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Possible metabolic basis for the developmental anomaly observed in in vitro culture, called 'vitreous plants'

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Abstract. Previous studies have shown that the developmental anomaly encountered in meristem culture, known as 'vitreous plants', is due to deficient lignin synthesis. This anomaly can be cured by addition of phloridzin to the culture medium. This study examines the activities of some enzymes involved in the synthesis of lignins and of flavonoids in normal and in vitreous plants of two apple cultivars. The results showed that all enzymes were consistently less active in the vitreous plants. This agrees with previous studies made on the hydroxycinnamate:CoA ligase activity in *Prunus avium* (L.) meristem-derived plants. The study on the substrate specificity of the enzyme demonstrates that while its activity is lower in the vitreous plant, its conformation is identical with that of the normal plant; the substrate that is specific to enzyme extracts of both sources is para-coumaric acid.

Introduction

The propagation of plants by in vitro techniques has proven to be very successful. A number of problems have been encountered by researchers and practitioners in this field, however in most cases plants showing abnormal development are thrown away and counted as 'loss'. One such developmental is the anomaly named 'vitreous plants'. These plants 'have an abnormal development, resulting in numerous stems with short, almost nonexisting internodes; the stems are dumpy, the leaves have broad basis, the laminae are narrow, longitudinally curved outwardly and less chlorophyllous than normal leaves, ... all parts, the peduncles and the laminae of the leaves exhibit a water, translucent aspect...' [14]. The disorder has been encountered with about fifteeen species, and was at first thought to affect only woody plants, but later found to occur in herbaceous plants as well (e.g. several species of *Allium, Brassica, Gerbera, ...*).

Hegedus and Phan [7] reported that the content of phloridzin was very low in 'vitreous' apple plants. Vieth et al. [16, 17] showed, by a microscopic study, that the anatomical structure of the stem of 'vitreous' plants exhibited an abnormal process of development, particularly of lignification. Most cells of the xylem were not lignified, and some lignified cells were found in the peripheral regions of the cortex. These observations, made on apple-plants, were corroborated by quantitative data obtained on *Prunus avium*

and *Salix babylonica* by Legouzé and his co-workers [9, 10, see also Beauchene, 2]. More recently, Phan and Letouzé [14] showed that the activity of hydroxycinnamate:CoA ligase, a key enzyme in phenol metabolism, was very low in 'vitreous' plants of *Prunus avium*, (L.) They also reported that the levels of proteins and chlorophylls were lower, while the concentration of soluble phenolics was high, suggesting that they were not used for lignification and other synthetic processes.

In the present work, we attempt to verify these data for apple-plants on hydroxycinnamate:CoA ligase, as well as on phenylalanine ammonia-lyase, hydroxycinnamoyl-CoA reductase and hydroxycinnamyl alcohol dehydrogenase.

Hegedus and Phan [7] demonstrated that phloridzin and its components (phlorogucinol and p-coumaric acid) induced the abnormal plants to resume their normal appearance, when added to the culture medium at low concentrations. Because p-coumaroyl-CoA plays a key role in lignin synthesis [Gross, 6], and serves as the precursor to flavonoid synthesis, we hypothesized that phloridzin given to vitreous plants could be catabolized into p-coumaroyl-CoA. This compound could then be used as the precursor for the synthesis of lignin, and consequently 'cures' the abnormal plants.

A curing effect has also been reported by Boxus [3] who cold-treated vitreous plants. It has also been observed that plants that had been induced to root had a higher level of phenolic compounds and could cure themselves spontaneously. In order to test these findings, we have extended our study to examine the effect of phloridzin applications, of cold treatment, and of rooting treatment, on the activities of the above mentioned enzymes.

In addition, we have assayed the crude enzyme extracts from normal and vitreous plants with different substrates, in order to find whether the anomaly induced any conformational change in the hydroxycinnamate CoA ligase.

Materials and methods

The meristems used in this work were taken from 2–3 year old plants of M-26 and 0–3 cultivars grown in the Experimental Farm of Agriculture Canada, in Frelighsburg, Quebec. Twigs were cut in autumn, when the buds were dormant, stored in a cold room (4 C) for at least 4 weeks to completely break dormancy, then were brought to room temperature to force bud growth. Buds of about 1 mm were excised, inoculated and allowed to grow for three months in a Murashige–Skoog medium [13], with benzyladenine (BA) (0.1 mg/l) as the cytokinin. The BA concentration was then increased to 0.5 mg/l, for 2 to 3 months, during which time the plants developed 4 to 5 stems for inoculum. Rooting was then induced using a medium containing the major elements of M & S at 0.5 x normal concentration and replacing BA by 0.5 mg/l IBA (indolebutyric acid). The newly formed daughter-plants were

then individually inoculated in 25 x 160 mm tubes containing 6 ml of Murashige-Skoog medium. Subsequent inoculations were made at 28 day-intervals. For all these steps, the cultures were illuminated 16 hours per day, with Westinghouse Cool-white tubes, giving out an irradiance of 56.6 μE S^{-1} m^{-2}.

Phenylalanine ammonia lyase (PAL) was assayed according to Bartz et al. [1] using phenylalanine as the substrate and the evolved cinnannic acid was estimated by absorption spectroscopy (290 nm). Hydroxycinnate: CoA ligase activity was estimated by the method of Ranjeva et al. [15], using p-coumaric acid as the substrate. Caffeic and ferulic acids were used in the specificity assay. Flavanone synthase was assayed according to the Ranjeva et al. [15], and chalcone-flavanone isomerase, according to Moustapha and Wong [12]. Hydroxycinnamoyl-CoA: NADP reductase and hydroxycinnamyl alcohol dehydrogenase activities were determined by measuring the change in the concentration of NADP by its optical density at 332 nm (Luderritz and Griesbach [11]. All enzyme activities are expressed in nanokatal per kg protein. Proteins were determined by Bradford's technique [4] colouring the proteins with Coomassie blue and reading the optical density at 525 nm [4]. Bovine serum albumin was used as the standard. All chemicals were purchased from SIGMA, and the absorption measurements were made using a Beckman spectrophotometer (Model Acta III).

Phloridzin (0, 1 g) was dissolved in 5 ml ethanol, then made up to 100 ml with distilled water. This stock solution was added to the cold medium prior to autoclaving. For cold treatment, plants were placed at 4 C for 4 weeks, in continuous darkness.

Results and discussion

A. Enzyme activities in normal and vitreous plants

Table 1 shows the comparative activities of PAL (I), hydroxycinnamate: CoA ligase (II), flavanone synthase (III), hydroxycinnamoyl-CoA: NADP reductase (IV), and hydroxycinnamyl alcohol dehydrogenase (V). All enzymes except flavanone synthase exhibited a markedly lower activity in vitreous plants than in normal ones.

The lower activities of hydroxycinnamoyl-CoA: NADP reductase and of hydroxycinnamoyl alcohol dehydrogenase are in agreement with a lower lignification rate in the vitreous plants. The hydroxycinnamate: CoA ligase results confirm those obtained by Phan and Letouzé [14] on *Prunus avium.* The lower activity of this enzyme in the vitreous plants also suggests a weakened lignification process. PAL activity is also lower in vitreous than in normal plants; this could indicate a product inhibition of the enzyme by the hydroxycinnamic acids, which accumulated because of the lower activity of hydroxycinnamate: CoA ligase.

Table 1. Activity of five enzymes in normal and vitreous plants of apple cultivars M-26 and 0-3

Enz.	Day	M-26			0-3		
		Normal	Vitreous	V/N%	Normal	Vitreous	V6N%
I	0	4.39	0.47	10.7	2.34	0.13	5.6
PAL	3	5.04	0.47	9.1	4.05	0.14	3.5
	7	4.99	0.55	11.0	4.34	0.12	2.8
	10	5.06	0.67	13.2	4.33	0.17	3.9
	14	5.11	0.61	11.9	4.54	0.17	3.7
	21	5.20	0.73	12.1	4.69	0.21	4.5
	28	5.31	0.73	13.7	4.83	0.19	3.9
II	0	39	11	28	29.3	5.4	18.4
Hydroxycinnamate:	3	41	12	29.3	33.2	3.2	9.6
CoA	7	42	10	23.8	44.5	4.7	10.6
ligase	10	36	15	41.7	41.4	8.3	20.0
	14	43	19	44.2	39.6	7.9	19.9
	21	50	21	42.0	39.6	8.7	20.0
	28	65	17	27.2	45.3	10.1	22.3
III	0	0	0		0	0	
Flavanone	3	0	0		0	0	
Synthase	7	0	0		0	0	
	10	0.07	0		0	0	
	14	0.06	0		0	0	
	21	0.03	0		0	0	
	28	0	0		0.07	0	
IV	0	26.2	0		19.3	0.3	1.6
Hydroxicinnamoyl-	3	26.1	0		17.3	0.7	4.0
CoA NADP	7	26.3	1.0	4.7	17.4	1.1	6.7
reductase	10	29.4	1.2	4.1	18.3	1.8	11.0
	14	30.0	3.1	10.3	20.4	2.4	11.8
	21	30.9	3.3	11.0	21.8	3.1	14.2
	28	33.1	5.1	15.4	23.2	3.6	15.5
V	0	5.6	0.8	14.3	5.6	0	
Hydroxycinnamoyl	3	5.4	0.9	17.7	5.9	0	
alcohol	7	6.0	0.7	11.7	6.4	0	
dehydrogenase	10	8.8	1.3	14.8	7.4	0	
	14	14.8	3.3	22.3	17.1	0	
	21	20.0	8.8	44.0	17.1	0	
	28	25.0	13.3	53.2	17.0	8.75	51.5

