Gene-Transformation-Induced Changes in Chemical Functional Group Features and Molecular Structure Conformation in Alfalfa Plants Co-Expressing Lc-bHLH and C1-MYB Transcriptive Flavanoid Regulatory Genes: Effects of Single-Gene and Two-Gene Insertion

Ravindra G. Heendeniya and Peiqiang Yu *

Department of Animal and Poultry Science, College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, SK S7N5A8, Canada; rgy863@mail.usask.ca

* Correspondence: peiqiang.yu@usask.ca; Tel.: +1-306-966-4150

Academic Editor: Rosa M. Lamuela-Raventós

Received: 19 January 2017; Accepted: 2 March 2017; Published: 20 March 2017

Abstract: Alfalfa (Medicago sativa L.) genotypes transformed with Lc-bHLH and Lc transcription genes were developed with the intention of stimulating proanthocyanidin synthesis in the aerial parts of the plant. To our knowledge, there are no studies on the effect of single-gene and two-gene transformation on chemical functional groups and molecular structure changes in these plants. The objective of this study was to use advanced molecular spectroscopy with multivariate chemometrics to determine chemical functional group intensity and molecular structure changes in alfalfa plants when co-expressing Lc-bHLH and C1-MYB transcriptive flavanoid regulatory genes in comparison with non-transgenic (NT) and AC Grazeland (ACGL) genotypes. The results showed that compared to NT genotype, the presence of double genes (Lc and C1) increased ratios of both the area and peak height of protein structural Amide I/II and the height ratio of α-helix to β-sheet. In carbohydrate-related spectral analysis, the double gene-transformed alfalfa genotypes exhibited lower peak heights at 1370, 1240, 1153, and 1020 cm\(^{-1}\) compared to the NT genotype. Furthermore, the effect of double gene transformation on carbohydrate molecular structure was clearly revealed in the principal component analysis of the spectra. In conclusion, single or double transformation of Lc and C1 genes resulted in changing functional groups and molecular structure related to proteins and carbohydrates compared to the NT alfalfa genotype. The current study provided molecular structural information on the transgenic alfalfa plants and provided an insight into the impact of transgenes on protein and carbohydrate properties and their molecular structure’s changes.

Keywords: molecular structure; function groups; gene transformation; nutrition and structure interaction; alfalfa plant; molecular spectroscopy

1. Introduction

The alfalfa plant is the “queen” of foraging due to its high nutritive value, particularly its high level of protein. However, extremely high soluble protein content in alfalfa plants causes major issues when ruminant livestock graze on alfalfa pasture, which include bloating causing animal death, N-to-energy unsynchronization, and nutrients being under-utilized [1,2]. How can we solve these issues? It has been found that the soluble protein in alfalfa plants can bind with proanthocyanidin (PA) to form a complex. This complex can prevent protein from being degraded in the rumen but shifts protein...
from the rumen to small intestine. In this way, the three major issues can be solved. But how can we make alfalfa plants produce PA?

Recent attempts to engineer proanthocyanidin (PA) synthesis in alfalfa by transformation of transcription factors such as Lc-bHLH [3], LAP1-MYB, PAP1-MYB [4] and more recently TaMYB14 [5] have had varying degrees of success in improving alfalfa protein quality. However, the effects of these single-gene and double-gene insertion/transformation on alfalfa’s molecular structure feature changes and chemical functional groups that related to protein utilization and digestion have not been studied.

Vibrational molecular spectroscopy-Fourier transform infrared spectroscopy (VMS-Ft/IR) has been used in the past as a non-destructive analytical technique to study molecular structures associated with nutritive value of plant material [6–10].

Recently, the alfalfa genotype with both Lc-bHLH and C1-MYB co-expressed plants became available. To our knowledge, there are no studies on the effects of single-gene and two-gene transformation on chemical functional groups and molecular structure changes in these transgenic plants. The objective of this study was to determine chemical functional group intensity and molecular structure changes in alfalfa forage plants that co-express Lc-bHLH and C1-MYB transcriptive flavanoid regulatory genes.

