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Original Research Article

Effects of Transparent Testa8 (*TT8*) gene and Homeobox12 (*HB12*) gene silencing in alfalfa (*Medicago sativa* L.) on molecular structure spectral profile in relation to energy, degradation, and fermentation characteristics in ruminant systems

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is a legume forage that is widely cultivated owing to its high biomass yield and favorable nutrient values. However, alfalfa contains relatively high lignin, which limits its utilization. Downregulation of two transcriptional factors, Transparent Testa8 (*TT8*) and Homeobox12 (*HB12*), has been proposed to reduce lignin content in alfalfa. Therefore, silencing of *TT8* (*TT8*i) and *HB12 (HB12*i) in alfalfa was achieved by RNAi technology. The objective of this project was to determine effect of gene modification through silencing of *TT8* and *HB12* genes in alfalfa plants on lignin and phenolic content, bioenergic value, nutrient supply from rumen degradable and undegradable fractions, and in vitro ammonia production in response to the silencing of *TT8* and *HB12* genes in alfalfa. All gene silenced alfalfa plants (5 *TT8*i and 11 *HB12*i) were grown under greenhouse conditions with wild type as a control. Samples were analyzed for bioactive compounds, degradation fractions, truly digestible nutrients, energetic values and in vitro ammonia productions in ruminant systems. Furthermore, relationships between physiochemical, metabolic and fermentation characteristics and molecular spectral parameters were determined using vibrational molecular spectroscopy. Results showed that the *HB12*i had higher lignin, while *TT8*i had higher phenolics. Both silenced genotypes had higher rumen slowly degraded carbohydrate fractions and truly digestible neutral detergent fiber, but lower rumen degradable protein fractions. Moreover, the *HB12*i had lower truly digestible crude protein, energetic values and ammonia production compared with other silenced genotypes. In addition, in relation to the nutritive values of alfalfa, structural carbohydrate parameters were negatively correlated, whereas alpha/beta ratio in protein structure was positively correlated. Furthermore, good predictions were obtained for degradation of protein and carbohydrate fractions and energy values from molecular spectral parameters. In conclusion, silencing of the *TT8* and *HB12* genes decreased protein availability and increased fiber availability. Silencing of the *HB12* gene also increased lignin and decreased energy and rumen ammonia production. Moreover, nutritional alterations were closely correlated with molecular spectral parameters. Therefore, gene modification through silencing the *TT8* and *HB12* genes in alfalfa influenced physiochemical, metabolic and fermentation characteristics.

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1. Introduction

Alfalfa (*Medicago sativa* L.), also known as the "queen of forage", is one of the most cultivated legume forage crops in the world thanks to its agronomic and nutritional merits. Firstly, alfalfa forage has high nutritive values and good palatability, making alfalfa an important fodder for dairy cows (Sánchez-Duarte and García, 2017).

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Secondly, alfalfa possesses a deep root system that allows it to grow under adverse soil and climate conditions, including arid and semi-arid areas (Hamidi and Safarnejad, 2010). Furthermore, the symbiotic relationship of alfalfa with rhizobia reduces the need for nitrogen fertilizers (Zhang et al., 2016). However, the utilization of alfalfa is limited by its rapidly degradable protein and relatively high lignin content (Lei et al., 2017, 2018a). Rapid ruminal protein degradation can lead to rumen bloat in grazing cows, resulting in huge economic losses to farmers (Jonker et al., 2012); whereas the high lignin content of alfalfa hinders the degradation and digestion of carbohydrates and other nutrients (Lei et al., 2017).

Lignin is an important aromatic compound in the plant cell wall (Boerjan et al., 2003), and is synthesized via the phenylpropanoid pathway (Lei et al., 2017). Transparent Testa (*TT8*) and Homeobox 12 (*HB12*) are two transcriptional factors in this pathway. Observation from *Brassica napus* showed a negative relationship between lignin content and gene expression of *TT8* and *HB12* (Li et al., 2016a; Lei et al., 2018a, 2018b, 2019). Therefore, we transformed alfalfa with *TT8* and *HB12* RNAi constructs to silence these 2 genes in alfalfa. Our previous publications showed that such genetic modifications affected the inherent molecular structures of alfalfa (Li et al., 2016a, 2018a). Silencing of *HB12* and *TT8* increased most structural carbohydrate (STC) spectral parameters and multivariate analyses of spectra successfully distinguished wild type alfalfa from gene silenced alfalfa (Lei et al., 2018a).

In our previous studies, we reported (1) how gene silencing affected chemical profiles and total in vitro gas production relating to molecular structures of alfalfa plants (Lei et al., 2018b) and (2) how gene silencing affected protein degradation and digestion, microbial protein synthesis, and metabolic protein in relation to molecular structures of alfalfa (Lei et al., 2019). The specific objectives of this study were to determine the effect of gene modification through silencing of *TT8* and *HB12* genes in alfalfa on (1) lignin and phenolic content, (2) bioenergic value, (3) carbohydrate and protein rumen degradable and undegradable fraction supply, and (4) in vitro ammonia production in response to silencing of *TT8* and *HB12* genes in alfalfa. Moreover, the relationships between these ruminant relevant nutritional profiles and spectral parameters were also determined. We hypothesized that such gene transformations would affect lignin and phenolic compounds, rumen degradations of the Cornell Net Carbohydrate and Protein System (CNCPS) fractions, bioenergetic values and in vitro ammonia production of alfalfa. It was hypothesized that the alterations in the nutritional profiles would also be closely related to molecular spectral parameters of alfalfa.

2. Materials and methods

2.1. Animal ethics statement

The University of Saskatchewan Animal Care Committee approved the animal trial under the Animal Use Protocol No. 19910012 and animals were cared for and handled in accordance with the Canadian Council of Animal Care (CCAC, 1993) regulations. Authors confirm that Canadian standards for the protection of animals and/or feed legislation have been met.

2.2. Gene silencing, alfalfa transformation, and plant harvests

Alfalfa plant samples were obtained from the Agriculture and Agri-Food Canada-Saskatoon Research and Development Centre (Saskatoon, Canada). The detailed processes of alfalfa transformation, growth conditions and harvest information were previously described (Li et al., 2015, Lei et al., 2018a, 2018b, 2019). Briefly, to make RNAi constructs, cDNA was synthesized from total alfalfa RNA that was extracted from the fourth internode. Fragments of *HB12* and *TT8* genes were amplified and used to make RNAi constructs as described previously (Li et al., 2015). The RNAi constructs were then transferred to *Agrobacterium tumefaciens* via electroporation, and the resulting *A. tumefaciens* strains were used to transform alfalfa explants according to Aung et al. (2015a).

TT8-silenced (*TT8*i) and *HB12*-silenced (*HB12*i) transgenic plants were grown with untransformed wild type control (WT) in a greenhouse under conditions of 21 to 23 °C, 16 h light daily and 70% humidity. Alfalfa plants were harvested at early-to-mid vegetative stage, freeze-dried and ground through a 1-mm screen. Due to the regulation and restriction on transgenic plant DNA research by the Canadian Food Inspection Agency (CFIA), we could not allow transgenic alfalfa to flower, which is why we harvested alfalfa at the early-to-mid-vegetative stage. There were 11 replicates of *HB12*i, 5 replicates of *TT8*i and 4 replicates of WT plants. Samples were ground through a 1-mm screen (Retsch ZM 200, Retsch Inc., Newtown, PA, USA) for chemical analysis and in vitro fermentation and through a 0.02-mm screen for spectra collection with attenuated total reflection (ATR) -Fourier transform infrared (ATR-FTIR) spectroscopy (Lei et al., 2018a, 2018b).

