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Phenylalanine ammonia lyase and syringaldazine oxidase activities in relation to lignin deposition in legumes

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ABSTRACT

Temporal differences in maximum enzyme activities were evaluated in relation to cell wall (CW) and lignin deposition in maturing legume stems. Three forage legumes, alfalfa (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.), and red clover (*Trifolium pratense* L.), were established in a greenhouse and basal stem regrowth was sampled biweekly for 10 weeks. Tissue was analyzed for phenylalanine ammonia lyase (PAL) and syringaldazine oxidase (SAO) activities as well as CW components including lignin. Cell wall and lignin content increased sigmoidally; PAL activity increased, peaked, and decreased; and SAO activity increased, peaked, and leveled off or decreased as a function of regrowth days. Maximum deposition of lignin followed that of other CW components. Time of maximum PAL activity occurred 8 days before maximum lignin deposition in birdsfoot trefoil and red clover and 3 days after maximum lignin deposition in alfalfa. Time of maximum PAL activity always preceded time of maximum SAO activity. Neither PAL nor SAO activity on a protein basis were correlated with lignin content. However, on a per plant basis, PAL was correlated with lignin content across species ($r = 0.60$). Syringaldazine oxidase activity was correlated with lignin content in alfalfa ($r = 0.88$), birdsfoot trefoil ($r = 0.97$), and red clover ($r = 0.92$). These results suggest that, while PAL can explain variation in lignin content across species, SAO is more closely related to lignin deposition within species.

INTRODUCTION

Better conceptualization of plant secondary cell walls has been achieved through studies of cell wall (CW) constituent biosynthesis and molecular structure (1-5). During primary-wall development, polysaccharides, phenolic acids, and proteins are incorporated into the wall, but not polymeric lignin. Polysaccharides deposited early in grass and legume stems are richer in hemicelluloses than are those deposited later during secondary wall development (6,7). Lignin deposition in the secondary wall always follows wall thickening. The degree to which individual cells are lignified depends upon the tissue type. Among CW components, lignin deserves attention because it provides plants with mechanical strength (4), protection against pests (8,9), and is thought to provide resistance to digestion by ruminant animals (10).

Genetic variation in lignin concentration has provided incentive for studies of lignin manipulation (11). Lewis and Yamamoto (12) pointed out the need to elucidate basic mechanisms involved in lignification. Recent studies have revealed relationships between phenylpropanoid enzymes and lignification in maize (*Zea mays* L.) (13,14) as well as specific peroxidase activity and lignification as a result of natural maturation in fruit (5). More information is needed, however, to determine temporal relationships between the maximum activity of these enzymes and lignin deposition.

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5), a general phenylpropanoid enzyme, and syringaldazine oxidase (SAO, EC 1.11.1.7), a specific peroxidase, catalyze the first and last steps of the lignin biosynthetic pathway, respectively. Although positive correlations between PAL activity and lignification have been established (15-17), recent investigations demonstrated that PAL activity and lignification may not be correlated due to the time

lag between maximum PAL activity and maximum lignin deposition (13,14). Activity of SAO, on the other hand, should be positively related to lignin synthesis because SAO activity is more closely related to lignin deposition than PAL activity and SAO is committed to lignin polymerization (5,18). Activity of PAL and SAO on a per plant basis (total enzyme units) may be more closely related to lignin deposition than when expressed on a protein basis because it is the amount, as well as the activity of enzymes, that is attributable to the quantity of lignin accumulated.

Increased knowledge of the temporal relationships between enzyme activity and CW component deposition will improve understanding of secondary CW biosynthesis and structure (6,7) and provide a foundation for targeted manipulation of secondary CWs. Basal stem tissue of three maturing temperate legumes was used in this investigation to attain uniformity of sampling and take advantage of the large variation that exists during maturation of these tissues (19). The objectives of this investigation were to: 1) determine the temporal relationships of lignin deposition, other CW component deposition, and PAL activity in relation to SAO activity, and 2) determine if SAO activity, on a per plant basis, is more closely related to lignin content compared with PAL activity. This investigation includes new results from SAO data as well as previously-reported results from investigations of lignin and other CW components (6) and PAL activity (17).