2. Results and Discussion

2.1. Effect of Single-Gene and Double-Gene Insertion/Transformation on Changes Chemical Functional Groups and Molecular Structure Related to Protein Properties

In univariate analyses, there were no significant differences ($p > 0.05$) in overall comparison of Amide-II and β-sheet spectral heights among different alfalfa genotypes (Table 1). The Lc1 genotype has exhibited the lowest parameters associated with both primary and secondary protein molecular structures i.e., Amide-I height (0.024) and area (1.261), Amide-II area (1.729), total Amide area (2.990), α-helix height (0.023) and α-helix/β-sheet ratio (1.109). The comparison with parent non-transgenic (NT) alfalfa shows that both the intensity (spectral height) and extent (band area) of Amide-II are lower in transformed alfalfa, so it has a significantly higher Amide-I/II ratio. There was also a significantly higher Amide-I/II ratio in double gene alfalfa compared to NT alfalfa. As stated before, Amide-I and Amide-II vibrations are governed by stretching of C=O and in-plane bending of N–H, respectively [11].

The Amide-II region is also associated with protein conformation [12]. Taken together, these results indicate that gene transformation has changed the intrinsic structural make-up of proteins and protein conformity. However, since α-helix and β-sheet spectral data (heights and ratios) that represent protein secondary structures have not shown significant differences between NT and transformed alfalfa, gene transformation may not significantly influence the protein degradation. Similar results have been reported by Yu et al. [12], in their protein molecular study on Lc-transformed alfalfa using synchrotron radiation VMS-Ft/IR.

In multivariate analysis, Agglomerative hierarchical cluster analysis (AHCA) and Principal Component Analysis (PCA) methods were used to discriminate the IR spectrum in the Amide region 1720–1480 cm$^{-1}$. In this region the ACGL genotype clearly separated from other genotypes in AHCA (Figure 1I). The Amide region could not be separated for NT vs. single-gene or NT vs. double gene-transformed alfalfa populations by either AHCA or PCA (Figure III–VI). As mentioned before, there are no studies on the effect of single-gene and two-gene insertion on the molecular structure changes in these trans-plants (C1, Lc1, Lc3, Lc1C1 and Lc3C1). No comparison or discussion with published results could be conducted in this section.
Table 1. Effect of double gene and single gene transformation on protein-related molecular spectral characterization of alfalfa.

| Alfalfa Population          | Protein Amide Height | Protein Amide Area | Protein Fine Structure |
|-----------------------------|----------------------|--------------------|------------------------|
|                             | Amide I Height       | Amide II Height    | Ratio of Amide I/II Heights | Amide I Area | Amide II Area | Amide I and II Total Area | Ratio of Amide I/II Area | Amide I Area | Amide II Area | Amide I and II Total Area | Ratio of Amide I/II Area | Amide I Area | Amide II Area | Amide I and II Total Area | Ratio of Amide I/II Area |
| C1                          | 0.029                | 0.019              | 1.536                    | 1.598                | 2.197          | 3.795              | 0.725                  | 0.027                | 0.072          | 0.027                  | 0.983                | 0.027            |
| Lc1                         | 0.024                | 0.015              | 1.533                    | 1.261                | 1.729          | 2.990              | 0.729                  | 0.023                | 0.023          | 0.023                  | 1.109                | 0.020            |
| Lc3                         | 0.028                | 0.018              | 1.519                    | 1.518                | 2.041          | 3.558              | 0.744                  | 0.025                | 0.025          | 0.025                  | 1.088                | 0.023            |
| Lc1C1                       | 0.029                | 0.019              | 1.582                    | 1.658                | 2.160          | 3.817              | 0.768                  | 0.029                | 0.029          | 0.029                  | 1.048                | 0.024            |
| Lc3C1                       | 0.027                | 0.016              | 1.657                    | 1.452                | 2.017          | 3.469              | 0.718                  | 0.025                | 0.025          | 0.025                  | 1.045                | 0.024            |
| NT                          | 0.029                | 0.025              | 1.497                    | 1.579                | 2.279          | 3.858              | 0.694                  | 0.027                | 0.027          | 0.027                  | 1.012                | 0.027            |
| ACGL                        | 0.030                | 0.019              | 1.572                    | 1.589                | 2.122          | 3.711              | 0.750                  | 0.029                | 0.029          | 0.029                  | 1.319                | 0.022            |
| SEM                         | 0.0014               | 0.0032             | 0.0836                   | 0.0821               | 0.1133         | 0.1944             | 0.0074                 | 0.0014               | 0.006          | 0.006                  | 0.0302               |                 |
| p value                     | 0.08                 | 0.38               | 0.07                     | 0.04                | 0.04           | 0.049              | <0.01                  | 0.04                 | 0.04           | 0.04                   | <0.01                |                 |

Contrast p value

| Single vs. Double           | 0.23                 | 0.99               | 0.01                     | 0.21                | 0.33           | 0.29               | 0.15                    | 0.09                 | 0.11           | 0.64                    |
| NT vs. Trans                | 0.34                 | 0.02               | 0.08                     | 0.32                | 0.03           | 0.09               | <0.01                   | 0.36                 | 0.93           | 0.16                    |
| NT vs. Single               | 0.20                 | 0.03               | 0.44                     | 0.18                | 0.02           | 0.05               | <0.01                   | 0.15                 | 0.63           | 0.14                    |
| NT vs. Double               | 0.81                 | 0.04               | 0.01                     | 0.79                | 0.13           | 0.33               | <0.01                   | 0.96                 | 0.38           | 0.31                    |
| CI vs. Lc                   | 0.08                 | 0.63               | 0.83                     | 0.05                | 0.03           | 0.04               | 0.21                    | 0.13                 | 0.52           | <0.01                   |
| ACGL vs. Single             | 0.04                 | 0.66               | 0.35                     | 0.18                | 0.32           | 0.25               | 0.06                    | 0.01                 | 0.86           | <0.01                   |
| ACGL vs. Double             | 0.30                 | 0.67               | 0.31                     | 0.74                | 0.80           | 0.77               | 0.47                    | 0.21                 | 0.16           | <0.01                   |

*a–d Means with different letters within same row differ (p < 0.05); SEM: Standard error of means; ACGL = AC-Grazeland; CI = Single transgenic CI; Lc1 = Single transgenic Lc1; Lc1C1 = Double transgenic Lc1 + CI; Lc3 = Single transgenic Lc3; Lc3C1 = Double transgenic Lc3 + CI; NT = Non-transgenic parent plant.
2.2. Effect of Single-Gene and Double-Gene Insertion/Transformation on Changes in Chemical Functional Groups and Molecular Structure with Regard to Carbohydrate Properties

The spectral features of structural carbohydrate (StCHO), cellulosic compounds and total carbohydrates (TCHO) are shown in Table 2. In the overall comparison of StCHO, there was no significant difference among the different alfalfa genotypes in relation to peak 3 intensities. However, intensities of peak 1 and 2 as well as total StCHO areas tended to be different ($p < 0.10$) among genotypes. The contrast analysis has shown that peak 1 intensity in ACGL was significantly lower ($p < 0.05$) than both single and double gene-transformed alfalfa genotypes. The peak 2 intensities of...
all the transgenic alfalfa were significantly lower ($p < 0.05$) than that of NT alfalfa. Probably due to lower absorption heights at peaks 1 and 2, the total structural carbohydrate areas in transformed alfalfa were lower than NT alfalfa. There was no significant difference ($p > 0.05$) in spectral data of cellulosic compounds or total area of the TCHO region among alfalfa populations. However, significantly higher intensities of peak 1 ($p < 0.05$) and peak 3 ($p < 0.01$) for the TCHO spectral region were observed in NT alfalfa compared to Lc3CI genotype. In the TCHO region, significantly, the highest genotype variations ($p < 0.01$) were observed under the peak 3 intensity and peak 3 area with the lowest peak 3 intensity and extent recorded with the ACGL genotype. The spectral data are influenced by both quantitative and qualitative features of a molecule. The peaks (1 and 3) at wave numbers ~1410 cm$^{-1}$ and ~1240 cm$^{-1}$ are closer to typical wave numbers of $\beta$-glucans (~1420 cm$^{-1}$) and hemicellulose (1246 cm$^{-1}$) respectively [13–15]. Since there was no significant difference in the contents of StCHO (represented by NDF and ADF), the differences observed in relation to peaks and total areas of StCHO spectral region among NT and transgenic alfalfa imply that gene transformation has an effect on the strength and polarity of vibrating bonds associated with StCHO. On the other hand, peak 3 at 1020 cm$^{-1}$ of the TCHO region represents non-structural CHO such as starch [15]. The pattern of variation in peak 3 intensity and extent were similar to the starch content variations among the genotypes, indicating that the variations in TCHO peak 3 profiles in this experiment are influenced mainly by the respective starch contents.

The multivariate analyses of the IR spectrum related to different carbohydrate regions are shown in Figures 2–4. The cluster analysis has not revealed any discrimination of spectral data related to StCHO region (Figure 2I,III,V) of the IR spectrum among different alfalfa populations. In PCA however, there was a clear discrimination between NT and double gene-transformed alfalfa populations (Figure 2VI). This reflects the differences observed in the total areas of the StCHO region in these alfalfa populations (Table 2), probably as a result of differences at peak 2 (~1370 cm$^{-1}$) and peak 3 (~1240 cm$^{-1}$). The PCA of cellulosic region further reveals a clear discrimination of NT from both the single-gene and double-gene-transformed alfalfa populations (Figure 3IV,VI). Since there was no significant difference in NDF or ADF between NT and transgenic alfalfa ($p > 0.05$), the NT vs. double gene discrimination observed in PCA can be attributed to qualitative or “molecular–structural” differences in StCHO components. The NT alfalfa populations were also discriminated from all the transgenic alfalfa in the TCHO region (Figure 4III), particularly from double gene-transformed alfalfa. As stated before, the TCHO region mainly represents the non-structural carbohydrates, which include starch in the plant material. The discrimination of NT from transgenic alfalfa in PCA can be attributed to the differences in both content and molecular structure of starch.

Once again, there is no study on the effect of single-gene and two-gene insertion (C1, Lc1, Lc3, Lc1C1 and Lc3C1) on the molecular structure changes in these trans-plants. Therefore no comparison and discussion could be made regarding published results.
Table 2. Effect of double gene and single gene transformation on carbohydrate-related molecular spectral characterization of alfalfa.

| Alfalfa Population | Structural Carbohydrates (StCHO) Profile | Cellulosic Compound Profile | Total Carbohydrate (TCHO) Profile |
|--------------------|------------------------------------------|-----------------------------|----------------------------------|
|                    | Peak 1 Height (~1410) | Peak 2 Height (~1370) | Peak 3 Height (~1240) | Total Area | Peak 1 Height (~1153) | Peak 2 Height (~1080) | Peak 3 Height (~1020) | Peak 1 Area | Peak 2 Area | Peak 3 Area | Total Area |
|                    | Baseline | Baseline | Baseline | Baseline | Baseline | Baseline | Baseline | Baseline | Baseline | Baseline | Baseline |
| C1                 | 0.014 | 0.012 | 0.010 | 2.532 | 0.007 | 0.322 | 0.016 ab | 0.052 | 0.064 ab | 0.705 | 2.962 | 4.759 a | 8.426 |
| Lc1                | 0.013 | 0.011 | 0.010 | 2.318 | 0.008 | 0.363 | 0.015 ab | 0.053 | 0.064 abc | 0.695 | 3.120 | 4.629 ab | 8.444 |
| Lc3                | 0.014 | 0.012 | 0.010 | 2.539 | 0.007 | 0.359 | 0.016 ab | 0.054 | 0.066 ab | 0.746 | 3.192 | 4.727 a | 8.665 |
| Lc1C1              | 0.015 | 0.012 | 0.010 | 2.527 | 0.008 | 0.351 | 0.016 ab | 0.054 | 0.064 ab | 0.720 | 3.296 | 4.508 ab | 8.524 |
| Lc3C1              | 0.013 | 0.011 | 0.010 | 2.343 | 0.007 | 0.335 | 0.014 b | 0.047 | 0.055 abc | 0.654 | 2.809 | 4.152 ab | 7.614 |
| NT                 | 0.014 | 0.014 | 0.011 | 2.713 | 0.008 | 0.351 | 0.017 a | 0.053 | 0.068 a | 0.750 | 3.070 | 4.972 a | 8.810 |
| ACGL               | 0.012 | 0.011 | 0.010 | 2.233 | 0.008 | 0.375 | 0.015 ab | 0.051 | 0.051 e | 0.690 | 3.288 | 3.736 h | 7.713 |
| SEM                | 0.0007 | 0.0009 | 0.0005 | 0.122 | 0.0004 | 0.0185 | 0.0007 | 0.0024 | 0.0028 | 0.0319 | 0.1369 | 0.2153 | 0.3808 |

| p value | 0.07 | 0.09 | 0.49 | 0.07 | 0.61 | 0.48 | 0.04 | 0.37 | <0.01 | 0.34 | 0.13 | <0.01 | 0.16 |