2.3. Analysis of cell wall residue, lignin and phenolic compounds

Cell wall residue (CWR) of alfalfa samples was isolated with phosphate buffer and Triton X-100 method as described by Brinkmann et al. (2002). About 10 mg of CWR was used to determine lignin content of alfalfa samples using the thioglycolate-alkaline (TGA) method as descried by Brinkmann et al. (2002). Lignin content was quantified with the OD reading at 280 nm using a UV/VIS spectrophotometer (Helios Zeta, Thermo Scientific, Madison, WI, USA). A calibration curve ($R^2 = 0.9999$) developed with commercial lignin (Sigma–Aldrich, Oakville, ON, Canada) was used to calculate lignin content in alfalfa samples. Lignin content was calculated based on both CWR and dry matter (DM).

Total phenolic content was quantified with the Folin-Ciocalteu (F–C) method according to Aung et al. (2015b). About 50 mg of ground samples were incubated in 3.5 mL of 80% (vol:vol) methanol and vortexed for 20 min at 2,500 rpm using a multi-tube vortexer. Extracts were then centrifuged 500 × *g* at room temperature for 15 min and the supernatant was used for the quantification of total phenolic content. The supernatant was combined with F–C reagent and sodium carbonate for color development and then an OD reading was taken at 765 nm with a UV/VIS spectrophotometer (Helios Zeta, Thermo Scientific, Madison, WI, USA). A calibration curve ($R^2 = 0.9982$) developed with gallic acid in 50% methanol was used for the calculation.

2.4. Prediction of nutrient supply from ruminal degradations of CNCPS fractions

Chemical compositions and CNCPS fractions can be found in our previous publication (Lei et al., 2018b). The updated v6.5 of CNCPS was used to study nutrient supply from CNCPS carbohydrate and protein fractions in this study (Higgs et al., 2015; Van Amburgh et al., 2015).

In this study, nutrient supply from rumen degradable and undegradable CNCPS fractions were calculated based on their degradation rates and passage rates. The equation for ruminal degradation of each fraction was $D = K_d/(K_d + K_p)$, where D was the degradation; K_d was the fractional degradation rate; K_p was the passage rate out of rumen. The fractional degradation rates K_d of each fraction were obtained from the database of NDS professional (RUM&N, RE, Italy), and the passage rate K_p of alfalfa was set at 4.5% per hour (Tamminga et al., 1994; Jonker et al., 2010). Nutrient

supply of rumen degradable CNCPS fractions was then calculated as the products of CNCPS fractions (%DM) and their corresponding degradations. Rumen undegradable CNCPS fractions were calculated as the differences between CNCPS fractions and rumen degradable fractions. Rumen degradable total carbohydrate (RDCHO) and rumen degradable total crude protein (RDCP) were calculated as the sums of all degradable fractions, while rumen undegradable carbohydrate (RUCHO) and rumen undegradable crude protein (RUCP) were the sums of undegradable fractions.

2.5. Determination of truly digestible nutrients and energetic values

Truly digestible nutrients and energetic values of transformed and WT alfalfa were calculated according to NRC (2001). Truly digestible nutrients were truly digestible neutral detergent fiber (tdNDF), truly digestible non-fiber carbohydrate (tdNFC), truly digestible crude protein (tdCP) and truly digestible fatty acids (tdFA). Bio-energetic values were DE_{1x}, DE_{3x}, ME_{1x}, ME_{3x}, NE_{Lp}, NE_{m}, and NE_{g}. The subscripts 1x and 3x mean the feed intake level of the animal at 1 time and 3 times maintenance level, respectively. Three times the level of maintenance was regarded as the production level of feed intake (NRC, 2001). Subscripts of Lp, m, and g with NE mean the net energy for lactation at the productive level of intake (3× in this case), for maintenance and for body weight gain, respectively.

2.6. In vitro ammonia production

The in vitro fermentation was conducted at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre (Lethbridge, Canada) according to Wang et al. (2006). Approximately 0.3 g of ground samples were placed into F57 Ankom bags (Ankom technology Inc., Macedon, NY, USA), and incubated in 125 mL glass bottles with rumen fluid and buffer mixer (1:2, vol:vol) at 39 °C in an incubator for 2 experimental runs. Rumen fluid was collected from 3 rumen-cannulated Angus heifers and filtered with 4-fold cheese cloths. Duplicate bottles of each sample were withdrawn from the incubator after 2, 4, 8, 12, 24 and 48 h of incubation. At time points of 4, 12, 24 and 48 h, 1.6 mL of liquid sample was collected into 0.32 mL of 1% (vol:vol) sulphuric acid for ammonia analysis. In the previous study, total gas production affected by gene silencing was reported (Lei et al., 2018b). The use of Angus heifers in this study as rumen fluid donors was approved by Lethbridge Research and Development Centre according to the Canadian Council on Animal Care (CCAC).

2.7. Spectra collection and univariate spectral analysis

Spectra collection, univariate analysis procedures and spectral parameter data were previously reported by Lei et al. (2018a). Briefly, 5 spectra were collected for each alfalfa sample with JASCO FT/IR-4200 with ATR (JASCO Corp., Tokyo, Japan). Spectral parameters calculated in the univariate analysis included total carbohydrate peaks (TC1 to TC4) and area (TCA), cellulosic compounds peak (CEC) and area (CECA), structural carbohydrate peaks (STC1 to STC4) and area (STCA), amide peaks (amide I and II) and areas (amide I area, AIA; amide II area, AIIA; total amide area, AA), carbonyl C=O (CCO) region and (a)symmetric CH_2 and CH_3 peaks (asymmetric CH_2, $AsCH_2$; symmetric CH_2, $SyCH_2$; asymmetric CH_3, $AsCH_3$; symmetric CH_3, $SyCH_3$) and area (ASCCA). These molecular spectral data were used in this study for the relationship study with bioenergic value, nutrient supply from carbohydrate and protein rumen degradable and undegradable fraction, and in vitro ammonia production in response to silencing of *TT8* and *HB12* genes in alfalfa.

2.8. Statistical analyses

2.8.1. Correlations between nutritional profiles and spectral parameters

Correlations between nutritional profiles and spectral parameters were performed with R software (R Core Team, 2017) in Rstudio environment (Rstudio team, Inc., Boston, MA, USA). The rcorr( ) function from the HMISC package was used to determine the correlation efficient and significance level. Prior to correlation analysis, normality tests of variables were performed with the shapiro.test( ) function from the STATS package. Correlations between normally distributed variables were performed with the Pearson method, while between other pairs of variables were performed with the Spearman method. Finally, the Corrplot( ) function was used to plot the correlation matrix between nutrition profiles and spectral parameters. Significance level was set at $P < 0.05$.

