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Floral *Trifolium* Proanthocyanidins: Polyphenol Formation and Compositional Diversity

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Recent studies on the biosynthesis of proanthocyandins have identified key genes and enzymes in the formation of 2,3-*cis*-flavan-3-ols (epiafzelechin, epicatechin, and epigallocatechin). However, the enzymes that catalyze the polymerization of monomer units remain unknown. Studies of proanthocyanidin synthesis have involved the reference plant, *Arabidopsis thaliana*, forage legumes, tobacco, and grape. For this paper the floral proanthocyanidins of 10 *Trifolium* species were studied to identify candidates with contrasting proanthocyanidin chemistry, for the identification of factors involved in polymerization. Proanthocyandins were present in the floral portions (3.1−12.2 g/100 g of dry matter) of *Trifolium* spp. Thiolyic cleavage of proanthocyanidin fractions liberated flavan-3-ol extension units dominated by cis stereochemistry. The terminal units' stereochemistry of *T. michelianum* was exclusively trans (catechin), that of *T. hirtum* mixed trans (catechin and gallocatechin), and that of *T. vesiculosum* equal proportions of trans and cis (catechin and epicatechin). Compositional dispersion for oligomers was determined by MALDI-TOF MS, which showed a range of ions up to ≈2200 Da. The three *Trifolium* spp. highlighted, all annuals, may warrant investigation for insights into proanthocyanidins biosynthesis.

KEYWORDS: Proanthocyanidin; *Trifolium*; catechin; flavonoid; thiolysis; MAIDI-TOF

INTRODUCTION

The beneficial roles of proanthocyandins, which are also known as condensed tannins, in animal nutrition (*1*−*3*) and human health (*4*−*6*) have been the focus of much attention. The regulation of the flavonoid biosynthetic pathway from flavanones to flavan-3-ols through polymeric proanthocyandins end products has been reviewed (*7*−*9*).

Recent papers (*7*, *10*) have established that the flavan-3-ol stereoisomers catechin and epicatechin (procyanidins) are the products of biosynthetic conversion processes different from those of the common precursor leucocyanidin. The enzyme leucoanthocyanidin reductase (LAR) catalyzes the synthesis of catechin, from 3,4-*cis*-leucocyanidin (*10*), and is the first step in proanthocyandin biosynthesis. The B-ring hydroxylation pattern of the catechin/epicatechin pair is determined by the enzymes flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), which are cytochrome P_{450} monoxygenases that act early in the pathway (*11*). The presence of both F3′H and F3′5′H leads to the formation of gallocatechin/epigallocatechin (prodelphinidins). Studies on proanthocyanidin synthesis have involved the reference plant *Arabidopsis thaliana* (*12*−*15*), the important forage alfalfa (*Medicago sativa*) (*16*), tropical and temperate forage legumes, *Desmodium uncinatum* (*10*) and *Lotus* species (*17*), respectively, and tobacco petals (*18*).

Proanthocyanidins have not been detected [<0.2 g/100 g of dry matter (DM)] in the leaves of *Trifolium* species (*19*), in contrast to some other forage legumes; instead, low concentrations have been observed in the flowers. Apart from the agronomically important *T. repens* (white clover) and *T. pratense* (red clover), the diversity of proanthocyandins expressed in *Trifolium* spp. flower heads has been largely unexplored. The *T. repens* floral prodelphinidins (*20*) consist of terminal units with nearly equal proportions of epigallocatechin and gallocatechin and a similar pattern for the extender units. The *T. pratense* floral procyanidins (*21*) have terminating units dominated by catechin, contrasted by epicatechin as the abundant flavan-3-ol extension unit. The predominant 2,3-trans (catechin) and cis (epicatechin) stereochemistries of the terminal and extender units, respectively, observed for the *T. pratense* floral proanthocyanidins, are in contrast to the *T. repens* floral proanthocyanidins for which both extender and terminal units show a

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Table 1. Floral Proanthocyanidin Content of 10 *Trifolium* Species[a]