Contrast p value

| Single vs. Double | 0.60 | 0.85 | 0.68 | 0.81 | 0.94 | 0.78 | 0.16 | 0.32 | 0.08 | 0.34 | 0.76 | 0.07 | 0.22 |
| NT vs. Trans      | 0.54 | 0.02 | 0.05 | 0.03 | 0.42 | 0.76 | 0.07 | 0.51 | 0.09 | 0.15 | 0.97 | 0.05 | 0.21 |
| NT vs. Single     | 0.45 | 0.03 | 0.10 | 0.06 | 0.48 | 0.87 | 0.24 | 0.79 | 0.33 | 0.32 | 0.89 | 0.25 | 0.47 |
| NT vs. Double     | 0.78 | 0.03 | 0.05 | 0.04 | 0.45 | 0.69 | 0.02 | 0.28 | 0.02 | 0.08 | 0.91 | 0.01 | 0.08 |
| C1 vs. Lc         | 0.26 | 0.41 | 0.74 | 0.51 | 0.15 | 0.10 | 0.66 | 0.55 | 0.83 | 0.71 | 0.27 | 0.77 | 0.79 |
| ACGL vs. Single   | 0.04 | 0.24 | 0.81 | 0.11 | 0.37 | 0.21 | 0.18 | 0.42 | <0.01 | 0.50 | 0.22 | <0.01 | 0.08 |
| ACGL vs. Double   | 0.02 | 0.32 | 0.93 | 0.17 | 0.35 | 0.15 | 0.83 | 0.99 | 0.01 | 0.94 | 0.16 | 0.02 | 0.44 |

ab Means with different letters within same row differ (p < 0.05); SEM: Standard error of means; ACGL = AC-Grazeland; C1 = Single transgenic C1; Lc1 = Single transgenic Lc1; Lc1C1 = Double transgenic Lc1 + C1; Lc3 = Single transgenic Lc3; Lc3C1 = Double transgenic Lc3 + C1; NT = Non-transgenic parent plant.
Figure 2. Cluster (I,III,V) and principal component (II,IV,VI) analyses of spectrum detected with VMS-Ft/IR in the structural carbohydrate region obtained from different alfalfa populations. A = AC-Grazeland; N = Non-transformed parent plant; V = Single transformed C1; W = Single transformed Lc1; X = Single transformed Lc3; Y = Double transformed Lc1xC1; Z = Double transformed Lc3xC1; S = single-gene transformed; D = double-gene transformed.
Figure 3. Cluster (I,III,V) and principal component (II,IV,VI) analyses of spectrum detected with VMS-Ft/IR in the cellulosic compound region obtained from different alfalfa populations. A = AC-Grazeland; B = Non-transgenic parent plant; C = Single transgenic C1; D = Single transgenic Lc1; E = Single transgenic Lc3; L = Double transgenic Lc1xC1; M = Double transgenic Lc3xC1; N = Non-transformed; S = single-gene transformed; D = double-gene transformed.
Figure 4. Cluster (I, III, V) and principal component (II, IV, VI) analyses of spectrum detected with VMS-Ft/IR in the total carbohydrate region obtained from different alfalfa populations. A = AC-Grazeland; B = Non-transgenic parent plant; C = Single transgenic C1; D = Single transgenic Lc1; E = Single transgenic Lc3; L = Double transgenic Lc1xC1; M = Double transgenic Lc3xC1. N = Non-transformed; S = single-gene transformed; D = double-gene transformed.

3. Materials and Methods

3.1. Transgenic Alfalfa Plant Material

All the plant material was grown and maintained at the Saskatoon Research Center, Agriculture and Agri-Food Canada. The single-gene (Lc1, Lc3 and C1) and double-gene (Lc1C1 and Lc3C1) transformed alfalfa genotypes along with non-transformed parent genotype (NT) were grown from seeds initially in the greenhouse. The non-transgenic AC Grazeland (ACGL) plants for this experiment were propagated vegetatively from existing plant stock maintained at the greenhouse. The plants were tested for the presence of respective transgenes by PCR, repotted and transferred into a growth
chamber where the light intensity, duration of light/dark and the temperature were maintained throughout the trial period at 550 µE·m⁻²·S⁻¹, 16 h/8 h and 22 °C respectively. The alfalfa plants were harvested with shears 5 cm above soil level, when the plants reached late-bud stage [16]. The harvested material were immediately frozen at −20 °C and subsequently freeze-dried.