MATERIALS AND METHODS

'Arrow' alfalfa (*Medicago sativa* L.), 'Viking' birdsfoot trefoil (*Lotus corniculatus* L.), and 'Arlington' red clover (*Trifolium pratense* L.) were established in a greenhouse during the spring in 25-cm diameter pots with a capacity of 3.8 L. Pots were arranged in a randomized complete block design of the three species in four replicates. A split-plot arrangement was used with species as whole-plots and sample age as sub-plots. Three plants were maintained in each pot throughout all experiments and an average of the three plants, within each replicate, was used for each data point. Greenhouse conditions for the experiment were described by Bidlack and Buxton (6).

Sampling

All forage was cut at 3 to 5 cm in September and sampled from October through December at 14, 28, 42, 56, and 70 days after regrowth. The basal 10 cm of regrowth stem material was used for analyses. Five grams of fresh material was quick frozen in liquid N_2 for PAL and SAO analyses and the remaining material was used for fiber analyses.

Enzyme Extraction and Assay

Acetone powders were prepared from frozen stem material as described by Bidlack et al. (17). Twenty-five milligrams of acetone powder from individual samples were mixed with 25 mL of 100 mM borate buffer, pH 8.7 (for PAL) or 25 mL of 100 mM phosphate buffer, pH 7.5 (for SAO), at 0 to 2°C. Suspensions were shaken at least once every 5 min for 1 h and then filtered through Whatman No. 54 filter paper to give a clear, crude, enzyme extract. These extracts contained PAL associated with the endoplasmic reticulum (2) and SAO of the CW (20). Protein was determined (21), in duplicate, and multiplied by the acetone powder yield to obtain protein on a per plant basis. The PAL enzyme was assayed spectrophotometrically (17). One unit of PAL activity was expressed as the amount of cinnamic acid produced in μmol h^{-1}.

Syringaldazine oxidase was assayed through modification of spectrophotometric procedures (18). All reagents were prepared the same day as enzyme extraction. Within 5 s of beginning the assay, 0.10 mL of 2 mM syringaldazine (in dimethylsulfoxide) was mixed with 7.4 mL of 0.88 mM hydrogen peroxide (in 100 mM phosphate buffer, pH 7.5). This mixture was added to 1.5 mL of enzyme extract and observed at 525 nm for synthesis of tetramethoxy-azo-bis-methylenequinone (ϵ = 65,000 cm^{-1}) for 1 min. One unit of activity was expressed as the amount of quinone produced in μmol min^{-1}.

Both PAL and SAO assays were run in duplicate and activity was expressed on a protein basis. These activities were multiplied by the protein yield (in acetone powder) of each plant to express PAL or SAO on a units per plant basis.

Fiber analysis

Dried samples were weighed and ground to pass through a 1 mm screen in a Udy Mill. Sequential fiber analysis, outlined by Van Soest and Robertson (22), was used to determine the amount of lignin

and other CW components in the ground stem samples.

Statistical Analysis and Graphical Representations

Data analyses for least significant differences were performed by the general linear model procedure in SAS (SAS PROC GLM; 23). Least significant differences at the 0.05 level of probability were calculated from the pooled standard error of the mean for species X age interactions or from the standard error of the mean within species. Nonlinear regression R^2 values for the Gompertz function and the third-order quadratic were calculated by dividing the residual sum of squares (from SAS PROC NLIN) by the corrected total sum of squares and subtracting from one.

Dry weight, CW, hemicellulose, cellulose, and lignin were expressed on a per plant basis for relative comparisons. Graphical representations of these measurements as a function of regrowth days were performed by fitting data to the Gompertz function as reported previously (6). Graphs for dry weight (DW), CW, and CW component deposition, were constructed from the first derivative of the Gompertz function with respect to regrowth days.

Activities of PAL and SAO on a protein basis were represented by fitting data to cubic, $Y = at^3 + bt^2 + ct + d$, where Y = PAL or SAO activity, a, b, c, and d = coefficients estimated by SAS PROC NLIN (23), and t = time in days.

Times of maximum DW, CW, CW component deposition were determined by setting the second derivative of the Gompertz function equal to 0 and solving for t. Times of maximum PAL and SAO activities were determined from average values over replicates by setting the first derivative of the third-order quadratic equal to 0 and solving for t.