2.8.2. Multilinear regressions of predicting nutritional profiles from spectral parameters

Multilinear regressions of predicting nutritional profiles from spectral parameters were performed with R software (R Core Team, 2017) under Rstudio environment (Rstudio team, Inc., Boston, MA, USA). The lm( ) function from the STATS package was used to perform multilinear regression. Before that, the aliased predictors that were completely linearly correlated with other predictors, were detected by using the alias( ) function from the STATS package and removed from the model. Afterwards, the vif( ) function from the CAR package was used to detect the variance inflation factor (VIF) values of predictors. Predictors with the highest VIF values that were greater than 10 were removed one-by-one from the model until VIF values of all predictors left in the model were less than 10. Then, the step( ) function from the STATS package was used to select linear models with the option of "direction = both". Finally, the insignificant predictors were removed from the model with a significance level of $P < 0.05$.

2.8.3. Statistical analysis with SAS software

The Mixed procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA) was used to analyze data. The model for CWR, lignin, phenolics, CNCPS degradations and energy values was $Y_{ij} = \mu + Trt_i + \varepsilon_{ij}$, where Y_{ij} was the nutritive variable; μ was the population mean; Trt_i was the genotype effect; ε_{ij} was the random error. For ammonia production data, the model was $Y_{ijk} = \mu + Trt_i + \mathrm{Run_j} + \varepsilon_{ijk}$, where $\mathrm{Run_j}$ was the random effect of experiment run. Degree of freedom was estimated with the Kenward Roger method. Prior to variance analysis, outliers were detected and removed when studentized residuals were greater than 2.5. Contrast statement was used to determine differences between WT and transformed alfalfa, and the Tukey–Kramer method was used for multi-treatment comparison. The pdmix800 macro (Saxton, 1998) was used to letter-group treatment means. Normality test of residual data was performed using the Shapiro Wilk method by using Proc Univariate procedure with Normal and Plot options. Significance level was set at $P < 0.05$ and trend was set at $0.05 < P < 0.10$.

3. Results and discussion

3.1. Effect of silencing TT8 and HB12 on CWR, lignin and phenolics

Content of CWR, lignin (based on DM and CWR) and phenolics are shown in Table 1. In general, CWR accounted for about 70% of sample DM and there were no significant differences in CWR between alfalfa genotypes ($P > 0.05$). As for lignin content, *HB12*i had higher lignin both based on CWR and DM compared with WT and *TT8*i ($P < 0.05$), with no differences between WT and *TT8*i ($P > 0.05$).

Table 1
Effect of silencing Transparent Testa8 (*TT8*) and Homeobox12 (*HB12*) on cell wall residue, lignin and total phenolic content of alfalfa.

Item	WT	Transformed alfalfa[1]		SEM	*P*-value	Contrast[2]
		*HB12*i	*TT8*i			W vs. G
CWR, % DM	71.45	72.76	72.26	1.694	0.858	0.653
Lignin content						
Lignin, mg/g CWR	9.26[b]	14.04[a]	9.57[b]	0.952	0.001	0.067
Lignin, mg/g DM	6.62[b]	10.24[a]	6.94[b]	0.765	0.003	0.076
Phenolic extraction, mg/g DM						
Phenolics	2.77[b]	2.99[b]	3.28[a]	0.069	0.001	0.001

CWR = cell wall residue; WT = wild type; SEM = standard error of the mean.
Means with different letters in each row are significantly different at $P < 0.05$.
[1] Transformed alfalfa: 11 *HB12*i and 5 *TT8*i.
[2] Contrast between wild type and gene transformed alfalfa.

Table 2
Effects of silencing Transparent Testa8 (*TT8*) and Homeobox12 (*HB12*) on rumen degradable and undegradable CNCPS fractions of alfalfa (% DM).

Item[1]	WT	Transgenic alfalfa[2]		SEM	*P*-value	Contrast W vs. G[3]
		*HB12*i	*TT8*i			
Rumen degradable carbohydrate fractions						
RDCA4	3.78[b]	4.93[a]	4.55[ab]	0.258	0.02	0.01
RDCB1	7.51[a]	1.07[b]	1.34[b]	0.259	<0.001	<0.001
RDCB2	23.56[b]	27.63[a]	26.32[a]	0.491	<0.001	<0.001
RDCB3	8.64[b]	11.03[a]	11.63[a]	0.440	<0.001	<0.001
RDCHO	44.23	44.32	43.84	0.541	0.75	0.85
Rumen undegradable carbohydrate fractions						
RUCA4	0.42[b]	0.55[a]	0.51[ab]	0.029	0.02	0.01
RUCB1	1.12[a]	0.16[b]	0.20[b]	0.039	<0.001	<0.001
RUCB2	3.03[b]	3.55[a]	3.38[a]	0.063	<0.001	<0.001
RUCB3	5.55[b]	7.1[a]	7.47[a]	0.284	<0.001	<0.001
RUCC	10.47[b]	14.14[a]	12.64[ab]	0.635	<0.001	<0.001
RUCHO	20.45[b]	25.37[a]	24.21[a]	0.634	<0.001	<0.001
Rumen degradable protein fractions						
RDPA2	7.93	7.28	8.05	0.249	0.05	0.44
RDPB1	10.1[a]	7.26[c]	8.28[b]	0.249	<0.001	<0.001
RDPB2	0.82	0.95	0.91	0.068	0.45	0.26
RDCP	19.07[a]	15.61[c]	17.25[b]	0.398	<0.001	<0.001
Rumen undegradable protein fractions						
RUPA2	1.52	1.4	1.55	0.048	0.05	0.46
RUPB1	3.32[a]	2.39[c]	2.72[b]	0.082	<0.001	<0.001
RUPB2	0.53	0.61	0.59	0.044	0.49	0.31
RUPC	0.44[b]	0.71[a]	0.65[a]	0.029	<0.001	<0.001
RUCP	5.95[a]	5.05[b]	5.5[ab]	0.14	<0.001	<0.001

CNCPS = Cornell Net Carbohydrate and Protein System; WT = wild type.
Means with different letters in each row are significantly different at $P < 0.05$.
[1] CA4, water-soluble carbohydrate, sugar; CB1, starch; CB2, soluble fiber; CB3, digestible fiber; CC, indigestible fiber; PA2, soluble true protein; PB1, insoluble true protein, PB2, fiber-bound protein, PC, indigestible protein; CHO, carbohydrate; CP, crude protein; RD, rumen degradable fractions; RU, rumen undegradable fractions.
[2] Transformed alfalfa: 11 *HB12*i and 5 *TT8i*.
[3] Contrast between wild type and gene transformed alfalfa.

In contrast, both *HB12*i and WT had lower phenolic content compared with *TT8*i ($P < 0.05$). The lignin pattern between alfalfa genotypes was consistent with our previous publication (Lei et al., 2018b), in which lignin was analyzed as acid detergent lignin (ADL). To our surprise, silencing of *HB12* resulted in higher lignin content, while silencing *TT8* had no impacts on lignin synthesis. The *HB12* protein belongs to the Homeodomain-Leucine Zipper (HD-Zip) family and is inducible with abscisic acid (ABA) treatment and water stress (Olsson et al., 2004; Son et al., 2010; Park et al., 2011). In contrast, the *TT8* protein functions as a transcriptional factor in the phenylpropanoid pathway, which serves as the source of lignin, flavonoids, phenolics and other metabolites (Vogt, 2010). The *TT8* gene encodes a basic helix-loop-helix (bHLH) protein, which works with MYB protein and WD40 as a ternary complex to control expression of late genes in the phenylpropanoid pathway (Xu et al., 2015). The reason that silencing the *TT8* gene increased phenolic content might be the redistribution of intermediates in the phenylpropanoid pathway to synthesise flavonoids and phenolics rather than anthocyanins and proanthocyanins.

3.2. Effect of silencing TT8 and HB12 on nutrient supply of rumen degradations of CNCPS fractions

The nutrient supply of rumen degradable and undegradable CNCPS fractions of transgenic and WT alfalfa are shown in Table 2. In general, *HB12*i and *TT8*i alfalfa had higher degradable carbohydrate fractions and lower protein fractions. The *HB12*i had higher RDCA4 compared to WT ($P < 0.05$), but *TT8* was neither significantly different from *HB12*i nor WT ($P > 0.05$). Both *HB12*i and *TT8*i had lower RDCB1, but higher RDCB2 and RDCB3 in comparison with WT ($P < 0.05$). Similar patterns of results were also found in rumen undegradable carbohydrate fractions, which resulted from discrepancies in CNCPS fractions (Lei et al., 2018b). Both *HB12*i and *TT8*i were equally higher for RUCB2, RUCB3, RUCHO and lower for RUCB1 compared to WT ($P < 0.05$). The *HB12*i had higher RUCA4 and RUCC compared to WT ($P < 0.05$), while *TT8*i showed no significant difference relative to *HB12*i and WT ($P > 0.05$). In terms of ruminal degradation of protein fractions, both transgenic genotypes had lower RDPB1, RDCP and RUPB1 with *HB12*i even lower than *TT8*i, and both had higher RUPC compared with WT ($P < 0.05$). Moreover, *HB12*i had lower RUCP compared with WT ($P < 0.05$), while *TT8*i was not different from *HB12*i and WT ($P > 0.05$).

Our results suggested that both transgenic alfalfa genotypes provided more slowly degradable carbohydrates and less rumen degradable protein, which is consistent with our previous publication on chemical composition and CNCPS fractions (Lei et al., 2018b). Chemically, both transgenic alfalfa genotypes had higher fiber but lower CP content (Lei et al., 2018b). A previous pilot study conducted on a smaller alfalfa population ($n = 2$) reported on rumen degradable CNCPS carbohydrate fractions (Li et al., 2015) and found similar results in RDCB1 and RUCB1. However, the pilot study failed to detect the differences in RUCB2, RDCB2, RDCB3, RUCB3 and RUCHO between alfalfa genotypes (Li et al., 2015). Furthermore, lower RDCHO was observed in transgenic alfalfa in the pilot study, which was not found to be different between alfalfa genotypes in the current study. The discrepancies between our studies might be attributed to the differences in population size. The current study used a larger alfalfa population (5 *TT8*i, 11 *HB12*i and 4 WT) compared with that of the pilot study ($n = 2$ for each alfalfa genotype). Lei et al. (2018a) reported that the population size of samples had an impact on statistical results of spectral parameters in alfalfa. This effect of population size might be attributed to variations in the extent of genetic modification, as shown in miR156 studies (Aung et al., 2015a, 2015b).

3.3. Effect of silencing TT8 and HB12 on truly digestible nutrients and bioenergetic values

Truly digestible nutrients, total digestible nutrients and energetic values of transgenic and WT alfalfa are shown in Table 3. Transformed alfalfa had equally higher tdNDF and lower tdCP in comparison with WT ($P < 0.01$). Moreover, *HB12*i was lower than WT in tdFA ($P < 0.05$), while *TT8*i was not different from neither *HB12*i nor WT ($P > 0.05$). In terms of TDN and bioenergetic values, *HB12*i was lower compared with *TT8*i and WT ($P < 0.05$). The *TT8*i had comparable TDN and energy values to WT ($P > 0.05$), although *TT8*i was numerically lower. The increased tdNDF of *TT8*i compensated for its decreases in tdCP and tdFA, thereby maintaining energy level. Yu et al. (2003) reported the effect of alfalfa variety and

Table 3
Effect of silencing Transparent Testa8 (*TT8*) and Homeobox12 (*HB12*) on truly digestible nutrients and energetic values in alfalfa.

Item[1]	WT	Transgenic alfalfa		SEM[2]	*P*-value	Contrast W vs. G[3]
		*HB12*i	*TT8*i			
Truly digestible nutrients, % DM						
tdNFC	37.52	36.76	35.58	0.974	0.45	0.31
tdCP	24.49[a]	19.53[b]	21.99[a]	0.645	<0.001	<0.001
tdFA	1.18[a]	0.18[b]	0.66[ab]	0.155	<0.001	<0.001
tdNDF	10.46[b]	13.48[a]	13.87[a]	0.515	<0.001	<0.001
Total digestible nutrients, % DM						
TDN_{1x}	68.12[a]	63.19[b]	65.93[a]	0.757	<0.001	<0.001
Bio-energetic values, Mcal/kg						
DE_{1x}	3.2[a]	2.92[b]	3.07[a]	0.038	<0.001	<0.001
DE_{3x}	3.01[a]	2.82[b]	2.92[a]	0.025	<0.001	<0.001
ME_{3x}	2.59[a]	2.4[b]	2.5[a]	0.025	<0.001	<0.001
NE_{Lp}	1.63[a]	1.5[b]	1.57[a]	0.017	<0.001	<0.001
NE_m	1.71[a]	1.51[b]	1.62[a]	0.028	<0.001	<0.001
NE_g	1.1[a]	0.92[b]	1.02[a]	0.025	<0.001	<0.001

WT = wild type.
Means with different letters in each row are significantly different at $P < 0.05$.
[1] tdNFC, truly digestible non-fiber carbohydrate; tdCP, truly digestible crude protein; tdFA, truly digestible fatty acid; tdNDF, truly digestible neutral detergent fiber; TDN_{1x}, total digestible nutrients at 1 time of maintenance level; DE_{1x}, digestible energy at 1 time maintenance level; DE_{3x}, digestible energy at 3 times maintenance level; ME_{3x}, metabolizable energy at 3 times maintenance level; NE_{Lp}, net energy for lactation at production level of intake; NE_m, net energy for maintenance; NE_g, net energy for growth; 3x represents the production level of feed intake.
[2] Transformed alfalfa: 11 *HB12*i and 5 *TT8*i.
[3] Contrast between wild type and gene transformed alfalfa.

maturity on energetic values of alfalfa, and energy values of those alfalfa samples were lower than our results. This difference might have resulted from the differences in harvest time. The earliest harvest date in Yu's study was early bud stage, while our samples were harvested at early-to-mid vegetative phase. Overall, the estimated energetic values of alfalfa in the present study were equivalent to published values (Belyea et al., 1999; Vahdani et al., 2014). In the pilot study (Li et al., 2016a), both tdNFC and tdCP were lower in transgenic alfalfa, and no differences were found in tdFA and tdNDF. Moreover, there were no differences between alfalfa genotypes in truly digestible nutrients and energetic values in the pilot study, although energy values were numerically lower in transgenic alfalfa, especially in *HB12*i (Li et al., 2016a). The differences in energy values between the two studies again could also be attributed to population size.

