examined ($p>0.33$ for all studies; one-way ANOVA with a

student's t-test as the post-hoc analysis; SAS software). Mice from each treatment were also injected with saline. Aberrant crypts were observed in 8% of saline treated mice (4/50), but were not confined to any single treatment.

Table 3: The addition of β-glucosidase activity to transgenic alfalfa diets reduced the number of ACF in the colon of CF- mice.

Treatment Comparison	Study	Without Enzyme N	Without Enzyme Mean ± SE (Total colon)	Without Enzyme Mean ± SE (Distal 2 cm)	With Enzyme N	With Enzyme Mean ± SE (Total colon)	With Enzyme Mean ± SE (Distal 2 cm)	p-value* (Total colon)	p-value* (Distal 2 cm)
			BD	**BD**		**BD+E**	**BD+E**		
BD vs. BD+E	1	10	6.9 ± 1.5	3.8 ± 0.9	10	9.9 ± 1.5	4.6 ± 0.92	> 0.1 (0.2, 0.1)	0.7 (0.5, 0.6)
	2	10	8.6 ± 1.3	5.9 ± 0.1	9	11.7 ± 1.4	6.6±1.0		
			CA	**CA**		**CA+E**	**CA+E**		
	1	10	3.3 ± 1.5	1.5±0.93	10	5.1 ± 1.5	2.2 ± 0.93		
CA vs. CA+E								>0.3	>0.5
	2	4	10.3 ± 2.4	7.3 ± 1.6	4	8.0 ± 2.1	5.3±1.6	(0.3, 0.5)	(0.6, 0.4)
			TA	**TA**		**TA+E**	**TA+E**		
	1	10	3.0 ± 0.9	1.9±0.8	10	0.8 ± 0.9	0.6 ± 0.4	< 0.01	<0.01
TA vs. TA+ E								(0.06,	(0.1,
	2	3	10.4 ± 2.4	8.7 ± 1.8	3	1.3 ± 2.4	1.0±1.8	<0.01)	<0.005)

Values represent mean ± SE. Diets are defined as follows: BD= basal diet, CA=control alfalfa, TA=transgenic alfalfa, E= α-galactosidase and R= resveratrol-aglycone. Diets with α-galactosidase contained 2600 α-galactosidase U/kg diet and 860 β-glucosidase U/kg diet. *Comparing no enzyme vs. enzyme treatments p-values were derived using Fisher's combination of tests of significance analysis. P-values for individual studies are listed in the parantheses under the combined p-values for each comparison.

Figure 1: β-glucosidase activity of α-galactosidase (National Enzyme Company) and β-glucosidase (G0395, Sigma Aldrich). B-glucosidase activity was expressed as a function of 4-methylumbelliferone formed after incubating the enzymes with the substrate 4-methylumbelliferone- β-glucoside for 30 minutes @ 37ºC. Values represent means ± SE (N=6). Linear parameters were as follows: (for α-galactosidase) slope= 2 x 10^{-5}, y-intercept= 0.0007 and R^2=0.9993; (for β-glucosidase) slope=3 X 10^{-6}, y-intercept=--.0001 and R^2=0.997. Fluorescent units (recorded at 360 nm excitation and 450 nm emission) were converted into µmoles of 4-methylumbelliferone formed based on the relationship between fluorescence and concentration of 4-MU authentic standards (see insert). This relationship was linear in the concentration range of 0.003-0.05 umoles of 4-MU (linear parameters were: slope= 34142, y-intercept = 136.89 and R^2= 0.9683).

Figure 1: