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Translocation of Coumarin Across a Graft Union m Sweetclover

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Translocation of Coumarin Across a Graft Union in Sweetclover[1]

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DESPITE several recent investigations of the biosynthesis of coumarin and related compounds in sweetclover (1, 7, 8, 12), little is known of the site or sites within the plant at which coumarin is formed. Weygand and Wendt (12) reported coumarin formation in root cultures of *Melilotus officinalis* (L.) Lam. when suitable precursors were supplied. Mothes and Kala (9) concluded that scopoletin and umbelliferone, compounds closely related to coumarin, can be synthesized by root cultures of *Atropa belladonna* L. The experiments cited (9, 12) gave no indication that the roots are the preferred site of synthesis or that synthesis takes place at all in the roots of intact plants. Neither of the studies excluded the possibility of synthesis in other organs.

If one assumes that coumarin is not translocated within the plant and that the use or destruction of the compound proceeds at relatively equal rates in all organs, the coumarin contents of the various plant parts might then be used as an indication of amounts of synthesis occurring at the respective sites. The distribution of coumarin in leaves, stems, and roots of ungrafted plants of sweetclover has been investigated by Brink,[3] Goplen et al. (4), Schaeffer et al. (11), and Akeson.[4] In this paper, data on the coumarin content of grafted sweetclover plants are presented and the significance of this information is discussed.

The term *coumarin*, as used in this paper, needs some clarification since recent work (6, 10) has disclosed that intact sweetclover plants contain little or no coumarin in the free, molecular form. On the other hand, it is now clear that glycosides of both *o*-coumaric acid (*trans-o*-hydroxycinnamic acid) and coumarinic acid (*cis-o*-hydroxycinnamic acid) are present in sweetclover tissue (7, 8, 10). Hydrolysis of these glycosides releases coumaric and coumarinic acids, both of which contribute to coumarin equivalence values in the fluorometric procedure used. The term coumarin is retained in this paper for convenience and because standards of pure coumarin were used in the assay procedure. It should be emphasized, however, that the glycosides of o-hydroxycinnamic acid, not molecular coumarin, are present in the intact plant.

MATERIALS AND METHODS

Plant Materials

The *Melilotus alba* Desr. plants used in making the grafts were homozygous with respect to the alleles *Cu/cu* and *B/b*. The effects of these two pairs of alleles on level and form of coumarin in sweetclover have been described in earlier publications (3, 6). Coumarin content is high in *Cu* plants and low in *cu* plants. Preparations of *B* plants display β-glucosidase activity while activity is virtually absent in preparations of *b* plants. All plants were derived from a cross of an individual of the *cucuBB* genotype and a *CuCubb* plant. The original cross was followed by nine generations of self-pollination of doubly heterozygous individuals. An additional generation of self-pollination was then required for the isolation of the four homozygous genotypes. Therefore, the lines used approached isogenicity for all genes except *Cu/cu* and *B/b*. Plants were grown in the greenhouse. A single plant of each genotype was used as the source of scions. Stocks used were clones of single plants in many cases or consisted of closely related F_{11} plants obtained from a single F_6 plant. Control grafts for each of the four genotypes consisted exclusively of stocks derived from cuttings from a single plant with scions obtained from the same plant as the stocks.

Grafting Procedures

Scions consisted of portions of stem approximately 1 cm. in length obtained from the terminal growing point of either the main stem or the side branches. With a razor blade the basal portion of the scion was gently trimmed to a wedge shape and quickly inserted into a longitudinal slit cut in a young, actively growing branch of the stock plant within 5 to 8 cm. of the apex of the branch. The slit, approximately 0.5 cm. in length, was made completely through the center of the stem. The scion was positioned in the slit at an upward angle and was held in place by wrapping with thin strips of self-sticking latex rubber. The portion of stem of the stock plant immediately above the scion was removed only after the scion had become established and was beginning to grow. Side branches from the stock plant were kept trimmed to give the scion increased opportunity for growth. Six or more plants of each graft combination were prepared so that at least four successful grafts of each type would be available for assay. Grafts were made during March 1960 and, where necessary, replacements were prepared during April. On May 26, all scions except those involving high-coumarin scions on low-coumarin stocks and *CuCuBB* scions on *CuCubb* stocks were cut off just above the first node. The resultant activation of the axillary bud in this node produced fresh, young growth that could be sampled close to the graft union. The high-coumarin scions were left intact since it was thought that this procedure would provide maximum amounts of coumarin for possible translocation to the stocks.