3.4. Effect of silencing TT8 and HB12 on in vitro ammonia production

The ammonia production during in vitro fermentation is shown in Table 4. Ammonia production was calculated both as milligram per gram DM sample fermented (mg/g DM) and milligram per gram nitrogen fermented (mg/g N). At the 4 h point, *HB12*i had lower ammonia production both based on DM and N compared with WT and *TT8*i ($P < 0.05$). At the 24 h point, *HB12*i had lower production than WT when calculated based on DM ($P < 0.05$). At the 48 h point, *HB12*i had the lowest ammonia production based on DM, while *TT8*i was the highest ($P < 0.05$). When calculated based on N, there were no differences between WT and *TT8*i, both of which were higher than *HB12*i ($P < 0.05$).

Our ammonia production results of alfalfa were comparable to those in a previous study (Wang et al., 2006). The lower ammonia production of *HB12*i could be explained by its low content of rumen degradable protein. When feed particles reach the rumen, dietary proteins are degraded by microbes eventually to ammonia nitrogen, which serves as the nitrogen source for most ruminal bacteria (Yang et al., 2010). Therefore, lower ammonia production was

Table 4
Effects of silencing Transparent Testa8 (*TT8*) and Homeobox12 (*HB12*) on ammonia production of alfalfa during in vitro fermentation: Comparison of gene transformed and wild type (WT) alfalfa.

Time	WT	Transgenic alfalfa		SEM[1]	*P*-value	Contrast W vs G[2]
		*HB12*i	*TT8*i			
Ammonia production, mg/g DM						
4 h	9.54[a]	4.90[b]	9.55[a]	2.078	<0.001	0.076
12 h	7.75	6.27	6.58	2.685	0.592	0.373
24 h	13.46[a]	8.68[b]	11.04[ab]	1.737	0.010	0.028
48 h	22.37[b]	11.39[c]	32.12[a]	2.358	<0.001	0.847
Ammonia production, mg/g N						
4 h	244.40[a]	150.48[b]	267.10[a]	63.405	0.006	0.334
12 h	193.00	193.26	176.00	79.847	0.928	0.848
24 h	335.45	264.32	305.15	49.956	0.271	0.259
48 h	624.11[a]	350.85[b]	879.63[a]	70.483	<0.001	0.925

Means with different letters in each row are significantly different at $P < 0.05$.
[1] Transformed alfalfa: 11 *HB12*i and 5 *TT8*i.
[2] Contrast between wild type and gene transformed alfalfa.

observed for *HB12*i as less rumen degradable protein was provided. When ammonia production exceeds the requirement of bacteria, excessive ammonia will be absorbed via rumen epithelium and converted to urea in the liver (Tas et al., 2006). Unlike the in vivo environment, the in vitro incubator does not absorb extra ammonia during the fermentation, which could lead to an increase in ammonia concentration over time.

3.5. Correlations between spectral parameters and nutritional profiles of alfalfa

Correlations between rumen degradation of CNCPS carbohydrate fractions and spectral parameters are shown in Fig. 1. TC2 and TC3 were positively correlated with RDCA4, RDCB2, RUCA4 and RUCB2 ($r > 0.6$, $P < 0.05$), but negatively correlated with RDCB1 and RUCB1 ($r = -0.5$, $P < 0.05$). STC parameters (except for STC2) were positively correlated with RDCA4, RDCB2, RUCA4, RUCB2 and RUPC ($r > 0.5$, $P < 0.05$), but negatively correlated with RDCB1, RDPB1, RDCP, RUCB1, RUPB1 and RUCP ($r < -0.5$, $P < 0.05$). On the contrary, ratios of amide I to amide II (amide I/II) and alpha to beta (alpha/beta) were positively correlated with RDCB1, RDPB1, RDCP, RUCB1, RUPB1 and RUCP ($r > 0.6$, $P < 0.05$). As for ASCC parameters, they were positively correlated with RDCA4, RDCB3, RUCA4 and RUCB3 ($r > 0.5$, $P < 0.05$), but negatively correlated with RDCP, RUCB1 and RUCP ($r < -0.5$, $P < 0.05$).

In general, rumen degradable carbohydrate (RDC) fractions had similar correlations to rumen undegradable carbohydrate fractions with spectral parameters. This similarity largely resulted from modeling calculations of rumen degradable and undegradable CNCPS fractions. Both fractions are highly dependent on the original contents of CNCPS fractions (Lei et al., 2018b). The pilot study found positive correlations between STC3 and RDCB1 and RUCB1, which was the opposite to our study (Li et al., 2015). CB1 is the starch fraction in the carbohydrate pool (Higgs et al., 2015), which was negatively correlated with STC peaks in the current study. Yari et al. (2013) reported that the PB1 fraction (insoluble protein) of alfalfa was positively correlated with the alpha/beta ratio, but negatively correlated with the amide I/II ratio. Conflicting results have also been reported in terms of relationships between PB1 and protein spectral parameters for different feedstuffs (Doiron et al., 2009; Yu and Nuez-Ortín, 2010; Theodoridou and Yu, 2013; Li et al., 2016b). This conflict might be attributed to differences in spectral processing methods and types of ingredients. Spectra were not normalized in previous studies, which could lead to errors in the determination of spectral parameters. The normalization process in spectral analysis could remove the