Significance of correlations, across and within species, was determined from means of the four replicates by the SAS PROC CORR program (23). Selected results from Bidlack and Buxton (6) and Bidlack et al. (17) are presented to show temporal relations. Linear regressions for lignin content vs SAO activity were performed from the means and graphed by SigmaPlot.

RESULTS AND DISCUSSION

Activities of PAL (top panel of Figure 1; from Bidlack et al. 1995) and SAO (middle panel of Figure 1) initially increased followed by a decrease or plateaued activity as a function of regrowth days. Significant decreases after peak activity of PAL were more apparent than the slight decreases of SAO activity after 25 days of regrowth. Tissue culture studies of *Zinnia elegans* L. have shown that peroxidase activity increases linearly or hyperbolically as a function of culture time (1), whereas PAL activity increases quickly after wounding and decreases thereafter or increases again during lignification. Dramatic increases in PAL activity during early growth, followed by a leveling-off or decrease, as shown in Figure 1 were

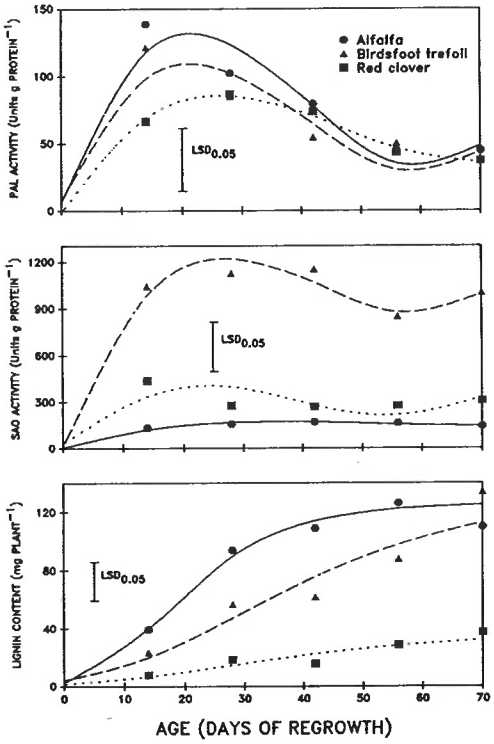

Figure 1. Phenylalanine ammonia lyase (PAL) activity of alfalfa, birdsfoot trefoil, and red clover with R^2 values of 0.91, 0.83, and 0.99, respectively; syringaldazine oxidase (SAO) activity of alfalfa, birdsfoot trefoil, and red clover with R^2 values of 0.98, 0.98, and 0.77, respectively; and lignin content of basal stem regrowth from alfalfa, birdsfoot trefoil, and red clover with R^2 values of 0.98, 0.92, and 0.90, respectively, as a function of regrowth days. Each point represents the mean of four values.

also shown in rice (*Oryza sativa* L.) callus cultures (24), wheat (*Triticum aestivum* L.) grain development (25) and maize stalk maturation (13).

A third order quadratic explained 83 to 99% of the variation in PAL activity for the three legumes (17) and explained 77 to 98% of the variation in SAO activity of alfalfa ($Y = 0.00147t^3 - 0.236t^2 + 11.3t + 3.64$; $R^2 = 0.98$), birdsfoot trefoil ($Y = 0.0211t^3 - 2.76t^2 + 104t + 13.0$; $R^2 = 0.98$), and red clover ($Y = 0.00909t^3 - 1.08^2 + 35.1t + 30.3$; $R^2 = 0.77$), as a function of regrowth days. Similarities of the first three coefficients for PAL activity (17) among species indicated that the shapes of the curves were similar and the fourth coefficient (Y-intercept) indicated that detectable PAL activity occurred between 0 and 7 d. Equations for SAO activity among species demonstrated less similarity in the first and third coefficients, because activity of SAO accelerated faster and reached higher activity in birdsfoot trefoil than in the other species. The process of lignification in birdsfoot trefoil may be different from the other species. Lignin in birdsfoot trefoil has a uniquely low sinapyl:coniferyl alcohol ratio compared with high sinapyl:coniferyl ratios in alfalfa and red clover (19). The fourth coefficient for SAO activity indicated that the enzyme activity could be detected between 4 and 30 days.