						% terminal				% extender				
						trans		cis		trans		cis		
Trifolium spp.	extracted	fiber	protein	total Bu–HCl	mDP	C	GC	EC	EGC	C	GC	EC	EGC	PC:PD
T. michelianum	5.6	0.5	0	6.1	9.1	100	0	0	0	10	0	90	0	100:0
T. resupinatum	5.2	0.1	0.3	5.6	8.6	54	0	46	0	7	0	93	0	100:0
T. vesiculosum	3.6	0.3	0.1	4.0	10.1	55	0	45	0	4	0	88	8	93:07
T. pratense	2.1	0.2	1.4	3.7	9.3	95	0	5	0	6	6	81	6	80:20
T. alexandrinum	7.8	0.5	0.4	8.7	12.2	86	10	4	0	13	7	59	21	73:27
T. incarnatum	3.1	0.1	0.2	3.4	12.4	48	6	46	0	1	15	68	16	72:28
T. hirtum	2.6	0.2	0.2	3.1	13.9	61	39	0	0	1	24	8	66	13:87
T. repens	7.6	1.2	0.7	9.5	10.5	0	57	0	43	0	36	1	63	1:99
T. fragiferum	10.6	0.6	1.1	12.2	11.9	0	68	0	32	0	35	1	64	1:99
T. ambiguum	9.0	0.9	1.5	11.4	9.2	0	70	0	30	0	24	1	75	1:99

[a] Concentration in plant material (g/100 g of DM) estimated by the butanol–HCl colorimertic assay; thiolysis reaction products of the proanthocyanidin fraction; mean degree of polymerization, % contributions of terminal and extender flavan-3-ols; and ratio of procyanidin to prodelphinidin units. Bu–HCl (g/100 g of DM), butanol–hydrochloric acid assay; mDP, mean degree of polymerization; C, catechin; EC, epicatechin; GC, gallocatechin; EGC, epigallocatechin; PC, procyanidin; PD, prodelphinidin.

similar mixture of cis and trans (epigallocatechin and gallocatechin) stereochemistries and to the proanthocyanidins of *Arabidopsis*, for which both terminal and extender units have recently (*18*) been shown to be exclusively of the cis (procyanidin) stereochemistry. Thus, the chemical characteristics of a more varied selection of *Trifolium* spp. floral proanthocyanidins were evaluated to determine their structural diversity and identify possible candidates with contrasting proanthocyanidin composition, for the identification of possible differences in flavonoid biosynthetic pathways and future targets for the identification of a key enzyme and/or gene involved in polymerization.

Eight *Trifolium* spp. in addition to *T. pratense* and *T. repens* were included in our study: arrowleaf (*T. vesiculosum*), balansa (*T. michelianum*), berseem (*T. alexandrinum*), Caucasian (*T. ambiguum*), crimson (*T. incarnatum*), Persian (*T. resupinatum*), rose (*T. hirtum*), and strawberry (*T. fragiferum*) clovers. In this study we have applied colorimetric assays, thiolyic cleavage, and MALDI-TOF to characterize the chemical composition of the proanthocyanidin oligomers and polymers of the flowers of both annual and perennial legumes from the *Trifolium* spp. and compared the results with those for the floral proanthocyanidins of the major forage legumes, *T. repens* and *T. pratense*.

MATERIALS AND METHODS

Plant Lines. Plants of arrowleaf cv. Sari (*T. vesiculosum* Sari.), balansa cv. Bolta (*T. michelianum* Savi.), berseem cv. Attlia (*T. alexandrinum* L.), Caucasian cv. Monaro (*T. ambiguum* Bieb.), crimson cv. Tibbee (*T. incarnatum* L.), Persian cv. Marai (*T. resupinatum* L.), red cv. Broadway (*T. pratense* L.), rose cv. Hykon (*T. hirtum* All.), strawberry cv. Onwards (*T. fragiferum* L.), and white cv. Demand (*T. repens* L.) clovers were grown at Grasslands Research Centre, Palmerston North, or Lincoln Research Centre, Christchurch, New Zealand. The flowers were harvested in spring 2005, transferred to plastic bags, and frozen at −20 °C.

Extraction and Isolation. Frozen flowers (200 g) were extracted with acetone/water (7:3; 1 L) containing ascorbic acid (1 g/L) in a VCM62 Varning blender (AB Hallde Maskiner, Kista, Sweden) for 30 min. Additional acetone/water (500 mL) was added, and after 1 h, the extract was strained through cheesecloth to remove plant material. The extract was concentrated in vacuo (40 °C) to remove acetone and the aqueous solution partitioned with dichloromethane (3 × 500 mL). The aqueous layer was concentrated in vacuo and subsequently freeze-dried to yield a crude proanthocyanidin extract (PAE).

Purification of Proanthocyanidins. Freeze-dried PAE (4.5 g) was dissolved in methanol/water (1:1; 50 mL), filtered through a Büchner funnel using Whatman no. 40 filter paper, and centrifuged (15 min, 1000*g*). The PAE solution was applied to an SR 25/45 Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) packed in methanol/water (1:1) connected to a Pharmacia GradiFrac system. Three fractions were eluted from the column with methanol/water (1:1; 1 L) at a flow rate of 7 mL/min. Elution (7 mL/min) with acetone/water (7:3; 800 mL) yielded a proanthocyanidin fraction IV. A further acetone/water fraction (200 mL) was collected to confirm complete elution of proanthocyanidins from the column. The fractions were concentrated in vacuo (40 °C) and freeze-dried.

Chemical Analyses. Floral proanthocyanidin concentrations were determined with a three-step extraction procedure for free, fiber-bound, and protein-bound proanthocyanidins (*22*) from ground freeze-dried plant material. Measurements were performed in duplicate; free proanthocyanidins were extracted with acetone/water, and fiber-bound and protein-bound proanthocyanidins were extracted with a sodium dodecyl sulfate/mercaptoethanol solution. Determination of the proanthocyanidin concentrations at each step used the butanol−HCl procedure of Porter et al. (*23*). The standard for spectrophotometric calibration for each floral *Trifolium* spp. was the corresponding proanthocyanidin fraction IV purified by Sephadex LH-20 chromatography.

A method based on that described by Guyot et al. (*24*) was utilized to perform the thiolysis reaction. The proanthocyanidin reaction products were analyzed immediately by RP-HPLC, and concentrations of terminal flavan-3-ols and the extender flavan-3-ol thiol adducts were estimated as described in detail by Sivakumaran et al. (*21*). Responses relative to dihydroquercetin (Sigma, St. Louis, MO) were in accordance with published values from Gu et al. (*25*).

MALDI-TOF MS Analyses of Proanthocyanidin Oligomers. Mass spectra were collected on a Micromass MALDI LR TOF mass spectrometer, equipped with delayed extraction and a N_2 laser, set at 337 nm in positive reflectron mode with an accelerating voltage of 15 kV and a reflectron voltage of 2 kV. The proanthocyanidin fraction (IV), 0.5 mg/mL, was reconstituted in acetone/water (8:2) and mixed with a 10 mg/mL matrix solution of 2,5-dihydroxybenzoic acid (Sigma) in acetone/water (8:2) at a volumetric ratio of 1:1. The proanthocyanidin−matrix solution was deionized on a cation exchange cartridge (Strata SCX, 55 μm, 70A, 100 mg of sorbent, purchased from Phenomenex, Torrance, CA) preconditioned with 0.1 M HCl (1 mL), Milli-Q water (5 mL), and finally acetone/water (8:2; 2 mL). The deionized proanthocyanidin−matrix solutions were spiked with a 0.1 M NaCl solution (0.5 μL) to promote the formation of single ion adducts ($[M + Na]^+$), and the solution (1 μL) was applied to a stainless steel target plate and crystallized at room temperature prior to MS analysis.

RESULTS AND DISCUSSION

Butanol−HCl Colorimetric Proanthocyanidin Assay. The estimated proanthocyanidin concentrations (*22*, *23*) in *Trifolium* flowers are given in **Table 1**. The total floral proanthocyanidin content ranges from 3.1 to 12.2 g/100 g of DM with the major contribution from extractable proanthocyanidins. The fiber-bound content was <1.0 g/100 g of DM, with the exception of

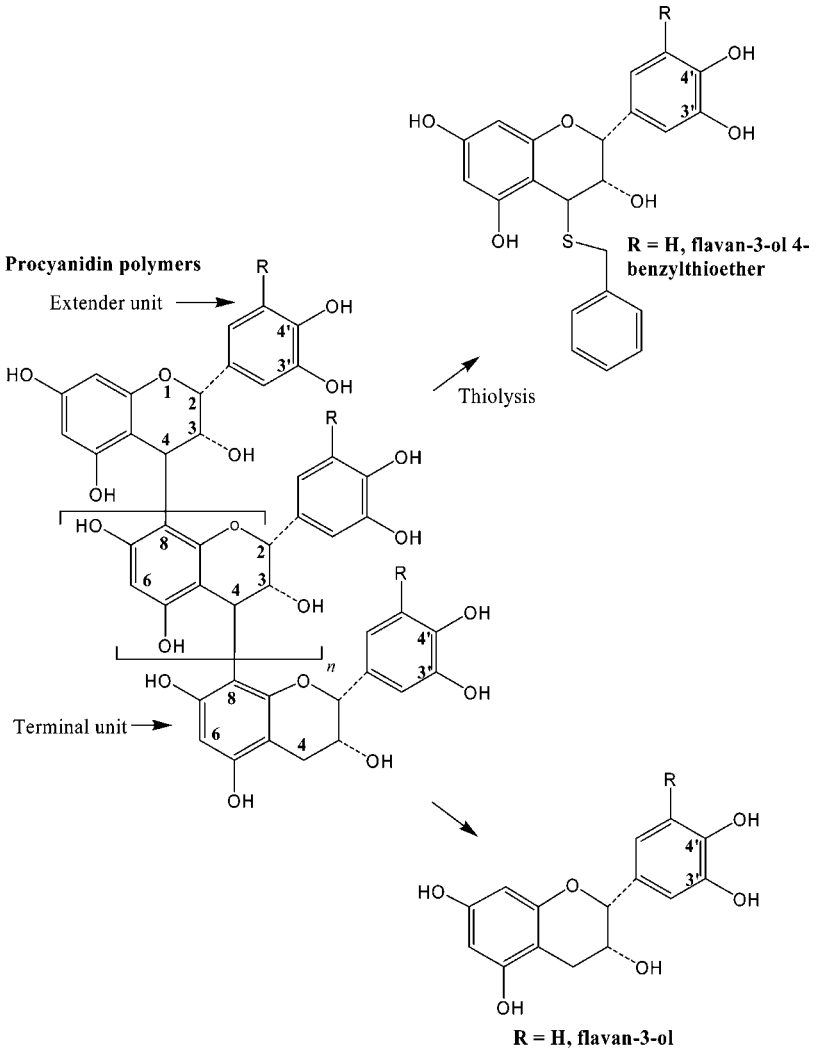

Figure 1. Products of the thiolyic cleavage reaction for proanthocyanidin fractions consisting of procyanidin and prodelphidinin units. The trans stereochemistry is associated with catechin/gallocatechin flavan-3-ols, whereas the cis stereochemistry is associated with epicatechin/epigallocatechin flavan-3-ols. All of the terminal units were released as flavan-3-ols and the extender units as flavan-3-ol benzyl thioethers. R = H, catechin/epicatechin; R = OH, gallocatechin/epigallocatechin.

T. repens (1.2 g/100 g of DM), and the protein-bound content was <1.0 g/100 g of DM, with the exception of *T. ambiguum* (1.5 g/100 g of DM), *T. pratense* (1.4 g/100 g of DM), and *T. fragiferum* (1.1 g/100 g of DM). White coloration appears to correspond to higher proanthocyanidin concentrations >8 g/100 g of DM (*T. alexandrinum*, *T. ambiguum*, *T. fragiferum*, and *T. repens*), and those flowers with red or pink coloration (*T. hirtum*, *T. incarnatum*, *T. michelianum*, *T. pratense*, *T. resupinatum*, and *T. vesiculosum*), indicating the presence of anthocyanidins, have lower proanthocyanidin concentrations of 3.0−6.0 g/100 g of DM. There is concern about the estimation of proanthocyanidin concentration by colorimetric methods (*7*, *22*, *26*). However, for each butanol−HCl assay performed, the corresponding Sephadex LH-20 purified proanthocyanidin fraction was used for calibration as recommended (*7*). There may be competition between the anthocyanidin and prothanthocyanidin pathways within a particular tissue. The trend observed for *Trifolium* spp. floral proanthocyanidins was analogous to that observed for *Vitis* spp. (grape), where high proanthocyanidin concentrations were detected in flower and seed compared to low leaf concentrations (*27*). Tobacco flower petals expressing the BAN gene (*18*) were observed to lose their pink flower pigmentation characteristic of wild-type plants, which resulted in the loss of anthocyanins and accumulation of proanthocyanidins. Extraction with butanol−HCl confirmed the presence of proanthocyanidins in tobacco petals, compared with the absence in control petals (*18*).