Sampling and Assaying Procedures

Leaves—Leaves were sampled 12 days after the original scions were cut back. Each sample consisted of the youngest, fully expanded leaf taken at the apex of a branch as close as possible to the graft union. The average distance from the graft union to the leaves sampled on the various scions was 6.5 cm. Because young leaves could not be found near the graft on the main stem of stocks, samples were obtained from short branches arising as close as possible to the graft unions. These branches arose at an average distance of 2.3 cm. below the graft union, with an additional distance of 6.2 cm. up the branch to the leaf to be sampled. In some cases, two leaves were used to increase sample size. In grafts involving high- and low-coumarin genotypes, leaves were taken from only the low-coumarin part of the graft. Samples from grafts of *CuCuBB* and *CuCubb* plants consisted of the *CuCubb* portion of the graft. In all control grafts, leaves were taken both above and below the graft union. Leaves were harvested simultaneously from the four plants representing one graft combination and immediately were placed in a small high-humidity chamber in which they were carried to the laboratory. Leaflets were then weighed to the nearest 0.1 mg. on a direct-reading balance. The mid-leaflet, used for the determination of dry matter, was dried overnight at 100° C. The two side-leaflets, weighed as one sample, were used for the determination of coumarin content. The extraction and assay procedures used were described earlier (5).

Stems—Stem samples, taken approximately 3½ weeks after cutting back the scions, were harvested from the same plants from which leaf samples were taken. Grafted stems were removed from

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[4] Akeson, W. R. Effect of genotype on level and distribution of melilotic acid and related compounds in *Melilotus*. Unpublished M.S. thesis, University of Nebraska, 1961.

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the plant as far as possible below the graft union and the cut ends of the stems were immediately submerged in water in a small test tube. Leaves and branches above and below the graft union, and the latex rubber surrounding the graft union, were carefully removed. Samples for coumarin assay consisted of a 1-cm. section of stem which included the graft union, and 5-cm. sections of stem immediately above and below the graft union. Wherever possible, a second 5-cm. section, both above and below the graft, was used for the determination of dry matter. The samples were weighed to the nearest 0.1 mg. on a direct-reading balance. Samples for dry matter determination were dried overnight at 100° C. before reweighing. The procedures for the extraction and assay of coumarin in the three sections from each grafted stem were as described previously (5) except that the stem sections were cut into pieces approximately 0.5 cm. in length, no ethyl alcohol was added, and an autoclaving time of 30 minutes was used.

RESULTS AND DISCUSSION

Data in the tables are presented in terms of percentage coumarin based on oven-dry weight and as the percentage of the contents in the control grafts. In the latter case, values for control grafts, in which scions and stocks from the same plant were grafted together, are shown as 100% for the respective comparisons in the tables.

The coumarin contents of four different types of scions, grafted to various types of stocks, are presented in Table 1. For the detection of the upward translocation of coumarin, the graft combinations involving low-coumarin scions on high-coumarin stocks are the most pertinent. The coumarin contents of both leaves and stems from *cucubb* and *cucuBB* scions, grafted to high-coumarin stocks, were greater than the levels in comparable tissues of the control grafts. In leaves increases ranged up to 2.7-fold, and in stems increases of 2.5- to 4.4-fold were observed. Thus, some upward translocation across the graft union was indicated.

The influence of various types of scions on the coumarin contents of four different types of stocks is shown in Table 2. Leaves from low-coumarin stocks to which high-coumarin scions had been grafted were generally somewhat higher in coumarin content than leaves from control stocks, but results were not completely consistent. In stem sections of stocks increases of 2.3- to 5.8-fold were observed when low-coumarin stocks were grafted to high-coumarin scions. Therefore, the downward translocation of coumarin was similar in extent to the movement upward.

In Table 1, extremely low values are shown for stem tissues from *CuCuBB* scions grafted to both types of low-coumarin stocks. In similar grafts involving scions of the other high-coumarin genotype, *CuCubb,* a slight increase in coumarin content was observed. The reverse situation was indicated by the data shown in Table 2. A slight reduction in coumarin content of high-coumarin stocks was noted when low-coumarin scions were grafted to *CuCubb* stocks, but a slight increase was noted on *CuCuBB* stocks. The reason for these differences is not apparent at present.