N.B. All enzyme activities are expressed in nKatal/Kg protein

Table 2. Flavanone synthase activity* in normal (rooted and non-rooted) and in vitreous (non-rooted, rooted and phloridzin-treated) plants

		Vitreous			Normal	
	Days	Non-rooted	PZ-treated	Rooted	Non-rooted	Rooted
	0	0	0	0	0	0
	3	0	0	0	0	0
	7	0	0	0	0	0
M-26	10	0	0	0	0.07	0.07
	14	0	0	0	0.06	0.12
	21	0	0.04	0	0.03	0.15
	28	0	0.03	0.02	0	0.10
	0	0	0	0	0	0
	3	0	0	0	0	0
	7	0	0	0	0	0
0–3	10	0	0	0	0	0
	14	0	0	0	0.03	0.07
	21	0	0	0	0.02	0.04
	28	0	0.01	0.07	0	0.04

*In nKal/Kg protein

Table 3. Internal phloridzin concentration of apple-plants (mg/g f.w.)

	M-26			0–3		
Day	Normal	Vitreous	Vitreous PZ-treated	Normal	Vitreous	Vitreous PZ-treated
0	0.082	0.011	0.014	0.021	0.006	0.009
3	0.079	0.012	0.022	0.022	0.005	0.008
7	0.080	0.017	0.051	0.025	0.009	0.010
10	0.084	0.022	0.069	0.024	0.007	0.010
14	0.089	0.033	0.107	0.021	0.007	0.018
21	0.090	0.012	0.102	0.017	0.014	0.017
28	0.086	0.025	0.084	0.013	0.017	0.023

Note: PZ = phloridzin

Flavanone synthase activity was very low in apple plants, in general. It was slightly higher in normal plants than in vitreous plants. Phloridzin is a compound specifically found in the apple-tree and therefore the relative inactivity of the enzyme which synthesizes its precursor (naringenin) is surprising. Vitreous plants treated with phloridzin and normal rooted plants developed some activity of this enzyme (Table 2). This finding corroborates the higher internal level of phloridzin found in treated and rooted plants (Table 3).

Comparing the activity of the enzymes in vitreous and normal plants, we found two different behaviours. PAL and ligase activities in both types of plants (Table 1) remained practically unchanged with time. As a result the ratio V/N also remained almost constant. On the contrary, the activities of hydroxycinnamoyl-CoA reductase and hydroxycinnamyl alcohol dehydrogenase increased with the age of the plant. In addition, the activity of each

enzyme increased more rapidly in the vitreous plants, resulting in a temporal increase in V/N. This means that surviving vitreous plants could eventually reach a stage when their lignin synthesizing enzyme content is sufficient to allow them to resume normal development. This is what has sometimes been observed. Vitreous plants have been found to spontaneously cure themselves, becoming green and developing the appearance of normal plants. We have no explanation for this self-curing phenomenon.

B. Effects of curing treatments

1. Effect of rooting (Tables). In normal plants, those that were rooted had higher activities of all enzymes except for flavanone synthase (Table 2). Rooting led to considerable stimulation of the activity of all enzymes assayed in vitreous plants, however (Table 4).