Lignin content in alfalfa, birdsfoot trefoil, and red clover increased significantly as a function of regrowth days (bottom panel of Figure 1). Use of the Gompertz function to express increases in lignin content of alfalfa ($R^2 = 0.98$), birdsfoot trefoil ($R^2 = 0.92$), and red clover ($R^2 = 0.90$) accounted for at least 90% of the variation in lignin content.

Comparison of the curves for enzyme activity and the sigmoidal curves of lignin content revealed that peak activity of PAL and SAO coincided with logarithmic increases in lignin content. Significant increases in SAO and PAL activity during the first 25 days of regrowth coincided with the lag and log phases of lignin content. Significant decreases in PAL activity after 30 days prefaced the leveling-off phase in lignin content, whereas the sustained or slight decreases in SAO activity closely followed the leveling-off of lignification after 30 days of regrowth.

Curves in Figure 2, constructed from the first derivative of the Gompertz function, demonstrated similarities among DW, CW, and CW component deposition. Significant differences in component deposition were most noticeable between days 10 and 40, when maximum rates were attained and declined in all species. Analogous curve peaks for hemicellulose, cellulose, CW, DW, and lignin deposition within species indicate that these components accumulated in tandem.

Alfalfa, which consistently had the highest lignin content throughout the regrowth period, also exhibited faster and higher DW, CW, and CW component depositions compared with the other species. Activity of PAL was also higher in alfalfa compared with the other species, but SAO activity of birdsfoot trefoil was at least three-fold higher than that of alfalfa throughout the regrowth period (top two panels of Figure 1).

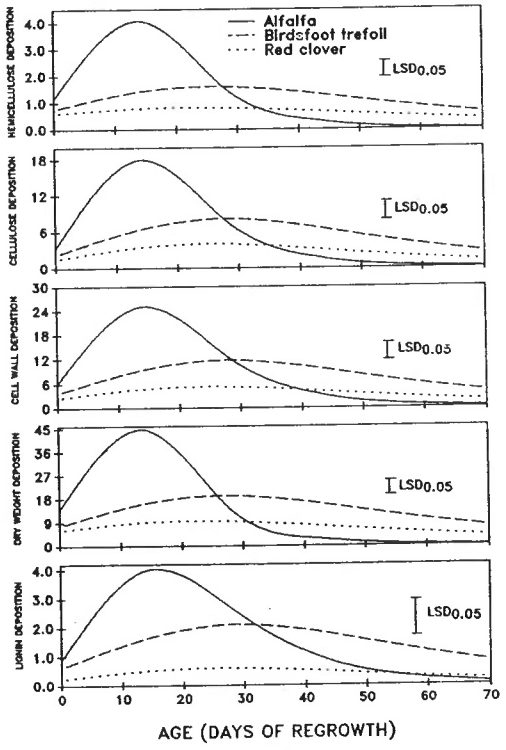

Figure 2. Hemicellulose, cellulose, cell wall, dry weight, and lignin deposition in stem bases of greenhouse-grown alfalfa, birdsfoot trefoil, and red clover as a function of regrowth days. Response curves were constructed from the mean of four values from the first derivative of the Gompertz function.

Time and extent of maximum DW and lignin deposition in red clover lagged behind maximum deposition of these components in alfalfa and birdsfoot trefoil (Table 1 and Figure 2). Additionally, time of maximum hemicellulose, cellulose, and CW deposition in red clover lagged behind deposition of these components in alfalfa. Other studies have shown that DW, CW, and CW component deposition accumulation in red clover lingers behind the same measurements in alfalfa and birdsfoot trefoil (26). In birdsfoot trefoil and red clover, time of maximum PAL activity (Table 1 and top panel of Figure 1) preceded time of maximum SAO activity (Table 1 and middle panel of Figure 1) and maximum activity of both enzymes preceded time of maximum lignin deposition (Table 1 and bottom panel of Figure 2). Time of maximum PAL activity in red clover occurred about 3 to 5 days later than alfalfa and birdsfoot trefoil, whereas time of maximum SAO activity in alfalfa occurred about 6 days later than birdsfoot trefoil and red clover (Table 1).

In general, maximum CW deposition occurred within 1 to 3 days of maximum DW deposition (Table 1). Among CW components, maximum hemicellulose deposition occurred first, followed by cellulose (1 to 3 days later) and then lignin (up to 14 days after maximum hemicellulose deposition). Time of maximum PAL activity occurred much earlier than time of maximum lignin deposition in birdsfoot trefoil and red clover and shortly after time of maximum lignin deposition in alfalfa. Time of maximum PAL activity always preceded time of maximum SAO activity.