3.2. Molecular Spectroscopy

The molecular structures of samples were analyzed using a JASCO Fourier-transformed infrared vibration spectroscopy (VMS-Ft/IR; JASCO Corporation, Tokyo, Japan, model-4200) with a ceramic IR light source. The VMS-Ft/IR consist of a deuterated L-alanine doped triglycine sulfate detector armed with a MIRacle™ attenuated total reflectance accessory module and equipped with a ZnSe crystal and pressure clamp (PIKE Technologies, Madison, WI, USA).

The transgenic and non-transgenic bio-forage samples were also tested using advanced synchrotron radiation-based Fourier transform IR microspectroscopy (SR-IMS) at the National Synchrotron Light Source in Brookhaven National Laboratory (NSLS-BNL, Upton, NY, USA) and Advanced Light Source in Berkeley National Laboratory (ALS-BNL, Berkeley, CA, USA) before using the VMS-Ft/IR approach.

3.3. Univariate Molecular Structure Spectral Processing and Analyses

JASCO spectra-manager II software was used to generate the spectra in the infrared range of 4000–800 cm⁻¹ at a resolution of 4 cm⁻¹. After noise elimination by JASCO spectra-manager™ II software (Tokyo, Japan), functional spectral bands were assigned for carbohydrates and proteins as per previous studies [12–14,17] and identified using software OMNIC version 8.2 (Thermo Fisher Scientific, Madison, WI, USA).

There are two primary frequency bands, i.e., structural Amide I (ca. 1700–1600 cm⁻¹) and Amide II (1560–1500 cm⁻¹) identifiable in the infrared spectrum resulting from vibration of C–O, C–N and N–H groups within the protein molecules. The Amide I band arising from stretching vibration of mainly the C–O group (80%) and to a lesser extent (20%) by C–N group shows a peak at ca. 1655 cm⁻¹ while Amide II band shows a peak at ca. 1550 cm⁻¹ due to bending vibration of N–H group (60%) and stretching vibration of C–N group (40%). The protein secondary structures α-helix and β-sheet peaks are located within the Amide I band and were identified using the second derivative function of OMNIC software [11].

The infrared spectrum of total carbohydrates (TCHO) that lies between wave numbers ca. 1180–900 cm⁻¹, arises from the stretching vibration of C–O and C–C and the deformation of C–OH. The spectral band of structural carbohydrates (StCHO; cellulose, hemicellulose and β-glucans) lies typically between ca. 1485–1188 cm⁻¹ with peaks at ca. 1410, 1370 and 1240 cm⁻¹ [18].

3.4. Multivariate Molecular Spectral Analysis on Intrinsic Structure Changes by Gene Inserting

Two different multivariate methods were employed in the current study to perform multivariate spectral analysis using Statistical 8.0 (StatSoft Inc., Tulsa, OK, USA). Agglomerative hierarchical cluster analysis (CLA), which uses Ward’s Algorithm method without parameterization for clustering, presents results as dendrograms [19–21]. Principal Component Analysis (PCA), which is the other multivariate analysis method, transforms all interrelated variances into new uncorrelated variances called principles components (PCs) [19–21]. The result of PCA is presented as a scatter plot using two main PCs, which took more than 95% of variance, in form of PC1 vs. PC2.

3.5. Statistical Analyses

The spectral intensity data were analyzed by PROC MIXED of SAS (2003) 9.3 version according to statistical model:

\[ Y_{ij} = \mu + P_i + \epsilon_{ij} \]
where, $Y_{ij}$ is the dependent variable, $\mu$ is the overall mean, $P_i$ is the fixed effect of alfalfa population ($i = 7$; NT, ACGL, C1, Lc1, Lc3, Lc1C1, Lc3C1) and $\epsilon_{ij}$ is the residual error.

The Tukey’s test was used for multiple population comparisons with letter groupings obtained using SAS pdmix800 macro [22]. The contrasts between means of different combinations of alfalfa populations were conducted using a statement from SAS. For all statistical analyses, significance was declared at $p < 0.05$ and tendency was declared at $0.05 < p < 0.10$.