2. Effect of phloridzin treatment. Hegedus et al. [7] have shown that vitreous plants had very low concentrations of phloridzin, and that phloridzin added to the culture medium, could reverse the trend and cause the vitreous plants to revert to normal. Table 3 shows that the applied phloridzin was absorbed by the treated plants, and Table 5 shows that it enhanced the activity of the two key-enzymes PAL and hydroxycinnamate:CoA ligase. There was a good correlation between the level of internal phloridzin and enzyme activity (Figure 1). The case of flavanone synthase is unique in that it increased in later developmental stages (Table 1 and 2). We can infer from these data that phloridzin is actively converted into its precursor(s). Also supporting this idea are the increased activities of hydroxycinnamoyl-CoA reductase and of hydroxycinnamoyl alcohol dehydrogenase, which indicate more active lignification. Flavanone synthase activity, when present, is very low, however. Moreover, there is a small but a definite increase in the activity of PAL and a sharp enhancement of hydroxycinnamate:CoA ligase. These data are indicative of a re-activation of the synthesis and the esterification by CoA of the hydroxycinamic acids. In other words, they indicate the 'normal' process of lignification. Phloridzin absorbed by treated vitreous plants might be used at the beginning of the processes, if it is used at all, but it does not seem to be the precursor of lignin in later stages.

3. Effect of cold treatment. The internal content of phloridzin in treated plants increased with age. The same trend was observed in the protein content, and the activities of PAL, hydroxycinnamate:CoA ligase and hydroxycinnamyl alcohol dehydrogenase (Table 6). This indicates that cold treatment triggers all the processes that contribute to a normal development of the plant. As pointed out by Hegedus et al. [7], however, the curing effect of cold treatment is short-lived, and the second generation of 'cured' plants tend to be again vitreous. On the contrary, the effect of phloridzin treatment is durable, and it is only in the fifth generation that some vitreous plants are encountered.

Table 4. PAL and Hydroxycinnamate:CoA ligase activities* in normal (rooted and non-rooted) plants and in vitrous (non-rooted, rooted, phlorizin treated) plants

PAL	Days	Vitrous non-rooted**	Vitreous PZ-treated	Vitreous rooted	Normal non-rooted	Normal rooted
	0	0.39	0.39	0.43	4.39	4.54
	3	0.47	0.35	0.49	5.04	4.77
	7	0.53	0.37	0.54	4.99	4.99[2]
m–26	10	0.55	0.49	0.79	5.06	5.04
	14	0.67	0.89	2.04[2]	5.11	5.23
	21	0.72	1.09	2.79	5.20	5.29
	28	0.81	1.83[1]	3.11[3]	5.31	5.36
	0	0.17	0.11	0.09	2.34	2.45
	3	0.14	0.11	0.14	4.05	2.71
	7	0.15	0.17	0.79	4.34	3.80[2]
0–3	10	0.20	0.34	0.83	4.33	3.83
	14	0.23	0.77	1.89	4.54	3.75
	21	0.22	0.89	1.93	4.69	3.80
	28	0.24	1.27	2.04	4.83	3.79
Ligase	Days	Vitreous non-rooted	Vitreous PZ-treated	Vitreous rooted	Normal non-rooted	Normal rooted
	0	11 ± 6	14 ± 5	9 ± 2	39 ± 5	44 ± 3
	3	14 ± 7	19 ± 4	11 ± 3	41 ± 2	43 ± 2
	7	17 ± 9	25 ± 7	29 ± 5	42 ± 4	56 ± 0
M–26	10	15 ± 4	39 ± 2	47 ± 1[2]	36 ± 3	65 ± 3
	14	19 ± 6	42 ± 11	54 ± 4	43 ± 4	89 ± 2[2]
	21	11 ± 6	41 ± 3	71 ± 5	50 ± 7	110 ± 5
	28	17 ± 5	48 ± 4[1]	84 ± 3[1]	65 ± 5	111 ± 4
	0	7.3	5.1	3.2	29.3	31.4
	3	6.7	4.3	7.5	33.2	37.4
	7	5.9	5.9	14.9	44.5	39.8
0–3	10	7.4	7.3	21.3[1]	41.4	59.7[2]
	14	6.9	14.5[1]	26.7	39.6	60.1
	21	6.1	18.0	41.7[2]	43.6	71.4
	28	7.3	17.1	55.6	45.3	77.7

[1]Plant apparently 'normal'
[2]Root initiation
[3]Plant totally "normal"
*in nKatal/kg protein
**Rooted: treated for rooting