In Tables 3 and 4 the effects of the high- and low-coumarin alleles *(Cu/cu)* on coumarin translocation are summarized. In Table 3 comparison of the value 0.047% for stem tissue from low-coumarin scions grafted to high coumarin stocks with 0.528% for the high-coumarin control scions and 0.014% for the low-coumarin control scions reveals that despite the increased level in the low-coumarin scions the content was still only about 9% of the content found in the high-coumarin control scions. Similarly, the values 0.028, 0.136, and 0.007% in Table 4 indicate that although the coumarin contents of stem tissue from low-coumarin stocks were increased by translocation from high-coumarin scions, the levels were still only about 20% of the contents observed in high-coumarin control stocks. Thus, it may be concluded that both upward and downward translocation of coumarin occurred, but the amount translocated appeared to be small in comparison with the amount of coumarin present in normal, high-coumarin tissue. This conclusion is in agreement with the preliminary observations reported by Brink.[3]

Table 1—Coumarin contents of scions grafted to various types of stocks.

Genotype		Coumarin content of scions			
Scion	Stock	Leaves		Stems	
		% ± S. E.*	% of control†	% ± S. E.*	% of control†
CuCuBB	CuCuBB	(5.59) ± 0.679		(1.074) ± 0.282	100
	CuCubb	-	-	0.516 ± 0.051	48
	cucuBB	-	-	0.200 ± 0.026	19
	cucubb	-	-	0.182 ± 0.014	17
CuCubb	CuCuBB	5.98 ± 0.409	71	0.252 ± 0.018	69
	CuCubb	8.46 ± 0.907	100	(0.366) ± 0.113	100
	cucuBB	-	-	0.532 ± 0.093	145
	cucubb	-	-	0.520 ± 0.102	142
cucuBB	CuCuBB	0.097 ± 0.013	102	0.037 ± 0.003	247
	CuCubb	0.254 ± 0.064	267	0.061 ± 0.007	407
	cucuBB	0.095 ± 0.025	100	0.015 ± 0.001	100
cucubb	CuCuBB	0.172 ± 0.043	232	0.053 ± 0.004	442
	CuCubb	0.173 ± 0.053	234	0.040 ± 0.003	333
	cucubb	0.074 ± 0.015	100	0.012 ± 0.001	100

* Values in parentheses are means of three replications; all other values are means of four replications. The percentages are expressed on an oven-dry basis.
† Control plants of each genotype were prepared by grafting together scions and stocks from the same plant.

Table 2—Coumarin contents of stocks grafted to various types of scions.

Genotype		Coumarin content of stocks			
Stock	Scion	Leaves		Stems	
		% ± S. E.	% of control	% ± S. E.	% of control
CuCuBB	CuCuBB	(5.74) ± 0.407	-	(0.103) ± 0.024	100
	CuCubb	-	-	0.106 ± 0.015	103
	cucuBB	-	-	0.137 ± 0.048	133
	cucubb	-	-	0.127 ± 0.030	123
CuCubb	CuCuBB	4.53 ± 0.552	61	0.196 ± 0.012	150
	CuCubb	7.49 ± 0.996	100	0.131 ± 0.023	100
	cucuBB	-	-	0.095 ± 0.010	73
	cucubb	-	-	0.084 ± 0.006	64
cucuBB	CuCuBB	0.309 ± 0.207	454	0.035 ± 0.004	583
	CuCubb	0.105 ± 0.023	154	0.028 ± 0.004	467
	cucuBB	0.068 ± 0.015	100	0.006 ± 0.001	100
cucubb	CuCuBB	0.083 ± 0.033	157	0.021 ± 0.001	233
	CuCubb	0.052 ± 0.015	98	0.029 ± 0.006	322
	cucubb	0.053 ± 0.027	100	0.009 ± 0.001	100

* See footnotes in Table 1 for basis of figures given as "%" and as "% of control."

Table 3—Summary of the effect of the *Cu/cu* gene pair on the coumarin content of scions grafted to various types of stocks.*

Genotype		Coumarin content of scions			
Scion	Stock	Leaves		Stems	
		% ± S. E.	% of control	% ± S. E.	% of control
CuCu	CuCu	6.77 ± 0.545	-	0.528 ± 0.101	100
CuCu	cucu	-	-	0.358 ± 0.054	68
cucu	CuCu	0.174 ± 0.026	207	0.047 ± 0.003	336
cucu	cucu	0.084 ± 0.014	100	0.014 ± 0.001	100

* See footnotes in Table 1 for basis of figures given as "%" and as "% of control."