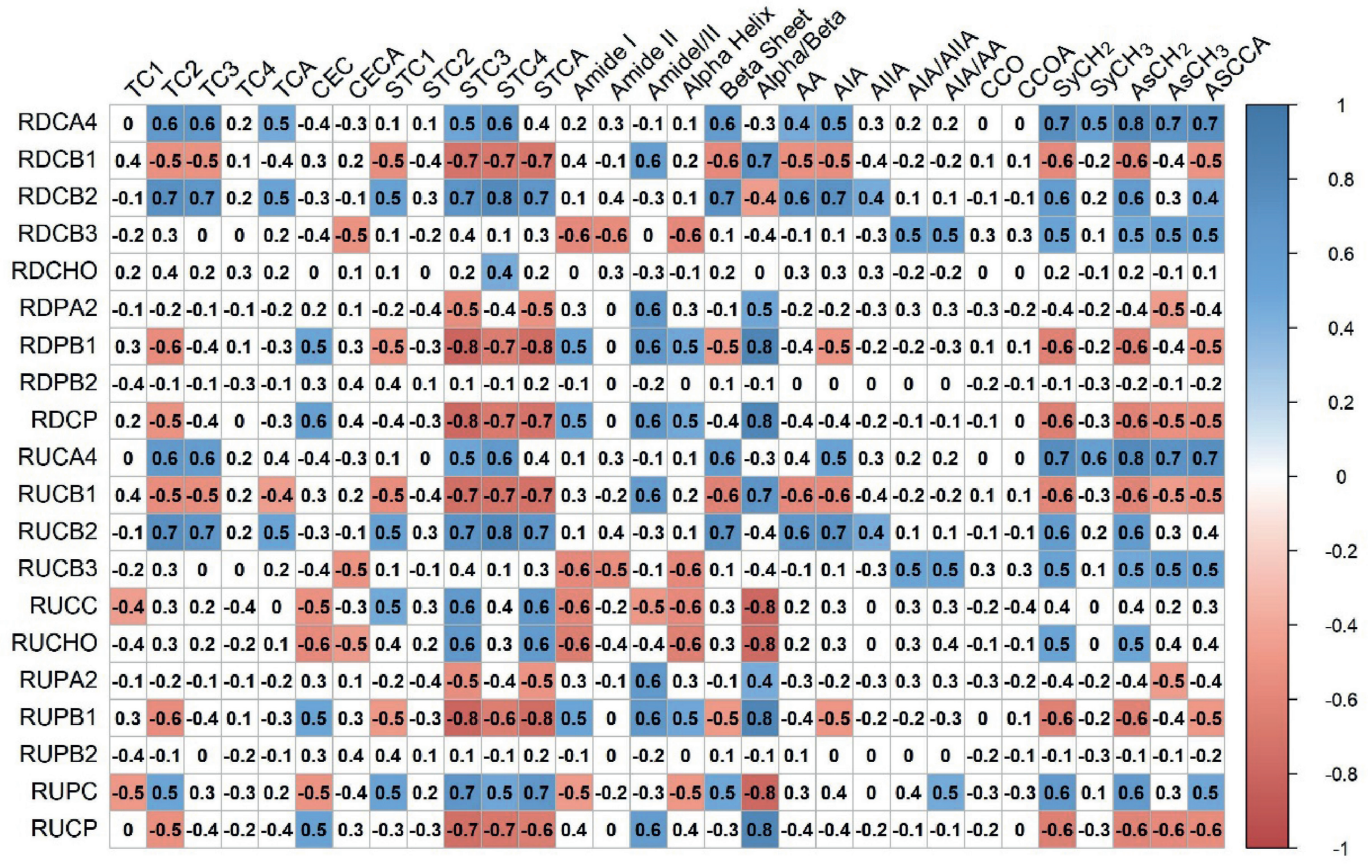

Fig. 1. Correlations between molecular structures and rumen degradations of CNCPS fractions of alfalfa. CNCPS = Cornell Net Carbohydrate and Protein System. **Color scales:** blue means positive correlation while red means negative correlation; colorless cells contain correlation coefficients that are not significant at $P < 0.05$. Values are correlation coefficients, and the deeper the color, the higher the absolute coefficient value. **CNCPS fractions:** CA4, water-soluble carbohydrate, sugar; CB1, starch; CB2, soluble fiber; CB3, digestible fiber; CC, indigestible fiber; PA2, soluble true protein; PB1, insoluble true protein; PA2, fiber bound protein; PC, indigestible protein; CHO, carbohydrate, CP, crude protein; RD, rumen degradable fractions; RU, rumen undegradable fractions. **Structural parameters:** TC1 to TC4, 4 major peaks in TC region at ca. 1,026 (TC1) 1,074 (TC2), 1,104 (TC3) and 1,149 (TC4) cm^{-1}, respectively; TCA, TC peak area (ca. 1,178 to 941 cm^{-1}); CEC, cellulosic compounds (ca. 1,237 cm^{-1}); CECA, CEC peak area (ca. 1,283 to 1,178 cm^{-1}); STC1-STC4, 4 major peaks at ca. 1,317 (STC1), 1,370 (STC2), 1,397 (STC3) and 1,453 (STC4) cm^{-1}, respectively; STCA, STC peak area (ca. 1,484 to 1,178 cm^{-1}); alpha/beta, ratio of alpha helix to beta sheet; AA, whole amide peak area (ca. 1,710 to 1,484 cm^{-1}); AIA, amide I peak area (ca. 1,710 to 1,575 cm^{-1}); AIIA, amide II peak area (ca. 1,575 to 1,484 cm^{-1}); CCO, carbonyl C=O (centers at ca. 1,733 cm^{-1}); CCOA, peak area of CCO region (baseline ca. 1,781 to 1,710 cm^{-1}); $SyCH_2$, symmetric CH_2 (ca. 2,850 cm^{-1}); $SyCH_3$, symmetric CH_3 (ca. 2,872 cm^{-1}); $AsCH_2$, asymmetric CH_2 (ca. 2,920 cm^{-1}); $AsCH_3$, asymmetric CH_3 (ca. 2,955 cm^{-1}); ASCCA, peak area of (a)symmetric CH_2 and CH_3 (ASCC, ca. 3,000 to 2,761 cm^{-1}).

impact of sample depth during spectral collection, allowing for comparison between spectra on a common intensity scale (Prates et al., 2018a).

Figure 2 shows correlations between spectral parameters and energetic profiles of alfalfa. The CEC, amide I, amide I/II, alpha helix and alpha/beta were positively correlated with tdCP, TDN and all energetic values ($r > 0.5$, $P < 0.05$), whereas STC parameters (except for STC2), SyCH2 and AsCH2 were negatively correlated with those nutritional profiles ($r < -0.5$, $P < 0.05$). Moreover, ASCC parameters (except for SyCH3) were positively correlated with tdNDF ($r = 0.5$, $P < 0.05$). Fig. 3 shows the correlations of CWR, lignin, phenolics and in vitro ammonia production with spectral parameters of alfalfa. Lignin content was positively correlated with STC parameters (except for STC2) ($r > 0.5$, $P < 0.05$), but negatively correlated with CEC and alpha/beta ratio ($r < -0.5$, $P < 0.05$). Although most spectral parameters were not significantly correlated with ammonia production, STC parameters and AIIA were negatively correlated with ammonia production at 4 h and 48 h of fermentation ($r < -0.5$, $P < 0.05$).

Samadi et al. (2013) reported that the alpha/beta ratio was negatively correlated with TDN and energy values in canola seed, which is the opposite to our results. However, other studies found positive correlations between alpha/beta ratio and TDN, although correlation coefficients were not significant (Zhang and Yu, 2012; Xin and Yu, 2013). In the pilot study (Li et al., 2016a), STC1 and STC2 were negatively correlated with tdNDF, while CEC was negatively correlated with tdFA. Once again, these discrepancies in relationships between spectral parameters and nutritive profiles might result from the spectral processing methods, ingredient types and population sizes. Notably, all previous studies only conducted correlations between few spectral parameters and their corresponding nutritive profiles, like amide parameters with protein profiles or carbohydrate parameters with carbohydrate profiles or lipid parameters with fat profiles (Yu and Nuez-Ortín, 2010; Xin et al., 2014; Refat et al., 2017). However, relationships between chemical compounds and spectral parameters are based on the absorption of light energy of chemical bonds (Stuart, 2004), which means spectral absorptions of chemical compounds are not limited to their experiential regions. This is because chemicals normally contain more than one type of chemical bond and one chemical bond exists in more than one compound. Therefore, we explored the correlation between nutritive profiles and spectral parameters from the whole mid-IR range (4,000 to 700 cm^{-1}) in the current study.