Table 5. HydroxycinnamoylCoA:NADP reductase and hydroxycinnamyl alcohol dehydrogenase* activities in normal (rooted and non-rooted) and in vitreous (non-rooted, rooted, phloridzin-treated) plants

Reductase	Days	Vitrous non-rooted	Vitreous PZ-treated	Vitreous rooted	Normal non-rooted	Normal rooted
	0	0	2.0	0	27.2	11.0
	3	0.4	2.0	2.1	27.1	27.1
	7	1.3	5.0	6.3	27.3	34.2
	10	4.1	7.1	9.4	29.4	43.0[1]
	14	7.4	12.7	18.2[1]	30.0	50.5
	21	6.7	11.4	24.8	30.1	46.4
	28	5.1	17.3	28.4	33.1	49.1
	0	0.7	2.1	0.7	19.3	16.4
	3	2.1	1.3	1.3	17.3	18.3
	7	2.4	3.2	3.7	16.4	19.2
	10	2.5	11.4	5.3	18.3	29.0[1]
	14	2.7	12.5	6.9	20.4	29.7
	21	2.5	11.4	19.2[1]	21.8	32.4
	28	2.9	17.3	22.9	23.2	37.9
Dehydrogenase	Days	Vitreous non-rooted	Vitreous PZ-treated	Vitreous rooted	Normal non-rooted	Normal rooted
	0	0.9	2.4	1.3	5.6	7.0
	3	1.3	2.1	2.4	6.0	8.6
	7	1.4	2.6	2.8	6.0	19.0[1]
	10	1.7	5.3	10.3[1]	8.8	21.5
	14	2.8	7.6	24.0	14.8	37.5
	21	4.3	10.3	28.8	20.0	37.5
	28	5.9	15.6	31.3	25.0	43.8
	0	0	0	0	5.6	6.3
	3	0	0	6.4	5.9	7.4
	7	0	1.1	11.2	6.4	7.9
	10	3.4	7.0	10.3	7.4	11.4
	14	3.0	11.1	17.9	17.1[1]	23.0[1]
	21	4.0	19.7	18.3	17.1	27.4
	28	6.9	23.4	19.3	17.0	29.4

*In nKatal/Kg protein
[1]Root initiation

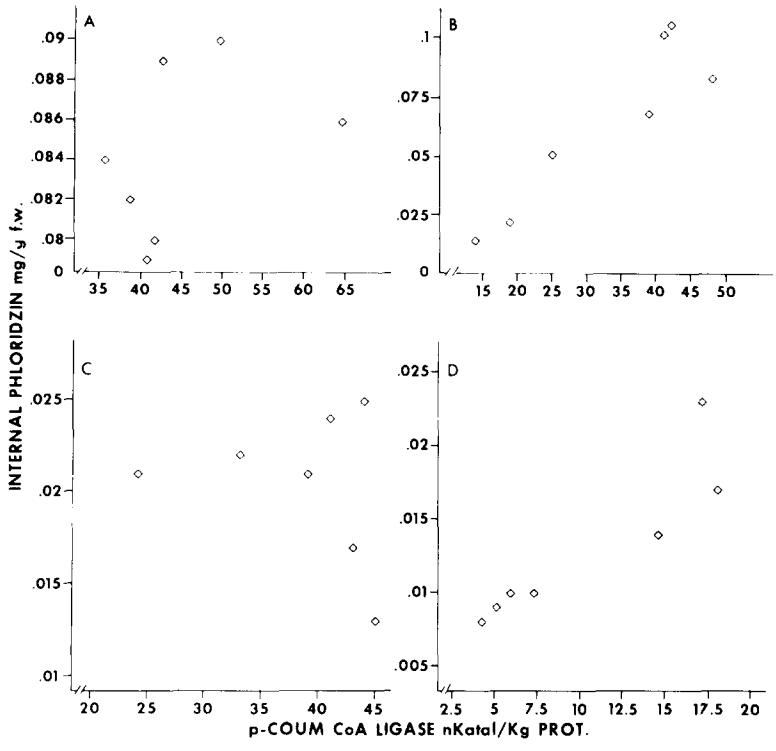

Figure 1. Correlation between internal level of phloridzin and activity of para-coumarate:CoA ligase. A. M-26 normal plant. B. M-26 vitreous, phloridzin-treated plant. C. 0–3 normal plant. D. 0–3 vitreous, phloridzin-treated plant.