4. Conclusions

The results in this study showed that the transformation of Lc and C1 genes have caused changes in the inherent molecular structures and chemical functional group intensity of both protein and carbohydrate chemical make-up and conformation, as revealed by advanced molecular spectroscopy with uni- and multivariate chemometrics. The biological and nutritional significance of these changes will be evaluated in further studies. The current study, for the first time, provided molecular structural information on transgenic alfalfa plants and insight into the impact of transgenes on protein and carbohydrate properties and their molecular structure.

Acknowledgments: Our “Bio-Forage Feeds” research projects have been supported by grants from the Ministry of Agriculture Strategic Research Chair Program, Natural Sciences and Engineering Research Council of Canada (NSERC-Individual Discovery Grant), and the Saskatchewan Agricultural Development Fund (ADF). The authors thank M Y. Gruber (Agriculture and Agri-Food Canada) for kindly helping with the development of single-gene and two-gene inserted transgenic alfalfa plants and Y. Wang, B. Coulman, D. A. Christensen, and J. J. McKinnon for sitting on the PhD student (RH) committee and Z. Niu in the Department of Animal and Poultry Science, the University of Saskatchewan for providing lab and animal trial assistance. The National Synchrotron Light Source in Brookhaven National Laboratory (NSLS-BNL, New York, NY, USA) and Advanced Light Source in Berkeley National Laboratory (ALS-BNL) are supported by the U.S. Department of Energy. The authors are grateful to Lisa Miller for synchrotron beamtime support at ALS and NSLS, discussions and collaborations, and Randy Smith (NSLS-BNL, New York, NY, USA) and Hans Bechtel (ALS, Berkeley, CA, USA) for helpful synchrotron data collection at ALS and NSLS.

Author Contributions: Peiqiang Yu designed the experiments; Ravindra G. Heendeniya performed the experiments, analyzed the data and Ravindra G. Heendeniya and Peiqiang Yu wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jonker, A.; Gruber, M.Y.; Mccaslin, M.; Wang, Y.; Coulman, B.; Mckinnon, J.J.; Christensen, D.A.; Yu, P. Nutrient composition and degradation profiles of anthocyanidin-accumulating Lc-alfalfa populations. Can. J. Anim. Sci. 2010, 90, 401–412. [CrossRef]
2. Jonker, A.; Gruber, M.Y.; Wang, Y.; Coulman, B.; Azarfar, A.; McKinnon, J.J.; Christensen, D.A.; Yu, P. Modeling degradation ratios and nutrient availability of anthocyanidin-accumulating Lc-alfalfa populations in dairy cows. J. Dairy Sci. 2011, 94, 1430–1444. [CrossRef] [PubMed]
3. Ray, H.; Yu, M.; Auser, P.; Blahut-Beatty, L.; McKersie, B.; Bowley, S.; Westcott, N.; Coulman, B.; Lloyd, A.; Gruber, M.Y. Expression of anthocyanins and proanthocyanidins after transformation of alfalfa with maize Lc. Plant Physiol. 2003, 132, 1448–1463. [CrossRef] [PubMed]
4. Peel, G.J.; Pang, Y.; Modolo, L.V.; Dixon, R.A. The LAPI MYB transcription factor orchestrates anthocyanidin biosynthesis and glycosylation in Medicago. Plant J. 2009, 59, 136–149. [CrossRef] [PubMed]
5. Hancock, K.R.; Collette, V.; Fraser, K.; Greig, M.; Xue, H.; Richardson, K.; Jones, C.; Rasmussen, S. Expression of the R2R3-MYB transcription factor TaMYB14 from Trifolium arvense activates proanthocyanidin biosynthesis in the legumes Trifolium repens and Medicago sativa. Plant Physiol. 2012, 159, 1204–1220. [CrossRef] [PubMed]
6. Doiron, K.; Yu, P.; McKinnon, J.J.; Christensen, D.A. Heat-induced protein structure and subfractions in relation to protein degradation kinetics and intestinal availability in dairy cattle. J. Dairy Sci. 2009, 92, 3319–3330. [CrossRef] [PubMed]
7. Xin, H.; Zhang, X.; Yu, P. Using synchrotron radiation-based infrared microspectroscopy to reveal microchemical structure characterization: Frost damaged wheat vs. normal wheat. Int. J. Mol. Sci. 2013, 14, 16706–16718. [CrossRef] [PubMed]
8. Yu, P.; Christensen, C.R.; Christensen, D.A.; McKinnon, J.J. Ultrastructural-chemical make-up of yellow-seeded (Brassica rapa) and brown-seeded (Brassica napus) canola within cellular dimensions, explored with synchrotron reflection FTIR microspectroscopy. *Can. J. Plant Sci.* 2005, 85, 533–541. [CrossRef]