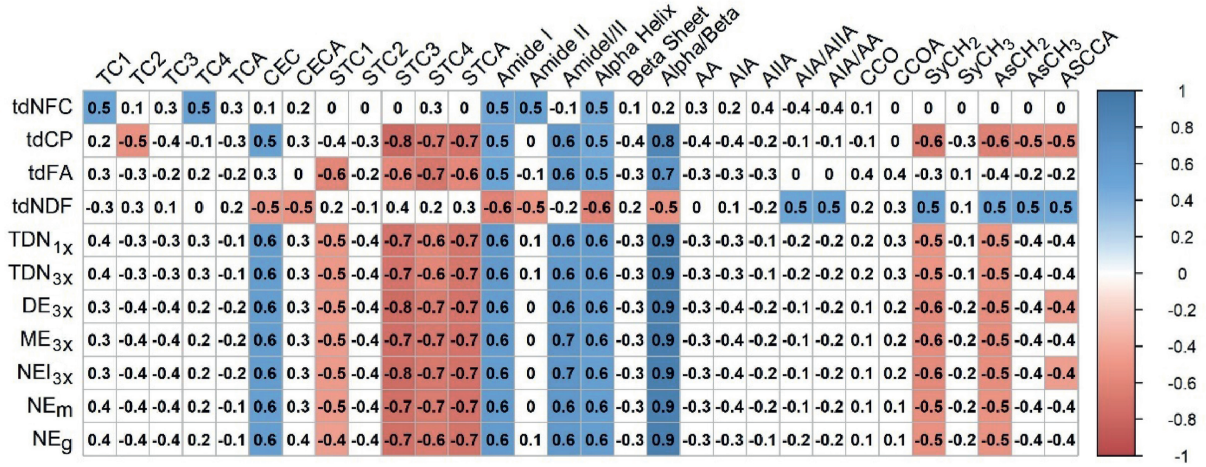

Fig. 2. Correlations between energetic profiles and structural parameters of alfalfa. **Color scales:** blue means positive correlation while red means negative correlation; colorless cells contain correlation coefficients that are not significant at $P < 0.05$. Values are correlation coefficients. The deeper the color, the higher the absolute coefficient value. **Energetic values:** tdNFC, truly digestible non fiber carbohydrate; tdCP, truly digestible crude protein; tdFA, truly digestible fatty acids; tdNDF, truly digestible neutral detergent fiber; TDN_{1x}, total digestible nutrients at one time of maintenance; TDN_{3x}, total digestible nutrients at 3 times of maintenance; DE_{3x}, digestible energy at 3 times of maintenance; ME_{3x}, metabolizable energy at 3 times of maintenance; NE_{L3x}, net energy for lactation at 3 times of maintenance; NE_m, net energy for maintenance; NE_g, net energy for growth. **Structural parameters:** TC1 to TC4, 4 major peaks in TC region at ca. 1,026 (TC1) 1,074 (TC2), 1,104 (TC3) and 1,149 (TC4) cm^{-1}, respectively; TCA, TC peak area (ca. 1,178 to 941 cm^{-1}); CEC, cellulosic compounds (ca. 1,237 cm^{-1}); CECA, CEC peak area (ca. 1,283 to 1,178 cm^{-1}); STC1 to STC4, 4 major peaks at ca. 1,317 (STC1), 1,370 (STC2), 1397 (STC3) and 1,453 (STC4) cm^{-1}, respectively; STCA, STC peak area (ca. 1,484 to 1,178 cm^{-1}); alpha/beta, ratio of alpha helix to beta sheet; AA, whole amide peak area (ca. 1710 to 1,484 cm^{-1}); AIA, amide I peak area (ca. 1,710 to 1,575 cm^{-1}); AIIA, amide II peak area (ca. 1,575 to 1,484 cm^{-1}); CCO, carbonyl C=O (centers at ca. 1,733 cm^{-1}); CCOA, peak area of CCO region (baseline ca. 1,781 to 1,710 cm^{-1}); $SyCH_2$, symmetric CH_2 (ca. 2,850 cm^{-1}); $SyCH_3$, symmetric CH_3 (ca. 2,872 cm^{-1}); $AsCH_2$, asymmetric CH_2 (ca. 2,920 cm^{-1}); $AsCH_3$, asymmetric CH3 (ca. 2,955 cm^{-1}); ASCCA, peak area of (a)symmetric CH_2 and CH_3 (ASCC, ca. 3,000 to 2,761 cm^{-1}).

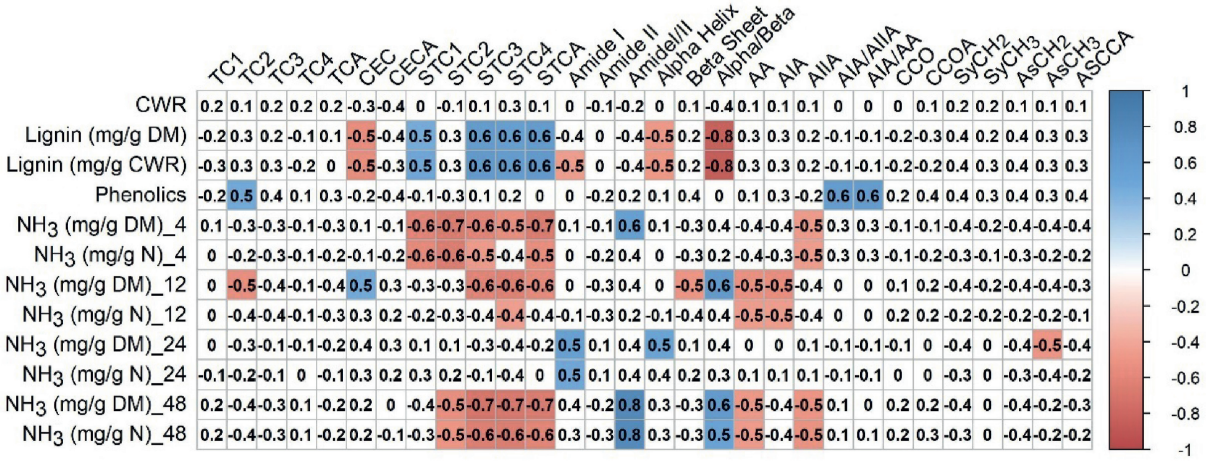

Fig. 3. Correlations between cell wall residue, lignin, phenolics and ammonia production during in vitro fermentation and structural parameters of alfalfa. **Color scales:** blue means positive correlation while red means negative correlation; colorless cells contain correlation coefficients that are not significant at $P < 0.05$. Values are correlation coefficients. The deeper the color, the higher the absolute coefficient value **Nutritional profiles:** CWR, cell wall residue; numbers at the end of each ammonia item means the time point (h) of in vitro fermentation. **Structural parameters:** TC1 to TC4, 4 major peaks in TC region at ca. 1,026 (TC1) 1,074 (TC2), 1,104 (TC3) and 1,149 (TC4) cm^{-1}, respectively; TCA, TC peak area (ca. 1,178 to 941 cm^{-1}); CEC, cellulosic compounds (ca. 1,237 cm^{-1}); CECA, CEC peak area (ca. 1,283 to 1,178 cm^{-1}); STC1 to STC4, 4 major peaks at ca. 1,317 (STC1), 1,370 (STC2), 1,397 (STC3) and 1,453 (STC4) cm^{-1}, respectively; STCA, STC peak area (ca. 1,484 to 1,178 cm^{-1}); Alpha/Beta, ratio of alpha helix to beta sheet; AA, whole amide peak area (ca. 1,710 to 1,484 cm^{-1}); AIA, amide I peak area (ca. 1,710 to 1,575 cm^{-1}); AIIA, amide II peak area (ca. 1,575 to 1,484 cm^{-1}); CCO, carbonyl C=O (centers at ca. 1,733 cm^{-1}); CCOA, peak area of CCO region (baseline ca. 1,781 to1,710 cm^{-1}); $SyCH_2$, symmetric CH_2 (ca. 2,850 cm^{-1}); $SyCH_3$, symmetric CH_3 (ca. 2,872 cm^{-1}); $AsCH_2$, asymmetric CH_2 (ca. 2,920 cm^{-1}); $AsCH_3$, asymmetric CH_3 (ca. 2,955 cm^{-1}); ASCCA, peak area of (a)symmetric CH_2 and CH_3 (ASCC, ca. 3,000 to 2,761 cm^{-1}).