Table 6. Effect of cold (4 C) treatment on the internal content of phloridzin protein and the activity* of PAL, hydroxycinnamate: CoA ligase and hydroxycinnamyl alcohol dehydrogenase of vitreous plants (M-26)

Days	mg/g f.w.	mg/g f.w.	PAL	Ligase	Dehydrogenase
0	0.011	8.6	0.26	7.4	9.4
7	0.018	10.1	0.43	11.2	11.2
14	0.025	11.7	1.14	19.8	11.4
21	0.043	21.3	3.41	44.3	25.4
28	0.073	29.7	4.67	55.5	27.9
Control, normal (28)		27.4	5.03	63.1	31.4
Control, vitreous (28)	0.019	9.4	1.66	16.4	13.3

*In nanoKatal/Kg protein

C. Substrate specificity of hydroxycinnamoyl-CoA ligase in normal and vitreous plants

The results in Table 7 show that the enzymes extracted from normal and vitreous plants of both cultivars have the same substrate specificity. The sole substrate used by the ligase was p-coumaric acid. It seems, therefore, that

Table 7. Activity* of hydroxycinnamate:CoA ligase in the presence of three different hydroxycinnamic acids as substrates

Substrate μmol	M-26		0-3	
	Normal	Vitreous	Normal	Vitreous
p-coumaric				
100	7 ± 3	4 ± 1	0	0
75	33 ± 2	5 ± 1	25 ± 3	2 ± 2
50	65 ± 5	17 ± 2	45 ± 2	10 ± 1
25	27 ± 1	8 ± 2	3 ± 1	0
10	9 ± 2	4 ± 1	0	0
Caffeic				
100	0	0	0	0
75	0	0	0	0
50	5 ± 2	0	0	0
25	4 ± 3	0	0	0
10	0	0	0	0
control[1]	65 ± 4	17 ± 2	65 ± 2	10 ± 1
Ferulic				
100	0	0	0	0
75	4 ± 2	0	0	0
50	5 ± 3	0	0	0
25	10 ± 1	0	0	0
10	0	0	0	0
control[1]	65 ± 5	17 ± 2	45 ± 2	10 ± 1

[1]p-coumaric acid, 50 μmol
*In nanoKatal/Kg protein

only the form I described by Grand et al. [5] is present in most of the plants assayed. Only a slight activity was detected with caffeic and ferulic acids, in normal M-26 plants, which could therefore contain a small proportion of each of the forms II and III. The data obtained thus do not indicate a change in the sub-unit assembly of the enzyme. The lower activity must be due to either a smaller amount of enzyme present, or the presence of a less active or inactivated enzyme.

Conclusion

From this study on the activities of various enzymes involved in the phenolic metabolism, the following tentative conclusions may be drawn. In normal plants, the lignin synthesis is active, and the activity of the enzymes involved in flavanone-chalcone synthesis is rather weak. These enzymes are not active in vitreous plants. This corroborates the findings of Hegedus et al. [8] that phloridzin, which is normally present in appletrees, is less abundant or absent in vitreous plants. The key-enzyme of both lines of synthesis (hydroxycinnamate:CoA ligase) has a lower activity in vitreous than in normal plants, which confirms previous results (Phan et al., [14]). This lower activity is not likely to be caused by a conformational change, because the enzyme in the plants assayed is only present in one form (form I of Grand et al., [2]).

The activities of hydroxycinnamoyl-CoA:NADP reductase and hydroxycinnamyl alcohol dehydrogenase were also lower in vitreous than in normal plants. The fact that their activity increased with age in all plants, however, could indicate that these enzymes are not primarily affected by the 'vitrification process' but that their lower activities in younger ages could be due to the depletion of their substrates through reduced ligase activity. The remedial effect of phloridzin indicates that this compound is converted by flavanone synthase back into its (normal) precursor (i.e. p-coumaroyl-CoA), which can be used as a precursor for lignin synthesis; p-coumaroyl-CoA was lacking because of the decreased activity of p-coumarate:CoA ligase. The 'emergency supply' of phloridzin also seems to re-activate the entire set of enzymes, those converting p-coumaroyl-CoA into lignin as well as those synthesizing p-coumaroyl-CoA (i.e. PAL and hydroxycinnamate:CoA ligase). Cold treatment resulted in an increase in the content of phloridzin as well as higher activities of the enzymes involved in phenolic metabolism. Unfortunately, our experiments did not suggest the mode of action of the low temperature treatment.

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