Thiolysis of Proanthocyanidins. Thiolytic degradation of proanthocyanidin fractions (**Figure 1**) has been found to provide good yields of cleavage products (*28*), with low levels of product degradation and epimerization (*25*). The mean composition and mDP of proanthocyanidins can be determined by strong acid total thiolysis and chromatography of the reaction products by RP-HPLC. The mean composition of the terminal units can be determined from the ratio of the released monomers, the mean composition of the extender units in the polymer chain from the ratio of benzylthioether adducts, and the mDP from the ratio of monomer to extender units. The flavan-3-ol composition of the proanthocyanidin fractions given in **Table 1** was estimated by chromatography (HPLC-PDA) of the thiolysis reaction products.

The thiolysis products indicated a consistent mean polymer chain length for all of the floral proanthocyanidin fractions with a range of 8.6−13.9 mDP. The *T. pratense* floral procyanidins consist of terminating units dominated by catechin (95%) and epicatechin (81%) as the abundant flavan-3-ol extension unit, identical to prior observations (*21*). The *T. repens* floral prodelphinidins consist of terminal units with nearly equal

Table 2. Ions Detected by MALDI-TOF MS for the 10 *Trifolium* Species Floral Proanthocyanidin Fractions with Trimer (DP3) to Heptamer (DP7) as $(M + Na)^+$ Adducts

polymer	PC	PD	calcd $(M + Na^+)$	*T. michelianum*	*T. resupinatum*	*T. vesiculosum*	*T. incarnatum*	*T. pratense*	*T. alexandrinum*	*T. hirtum*	*T. ambiguum*	*T. fragiferum*	*T. repens*
trimer	3	0	889	889.0	889.1	889.0	889.1	889.0	889.0				
	2	1	905				903.0	905.0	902.9	903.1	903.0	903.0	
	1	2	921						923.9	921.2			
	0	3	937								937.1	937.1	937.2
tetramer	4	0	1177	1177.0	1177.1	1177.0	1177.1	1177.1	1177.0				
	3	1	1193					1195.8	1193.0				
	2	2	1209							1209.1			
	1	3	1225							1225.1			1225.1
	0	4	1241							1241.1	1241.0	1241.0	1241.1
pentamer	5	0	1466	1465.0	1465.1	1465.0	1465.0	1464.9	1465.0	1466.0			
	4	1	1482					1486.9	1480.9				
	3	2	1498										
	2	3	1514						1513.0	1513.0			
	1	4	1530		1531.0					1529.0	1528.9	1528.9	1529.0
	0	5	1546							1546.0	1544.9	1544.9	1545.0
hexamer	6	0	1754	1753.0	1754.0	1753.0	1754.0	1752.9	1752.9				
	5	1	1770		1770.0								
	4	2	1786										
	3	3	1802										
	2	4	1818							1817.0			
	1	5	1834										
	0	6	1850								1848.8	1848.9	1849.0
heptamer	7	0	2042	2041.0	2042.1	2042.0	2041.9	2042.8	2043.0				
	6	1	2058										
	5	2	2074										
	4	3	2090										
	3	4	2106										
	2	5	2122										
	1	6	2138							2137.1			
	0	7	2154								2155.0	2153.9	2154.0

proportions of epigallocatechin (43%) and gallocatechin (57%) and extender units showing epigallocatechin (63%) and gallocatechin (36%), again consistent with prior observations (*21*). The dramatic difference in the stereochemistry of the terminal and extender units observed for the *T. pratense* floral procyanidins contrasts with the mixture of cis and trans stereochemistries observed for *T. repens* floral prodelphinidins. When a more varied range of *Trifolium* spp. was evaluated, which included annuals (*T. alexandrinum*, *T. hirtum*, *T. incarnatum*, *T. michelianum*, *T. resupinatum*, and *T. vesiculosum*), trends in proanthocyanidin composition observed previously (*21*) were confirmed and additional trends became apparent.

As observed in other plant species studied (*16*−*18*) the floral *Trifolium* flavan-3-ol extension units are dominated by the 2,3-cis stereochemistry, epicatechin for procyanidins and epigallocatechin for prodelphinidin fractions (**Table 1**). However, the terminating flavan-3-ols for the procyanidin fractions are dominated by catechin for *T. michelianum* (100%) and *T. alexandrinum* (86%), as found previously for *T. pratense* (95%), whereas *T. incarnatum*, *T. resupinatum*, and *T. vesiculosum* have equal proportions of epicatechin and catechin, with cis and trans sterochemistry, respectively, indicating that rigorous control of chain extension was observed for the procyanidin fractions, but the selection of the terminal group may be controlled by alternative mechanisms. The prodelphinidins consist of terminal flavan-3-ols dominated by gallocatechin for *T. ambiguum* (70%) and *T. fragiferum* (68%), and then nearly equal proportions of epigallocatechin (43%) and gallocatechin (57%) for *T. repens*, having cis and trans stereochemistry, respectively. A contrasting pattern is observed for *T. hirtum* with both catechin (61%) and gallocatechin (39%), with trans stereochemistry only. It may be that mechanisms for the selection of terminal groups for the *T. incarnatum*, *T. resupinatum*, and *T. vesiculosum* floral procyanidins, where equal portions of cis and trans flavan-3-ols are selected, are also present for *T. ambiguum*, *T. fragiferum*, and *T. repens* floral prodelphinidins. However, *T. hirtum* is the only floral *Trifolium* spp. found in which both the procyanidin and prodelphinidin terminal flavan-3-ols consist exclusively of the trans stereochemistry.

Thus, the thiolytic cleavage of the 10 selected *Trifolium* spp. floral proanthocyanidin fractions evaluated here suggested from the terminal units composition that three possible proanthocyanidin products from polymerization of the flavan-3-ols might occur. All *Trifolium* spp. floral proanthocyanidin fractions evaluated have similar proportions of the cis stereoisomers (epicatechin or epigallocatechin) for the extender unit composition. Diversity was found for the flavan-3-ol terminal groups of the proanthocyanidin fractions, which can consist solely of the trans stereochemistry (catechin; procyanidins only), nearly equal proportions of the cis and trans stereochemistries, or the trans stereochemistry with similar proportions of catechin and gallocatechin. Alternatively, there could be less rigorous control (random) of the selection of terminal units than chain extension units through the polymerization process during proanthocyanidin biosynthesis.

Proanthocyanidin Oligomers Structural Polydispersity. Mass spectrometry provides supporting information on the composition of proanthocyanidin oligomer mixtures in the form of ion masses corresponding to sets of oligomers of the same degree of polymerization (DP) and composition (*29*). MALDI-TOF MS of the proanthocyanidin fraction was carried out in positive ion mode under weakly acidic conditions (*30*). Oligomers (**Table 2**) were detected as singly charged sodium adducts of DP3−DP7 (**Figure 2**), slightly lower in range than the mDP

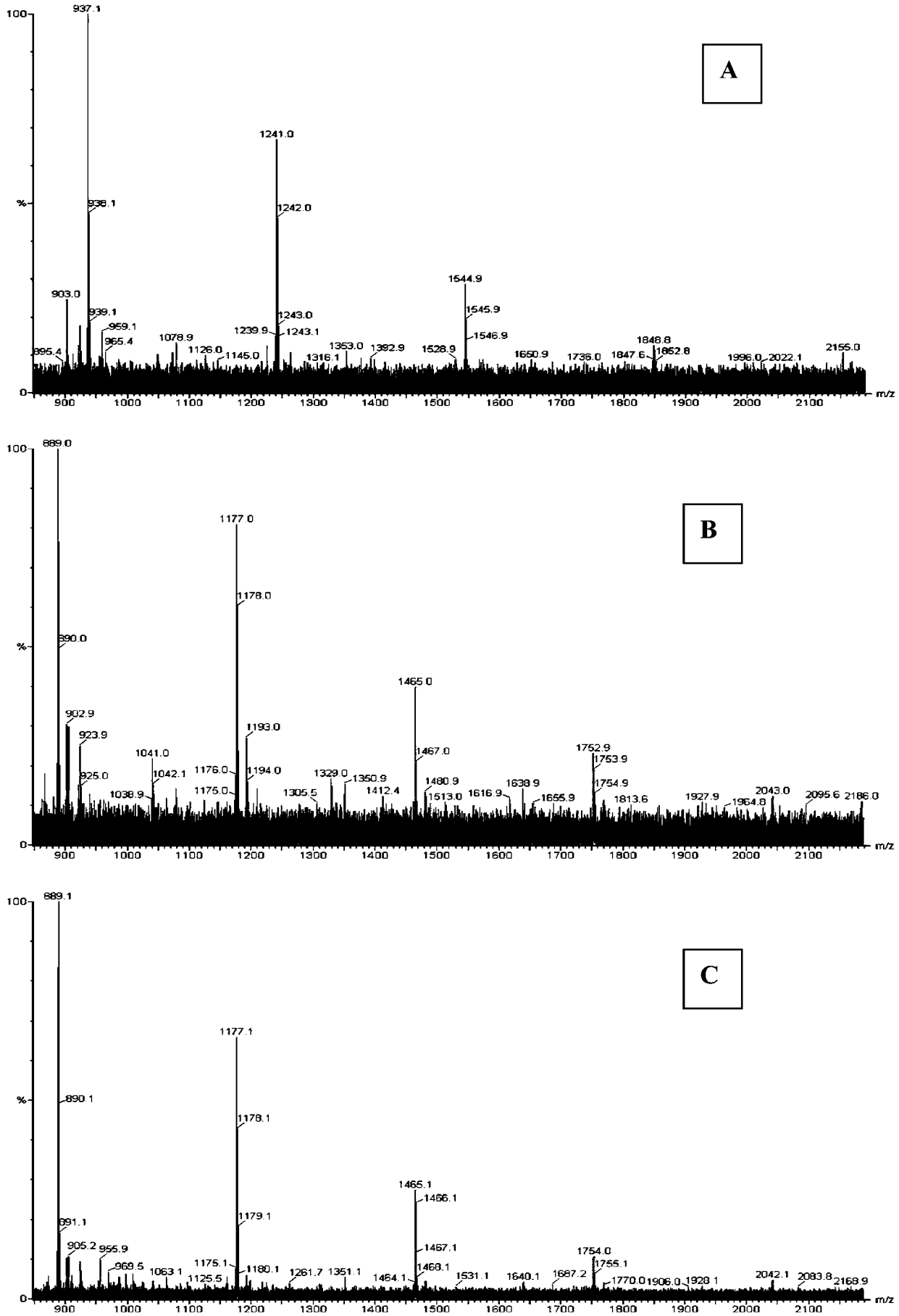

Figure 2. MALDI-TOF MS spectra of proanthocyanidin fractions for floral (**A**) *T. ambiguum* prodelphidinins, (**B**) *T. alexandrinum* hetero-proanthocyanidins, and (**C**) *T. resupinatum* procyanidins with $(M + Na)^+$ trimer (DP3) to heptamer (DP7) oligomer ions.

estimated by thiolysis (**Table 1**). MALDI-TOF spectra provide only the relative procyanidin to prodelphinidin composition of the lower DP oligomer ions and do not provide the detailed compositional information on cis to trans stereochemistry determined by thiolyic cleavage. The MALDI ions of *T. vesiculosum* and *T. michelianum* procyanidin fractions were comparable in composition (**Table 2**). However, the thiolysis data (**Table 1**) indicated contrasting terminal unit composition with *T. michelianum* dominated by catechin (100%) and *T. vesiculosum* with equal proportion of both epimers (catechin/epicatechin). The same trend was not observed for prodelphinidins with *T. ambiguum*, *T. fragiferum*, and *T. repens* having comparable MALDI-TOF ions (**Table 2**) and identical composition by thiolytic cleavage. However, the information provided by the thiolysis data (**Table 1**), indicating nearly equivalent proportion of both epimers (gallocatechin/epigallocatechin), cannot be deduced from the MALDI-TOF MS spectrum.

In each mass range, the species observed for *T. ambiguum* dominant prodelphinidins (**Figure 2A**) were derived from prodelphinidin homo-oligomers, namely, trimer species (*m*/*z* 937) to heptamer species (*m*/*z* 2155). All of the major signals were consistent with molecular ion masses of oligomers of the prodelphinidin-type, with the exception of trimer species (*m*/*z* 903), corresponding to two procyanidin units and one prodelphinidin unit. The proanthocyanidin oligomers for *T. alexandrinum* were hetero-proanthocyanidins (**Figure 2B**), namely, trimer (DP3; *m*/*z* 889, 903, and 924), tetramer (DP4; *m*/*z* 1177 and 1193), pentamer (DP5; *m*/*z* 1465, 1481, and 1513), hexamer (DP6; *m*/*z* 1753), and heptamer (DP7; *m*/*z* 2043) ions. These results are in agreement with those of the thiolyic cleavage of the proanthocyanidin polymer fraction presented (**Table 1**), which indicate a hetero-proanthocyanidin fraction, dominated by procyanidin units. The *T. resupinatum* procyanidins (**Figure 2C**) were derived from procyanidin homo-oligomers, namely, trimer species (*m*/*z* 889) to heptamer species (*m*/*z* 2042), with some ions representative of hetero-proanthocyanidin, namely, trimer species (*m*/*z* 905) and hexamer species (*m*/*z* 1770). All of the major signals were consistent with molecular ion masses of oligomers of the procyanidin type. However, as discussed above for *T. vesiculosum*, the thiolysis data (**Table 1**) indicate the terminal units consist of equal proportions of both epimers (catechin/epicatechin) for the *T. resupinatum* procyanidin fraction, information that is lost in the MALDI-TOF MS spectra (**Figure 2C**).