Except for SAO activity, measurements in alfalfa achieved their maximum earlier than in birdsfoot trefoil or red clover. In birdsfoot trefoil and red clover, time of maximum PAL activity occurred 2 to 5 days prior to maximum SAO activity and 8 days prior to maximum lignin deposition. Maximum PAL and SAO activities were achieved later than maximum lignin deposition in alfalfa.

Correlation of PAL and SAO activities on a protein basis with lignin content revealed correlation coefficients of $r = -0.35$ ($p > 0.05$) and $r = -0.01$ ($p > 0.05$), respectively. Because these coefficients were not significant, we concluded that the activities of PAL and SAO at any one moment could not be related to the amount of lignin present in basal stem tissues of the three temperate legumes. Even

Table 1. Estimated times of maximum dry weight (DW), cell wall (CW) and CW component deposition on a per-plant basis and maximum phenylalanine ammonia lyase (PAL) and syringaldazine oxidase (SAO) activities on a per-protein basis of stem base regrowth harvested from greenhouse-grown legumes. Times of maximum DW, CW, and CW component deposition were calculated by setting the first derivative of the Gompertz function equal to 0 and solving for t; times of maximum PAL and SAO activity were calculated by setting the second-order quadratic equal to 0 and solving for t.

	Species			
Measurement	Alfalfa	Birdsfoot trefoil	Red clover	$LSD_{0.05}$
	---------- Days ----------			
Hemicellulose	13.0	25.5	20.6	7.6
Cellulose	14.7	28.7	23.9	7.4
Cell wall	14.9	28.2	23.0	8.3
Stem base DW	12.7	27.2	27.7	9.6
PAL activity	20.3	21.5	25.3	3.7
SAO activity	32.1	26.4	27.3	20.0
Lignin	17.4	30.0	34.0	8.9
$LSD_{0.05}$	9.2	6.0	8.4	

though the specific activities of these enzymes could not be related to lignin content, we postulated that the amount of enzyme present in legume stem tissue could be related to lignin content. Thus, activity of PAL and SAO were evaluated on a per plant basis (enzyme total units) and presented in Table 2.

Activity of PAL on a per plant basis fluctuated randomly and SAO activity on a per plant basis increased as a function of regrowth days for all species. The increase in SAO activity on a per plant basis was significant in birdsfoot trefoil and red clover (Table 2). Throughout regrowth, PAL activity was significantly higher in alfalfa compared with birdsfoot trefoil and red clover. Across species, PAL per plant was significantly correlated with lignin content ($r = 0.60, p < 0.05$), whereas SAO per plant was not significantly correlated with lignin content ($r = 0.48, p > 0.05$). Greater variation in PAL and lignin across species, which was attributed to plant age and species differences, caused significance of the lignin versus PAL correlation.

Induction of PAL through fungal elicitors (27) and rubbing (15) also dramatize variations in PAL and provide a foundation for significant correlations between lignin and PAL. Correlation coefficients as high as 0.99 have been reported (16) for lignin content versus PAL in wheat as affected by light intensity. Even though the lignin versus PAL correlation may not show up as significant in some species (13,14), it is evident from this and other studies (17) that lignification is attributable to the precursory PAL enzyme. The SAO enzyme, on the other hand, is a specific peroxidase, and its activity may be more closely associated with lignification within species.

Within species, CW and CW component content were almost all significantly correlated with each other (Table 3). Activity of PAL on a per plant basis was not correlated with CW components or SAO activity within species. Other studies (13) have shown that, while PAL activity was not correlated with lignin concentration, enzymes further down the pathway of lignin biosynthesis were correlated. Table 3 shows that activity of SAO on a per plant basis was correlated with lignin in all species and, with exception of cellulose content in alfalfa, SAO was correlated with all CW components. These results demonstrated that, within species, CW and lignin content were closely associated with SAO on a per plant basis. Thus, the amount of the SAO enzyme was a good indication of the amount of lignin deposited in cool-season legume stem bases.