Table 4—Summary of the effect of the *Cu/cu* gene pair on the coumarin content of stocks grafted to various types of scions.*

Genotype		Coumarin content of stocks			
Stock	Scion	Leaves		Stems	
		% ± S. E.	% of control	% ± S. E.	% of control
CuCu	CuCu	5.90 ± 0.549	-	0.136 ± 0.013	100
CuCu	cucu	-	-	0.111 ± 0.014	82
cucu	CuCu	0.137 ± 0.051	225	0.028 ± 0.002	400
cucu	cucu	0.061 ± 0.015	100	0.007 ± 0.001	100

* See footnotes in Table 1 for basis of figures given as "%" and as "% of control."

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The values obtained for stem sections probably represent the maximum translocation attained in these plants, since the tissues sampled were immediately adjacent to the graft union. In comparison with stem samples, leaf samples generally gave slightly less indication of translocation, doubtless because the leaves assayed were farther from the graft union. Results from leaves were also somewhat more variable, probably because the distance from the graft union to the leaf sampled was not the same in all cases. In preliminary experiments, no evidence of translocation was detected in leaf samples considerably more distant from the graft union than the leaf samples whose coumarin contents are reported in the tables. Only as sampling techniques were improved could translocation be detected. From the foregoing discussion, it is apparent that the amount of coumarin translocation in grafts was either relatively small or that destruction of the translocated coumarin occurred in the low-coumarin tissue.

Assay of the 1-cm. sections of stem that included the graft union revealed coumarin percentages that were intermediate between the levels found in the scion and the stock. Thus, no accumulation of coumarin at the graft union was indicated.

No evidence of translocation was detected in several approach grafts between high- and low-coumarin plants. In this type of graft, very little of the stem tissue is damaged in the grafting procedure.

In a series of root grafts, the coumarin content of low-coumarin scions grafted to high-coumarin roots was no higher than comparable tissue from ungrafted low-coumarin plants. When high-coumarin scions were grafted to low-coumarin roots, the coumarin content of the scions was no lower than the levels found in comparable tissues from high-coumarin scions grafted on high-coumarin roots or in ungrafted high-coumarin plants. One can conclude from these findings that the root is of little or no importance in determining the coumarin content of the above-ground parts. This result is in marked contrast to the situation in tobacco where nicotine is synthesized primarily in the root and is actively translocated to the stems and leaves (2).

The recent investigations of Goplen et al. (4), Schaeffer et al. (11), and Akeson[4] on distribution of coumarin in the sweetclover plant are in agreement in indicating that young leaves are high in level of coumarin, stems are intermediate, and roots are low. Typical values reported by Akeson for greenhouse-grown material are 4.77% (dry weight basis) for young leaves, 0.83% for stems, and 0.22% for roots. Several possible explanations might be suggested for this pattern of distribution. Perhaps the two simplest possibilities are (a) that coumarin is made exclusively in the young leaves and then translocated in limited quantities to the other portions of the plant or (b) that coumarin is made at different rates in the various parts of the plant and translocation is relatively unimportant in determining the distribution pattern. The limited translocation indicated by the experiments reported here does not permit an unequivocal choice between these or other possible explanations. However, available data on distribution and translocation do provide convincing evidence that, whether or not all coumarin is made in the leaves, the leaves are certainly an important site of coumarin synthesis.

SUMMARY

Translocation of coumarin in sweetclover was studied by using graft combinations of plants differing with respect to the coumarin-conditioning genes *Cu/cu* and *B/b*. The assay of stem sections, taken immediately above and below the graft union, indicated that the coumarin contents of low-coumarin stocks and scions grafted to high-coumarin tissues were approximately 2 to 5 times as great as the levels in comparable low-coumarin tissues of the control grafts. However, the amount detected was relatively small compared with the amount of coumarin in comparable high-coumarin tissue. Results from leaves taken somewhat farther from the graft union than the stem sections were in general agreement with the data from stems except that slightly less evidence of translocation was observed. Translocation was not detected in tissues taken some distance from the graft union. Thus, evidence for translocation of coumarin was strongest in the stem sections, probably because the tissues sampled were immediately adjacent to the graft union.

Data from root grafts indicated that the root is apparently of little or no importance in determining the coumarin content of the above-ground parts. Assays of leaves, stems, and roots of high-coumarin sweetclover plants suggest a gradient from high levels in young leaves to extremely low levels in roots. This pattern of coumarin distribution, viewed in conjunction with the data pertaining to translocation, may be interpreted as indicating that the primary site of coumarin synthesis is in the young, actively growing leaves and that stems and roots are relatively unimportant in coumarin synthesis.

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