Structure, Stereochemistry, and Biosynthesis. The butanol−HCl assay estimation of the proanthocyanidin content of *Trifolium* flowers and characterization of proanthocyanidin fractions by both thiolyic cleavage and MALDI-TOF MS thus provide a consistent view of the floral proanthocyanidins evaluated in this study. The stereochemical findings for floral *Trifolium* proanthocyanidins are of interest in light of recently reported discoveries in flavan-3-ol biosynthesis (*15*, *17*, *18*). The observations presented here for floral *Trifolium* spp. contrast with proanthocyanidins of the reference plant *Arabidopsis*, for which both terminal and extender units have been shown to consist exclusively of the cis (procyanidin) stereochemistry (*13*, *18*).

Although *Arabidopsis* makes proanthocyanidin, there is no observed LAR orthologue in the *Arabidopsis* genome. This may be because *Arabidopsis* seems to produce only an epicatechin, rather than a dual catechin/epicatechin-based proanthocyanidin similar to that of many other plants (*10*). Transformation of the forage, *M. sativa*, which does not produce proanthocyanidins (*16*), with the anthocyanin regulatory genes of maize (Lc, B-Peru, and C1) stimulates the flavonoid pathway and only the Lc gene activated LAR in foliage with accumulation of proanthocyanidin polymer, but catechin monomers were not detected. The definitive research to date by Xie et al. (*18*) has been on the BANYULS (BAN) genes from *Arabidopsis thaliana* and *M. truncatula* that encode anthocyanidin reductase, which converts anthocyanidins to their corresponding 2,3-*cis*-flavan-3-ols. Expression of BAN in tobacco flower petals and *Arabidopsis* leaves resulted in the loss of anthocyanins and accumulation of proanthocyanidins. Strategies with dihydroflavonol reductase (DFR) and F3′5′H expression into *L. corniculatus* root cultures to modify the hydroxylation of proanthocyanidins polymers (*17*) were associated with increased concentrations, but there was also no alteration in polymer hydroxylation. These results (*17*) suggested that additional mechanisms might control the hydroxylation state of proanthocyanidins in this legume species. Grape genes encoding the enzymes LAR and ANR, recently isolated and functionally characterized by Bogs et al. (*31*), indicated that the flowers had high proanthocyanidin concentrations, and accumulation continued in skin and seeds from fruit set until the onset of ripening. The genes encoding LAR were expressed in developing fruit, particularly in seeds, but had low expression in leaves.

Although recent studies (*10*, *16*) have established that the flavan-3-ol stereoisomers catechin and epicatechin are the products of biosynthetic conversion processes different from that of the common precursor leucocyanidin, the molecular species involved in chain extension and the determinants of their stereochemistry remain a matter of conjecture (*7*, *9*, *18*).

The pattern of observations of floral proanthocyanidin composition detailed here indicates that the stereochemistry of the extension units and linkage between that of the terminal units may be independently regulated. This research highlights the benefits of dialogue between plant breeders and laboratory scientists connecting innovations in plant research to future downstream applications through plant genomics, as suggested by Delmer (*32*). This could lead to efforts to introgress valuable traits from new sources and to identify genes that control key traits. Therefore, selected floral *Trifolium* spp. may present future targets for investigation of genes and enzymes involved in the regulation of proanthocyanidin biosynthesis. These agricultural forage species may provide leads into proanthocyanidin biosynthesis with implications for a wider range of proanthocyanidin-containing plants with health benefits for animals and humans.

ABBREVIATIONS USED

DM, dry matter; mDP, mean degree of polymerization; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

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