Linear regression of lignin content versus SAO activity on a per plant basis indicated that the linear model accounted for 77 to 96% of the variation within each species (Figure 3). Extrapolation of regression lines to the Y-axis suggested that, in alfalfa and red clover, lignin was present in stem

Table 2. Phenylalanine ammonia lyase (PAL) and syringaldazine oxidase (SAO) activity on a per plant basis of stem base regrowth harvested from greenhouse-grown legumes. Least significant differences at the 0.05 level of probability from pooled standard error of the mean for PAL and SAO activity are 3.6 and 20.6, respectively.

Days of Regrowth	PAL Activity			SAO Activity		
	Alfalfa	Birdsfoot trefoil	Red clover	Alfalfa	Birdsfoot trefoil	Red clover
	-------- Units PLANT^{-1} ---------			--------- Units PLANT^{-1} ---------		
14	7.6	3.4	1.2	7.6	30.2	6.7
28	8.0	4.4	2.7	12.5	54.8	8.4
42	7.0	3.0	1.5	17.1	64.3	6.0
56	4.7	4.0	2.0	20.4	72.0	13.2
70	7.8	4.9	2.7	12.4	134.3	24.6

Table 3. Simple correlations among cell wall (CW) content, CW component content, and enzyme activity on a per plant basis of stem base regrowth of individual greenhouse-grown legumes.

	Species														
	Alfalfa					Birdsfoot trefoil					Red clover				
	CEL[a]	CW	PAL	SAO	LIG	CEL	CW	PAL	SAO	LIG	CEL	CW	PAL	SAO	LIG
HEM	0.93*	0.95*	-0.43	0.93*	0.95*	0.98**	0.98**	0.72	0.96**	0.99**	0.85	0.90*	0.83	0.94*	0.88*
CEL		1.00**	-0.62	0.82	0.99**		1.00**	0.65	0.96**	0.99**		0.99**	0.70	0.88*	0.98**
CW			-0.61	0.86*	1.00**			0.67	0.97**	1.00**			0.74	0.92*	0.99**
PAL				-0.54	-0.65				0.67	0.69				0.63	0.71
SAO					0.88*					0.97**					0.92*

[a]CEL = cellulose; CW = cell wall; PAL = phenylalanine ammonia lyase; SAO = syringaldazine oxidase; LIG = lignin; HEM = hemicellulose.
*, **, Pearson's rank coefficient differs significantly from zero, based on the 0.01 and 0.05 levels of probability, respectively.

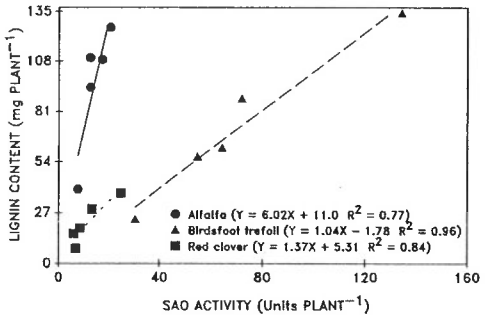

Figure 3. Relationship between lignin content and syringaldazine oxidase (SAO) activity on a per plant basis in stem bases of greenhouse-grown forage legumes as a function of regrowth days. Each point represents the mean of four values.

tissue before SAO reached detectable activity. In birdsfoot trefoil SAO activity appeared only one or two days before lignin could be quantified. Slopes from linear regression in Figure 3 indicated the relative dependence of lignin in individual species on SAO activity. Of the three species, alfalfa had the steepest slope. Alfalfa also had the highest DW yield and lignin content of the three species. Birdsfoot trefoil and red clover, on the other hand, had less DW yield, less lignin content, and thus had gradual slopes compared with the steep slope of alfalfa.

CONCLUSIONS

Time of maximum lignin deposition followed that of other CW components. Maximum activity of PAL occurred much earlier than lignin deposition for birdsfoot trefoil and red clover, but shortly after maximum lignin deposition for alfalfa. Maximum activity of PAL always preceded maximum activity of SAO. Neither PAL nor SAO activity on a protein basis were correlated with lignin content; however, PAL on a per plant basis was correlated with lignin content across species and SAO on a per plant basis was correlated with lignin content within species. These results demonstrate that PAL and SAO are related to lignin deposition in a cause-and-effect relationship. Activity of SAO was more closely related to lignification within legume species.

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