3.6. Prediction of nutritional profile from spectral parameters

Only peak heights were selected to predict nutritional profiles as peak areas had complete collinearities with peak heights. Predicted equations of nutritional profiles from spectral parameters with adjusted R^2 greater than 0.7 are shown in Table 5. The RDCB1 and RUCB1 could be predicted with great estimation power from TC1, TC2, STC1, amide II and AsCH3 ($R^2 = 0.945$ and 0.956, respectively). Moreover, predictions for RDCB3, RUCB3, RUCP and tdNDF were also obtained with adjusted R^2 higher than 0.81. In addition, predictions for RDCA4, RDPB2, RDCP, RUCA4, RUPB1, RUPC, tdCP and all energy values were also obtained with good estimation power ($R^2 > 0.7$). Numerous predictions with good estimation power of nutrient content and availability from spectral parameters have been previously published (Peng et al., 2014; Xin et al., 2014; Refat et al., 2017; Prates et al., 2018b). In the current study, spectral parameters of TC1, TC2, TC4, CEC, STC1, STC2, amide I, amide II and CCO were selected for prediction of rumen degradations of CNCPS

Table 5
Prediction equations for rumen degradable and undegradable CNCPS fractions, digestible nutrients and energy values from spectral parameters of alfalfa.

Item[1]	Prediction equation[2]	RSE[3]	Adjusted R^2	*P*-value
Rumen degradable CNCPS fractions, % DM				
RDCA4	$Y = 1.71 - 46.48$ STC2 $+ 20.29$ amide II $- 89.13$ CCO $+ 159.13$ $AsCH_3$	0.38	0.729	<0.001
RDCB1	$Y = 0.87 + 59.34$ TC1 $- 46.38$ TC2 $- 91.45$ STC1 $+ 26.43$ amide II $- 119.59$ $AsCH_3$	0.564	0.945	<0.001
RDCB3	$Y = 22.46 - 114.93$ TC4 $- 125.61$ CEC $+ 77.21$ STC1 $- 26.49$ amide II $+ 237.6$ CCO	0.609	0.827	<0.001
RDPB2	$Y = 1.55 - 4.76$ TC1 $+ 20.83$ CEC $+ 7.59$ STC1	0.113	0.734	<0.001
RDCP	$Y = 12.1 + 122.2$ CEC $- 85.51$ STC1 $+ 61.19$ amide I $- 39.31$ amide II $- 110$ CCO	0.942	0.753	<0.001
Rumen undegradable CNCPS fractions, % DM				
RUCA4	$Y = 0.2 - 5.39$ STC2 $+ 2.33$ amide II $- 9.94$ CCO $+ 17.95$ $AsCH_3$	0.042	0.736	<0.001
RUCB1	$Y = 0.08 + 9.79$ TC1 $- 6.89$ TC2 $- 16.46$ STC1 $+ 4.4$ amide II $- 10.55$ CCO $- 13.82$ $AsCH_3$	0.075	0.956	<0.001
RUCB3	$Y = 14.46 - 74.38$ TC4 $- 81.05$ CEC $+ 49.75$ STC1 $- 16.95$ amide II $+ 153.46$ CCO	0.394	0.826	<0.001
RUPB1	$Y = 4.07 - 11.34$ TC2 $+ 13.45$ amide I	0.246	0.703	<0.001
RUPC	$Y = 0.76 - 1.63$ TC1 $+ 2.34$ TC2 $+ 4.77$ STC1 $- 2.51$ amide I	0.066	0.718	<0.001
RUCP	$Y = 5.51 + 46.81$ CEC $- 29.16$ STC2 $+ 8.2$ amide I $- 31.23$ CCO	0.19	0.848	<0.001
Truly digestible nutrients, % DM				
tdCP	$Y = 15.05 + 166.49$ CEC $- 111.71$ STC1 $+ 77.44$ amide I $- 49.05$ amide II $- 143.44$ CCO	1.205	0.763	<0.001
tdNDF	$Y = 27.51 - 150.54$ TC4 $- 148.83$ CEC $+ 105.09$ STC1 $- 29.67$ amide II $+ 276.76$ CCO	0.754	0.811	<0.001
Energetic values, Mcal/kg				
DE_{3x}	$Y = 2.68 + 6.71$ CEC $- 6.75$ STC2 $+ 2.27$ amide I	0.049	0.754	<0.001
ME_{3x}	$Y = 2.24 + 6.69$ CEC $- 6.81$ STC2 $+ 2.34$ amide I	0.048	0.763	<0.001
NE_{L3x}	$Y = 1.4 + 4.76$ CEC $- 4.85$ STC2 $+ 1.61$ amide I	0.033	0.773	<0.001
ME	$Y = 2.23 + 7.5$ CEC $- 8.19$ STC2 $+ 2.85$ amide I	0.058	0.756	<0.001
NE_m	$Y = 1.38 + 6.26$ CEC $- 7.13$ STC2 $+ 2.52$ amide I	0.052	0.741	<0.001
NE_g	$Y = 0.77 + 5.65$ CEC $- 6.3$ STC2 $+ 2.3$ amide I	0.045	0.758	<0.001

CNCPS = Cornell Net Carbohydrate and Protein System.
[1] RD, rumen degradable fractions; RU, rumen undegradable fractions; CA4, water-soluble carbohydrate, sugar; CB1, starch; CB3, digestible fiber; PB1, insoluble true protein, PB2, fiber-bound protein, PC, indigestible protein; RDCP, rumen degradable crude protein; RUCP, rumen undegradable protein; tdCP, truly digestible crude protein; tdNDF, truly digestible neutral detergent fiber; DE_{3x}, digestible energy at 3 times maintenance level; ME_{3x}, metabolizable energy at 3 times maintenance level; NE_{L3x}, net energy at 3 times of maintenance level; NE_m, net energy for maintenance; NE_g, net energy for growth; 3x represents the production level of feed intake.
[2] TC1 to TC4, 4 major peaks at total carbohydrate (TC) region; CEC, cellulosic compounds; STC1 to STC4, 4 major peaks at structural carbohydrate region; CCO, carbonyl C=O; $AsCH_3$, asymmetric CH_3.
[3] RSE, residual standard error.

fractions, tdCP and tdNDF. As for energetic values, CEC, STC2 and amide I were selected for the prediction equations.

4. Conclusions

In summary, silencing *HB12* and *TT8* genes in alfalfa enhanced fiber supply and decreased protein supply to the animal. Moreover, silencing *HB12* increased lignin content and reduced energy values of alfalfa. In addition, the alterations in nutritional profiles of alfalfa by genetic modification were closely related to nutrient profiles. Prediction equations were obtained with good estimation powers for several nutrient profiles from spectral parameters, especially for rumen degradable and undegradable fractions.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. This article is part of a student graduate thesis.

Author contributions

Yaogeng Lei was a PhD student. **Dr. Abdelali Hannoufa** as AAFC research scientist developed the alfalfa plant material for **Yaogeng Lei** to study and provided support for his lab work. **Yaogeng Lei** carried out the experiments and measurements and wrote the thesis and the draft manuscript. **Peiqiang Yu** was PI, supervisor, corresponding author and designed the studies and acquired the fund.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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