9. Yu, P. Plant-based food and feed protein structure changes induced by gene-transformation, heating and bio-ethanol processing: A synchrotron-based molecular structure and nutrition research program. *Food Mol. Nutr.* 2010, 1535–1545. [CrossRef] [PubMed]

10. Yu, P.; Nuez-Ortín, W.G. Relationship of protein molecular structure to metabolisable proteins in different types of dried distillers grains with solubles: A novel approach. *Br. J. Nutr.* 2010, 104, 1429–1437. [CrossRef] [PubMed]

11. Jackson, M.; Mantsch, H.H. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol.* 1995, 30, 95–120. [CrossRef] [PubMed]

12. Yu, P.; Jonker, A.; Gruber, M. Molecular basis of protein structure in proanthocyanidin and anthocyanin-enhanced Lc-transgenic alfalfa in relation to nutritive value using synchrotron-radiation FTIR microspectroscopy: A novel approach. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* 2009, 73, 846–853. [CrossRef] [PubMed]

13. Wetzel, D.L.; Eilert, A.J.; Pietrzak, L.N.; Miller, S.S.; Sweat, J.A. Ultraspatially resolved synchrotron infrared microspectroscopy of plant tissue in situ. *Cell. Mol. Biol.* 1998, 44, 145–168. [PubMed]

14. Yu, P. Application of advanced synchrotron radiation-based Fourier transform infrared (SR-FTIR) microspectroscopy to animal nutrition and feed science: A novel approach. *Br. J. Nutr.* 2004, 92, 869. [CrossRef] [PubMed]

15. Yu, P.; Block, H.; Niu, Z.; Doiron, K. Rapid characterization of molecular chemistry, nutrient make-up and microlocation of internal seed tissue. *J. Synchrotron Radiat.* 2007, 14, 382–390. [CrossRef] [PubMed]

16. Fick, G.W.; Mueller, S.C. Alfalfa-quality, maturity and mean stage of development. *Inf. Bull.* 1989, 217, 1–16.

17. Yari, M.; Valizadeh, R.; Naserian, A.A.; Jonker, A.; Yu, P. Protein molecular structures in alfalfa hay cut at three stages of maturity and in the afternoon and morning and relationship with nutrient availability in ruminants. *J. Sci. Food Agric.* 2013, 93, 3072–3080. [CrossRef] [PubMed]

18. Yu, P. Short communication: Relationship of carbohydrate molecular spectroscopic features to carbohydrate nutrient profiles in co-products from bioethanol production. *J. Dairy Sci.* 2012, 95, 2091–2096. [CrossRef] [PubMed]

19. Yu, P. Applications of hierarchical cluster analysis (CLA) and principal component analysis (PCA) in feed structure and feed molecular chemistry research, using synchrotron-based fourier transform infrared (FTIR) microspectroscopy. *J. Agric. Food Chem.* 2005, 53, 7115–7127. [CrossRef] [PubMed]

20. Yu, P. Protein secondary structures (α-helix and β-sheet) at a cellular level and protein fractions in relation to rumen degradation behaviours of protein: A new approach. *Br. J. Nutr.* 2005, 94, 655–665. [CrossRef] [PubMed]

21. Yu, P. Synchrotron-based microspectroscopic analysis of molecular and biopolymer structures using multivariate techniques and advanced multi-components modeling. *Can. J. Anal. Sci. Spectrosc.* 2008, 53, 220–231.

22. Saxton, A.M. A macro for converting mean separation output to letter groupings in PROC MIXED. In *Proceedings of the 23rd SAS Users Group International*, Nashville, Tennessee, 22–25 March 1998; SAS Institute: Cary, NC, USA, 1998; pp. 1243–1246.