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Natural Photodynamic Activity of 5-Aminolevulinic Acid Produced by *E. coli* Overexpressing ALA Synthase from *Bradyrhizobium japonicum*

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ABSTRACT : The present study was conducted to determine plant growth and physiological responses of corn, barnyardgrass, and soybean to ALA (5-aminolevulinic acid). ALA effect on early seedling growth of test plants was greatly concentration dependant, suggesting that it inhibits at higher concentrations. No significant difference in herbicidal activity of two types of ALA on plant height and weight of test plants was observed. Barnyardgrass was the most sensitive to ALA and followed by corn and soybean, indicating that both crop plants were less affected by ALA concentration as well as different growth stages than barnyardgrass. Greatly reduced chlorophyll contents from leaves of three plant species were observed with increasing of ALA concentration. Compared with untreated controls, higher amounts of three tetrapyrroles were detected from three crop plants, indicating more accumulation in ALA-treated plants. The differential selectivity among plant species would be explained with the differences in tetrapyrrole accumulating capabilities, the susceptibility of various greening groups of plant species to the accumulation of various tetrapyrroles, and their metabolism in various plant tissues. The results indicate that negative biological potential of ALA exhibited differently on plant species, and that the photodynamic herbicidal activity against susceptible plants highly correlated with the extent of tetrapyrrole accumulation by the species.

Keywords: 5-aminolevulinic acid, herbicidal potential, tetrapyrrole accumulation, eco-friendly weed management

Porphyrin compounds play an essential role in plant metabolism. The porphyrin ring system is derived from 5-aminolevulinic acid (ALA). In plants, algae and a few bacteria, ALA is formed from the five-carbon skeleton of glutamate in unit of the C5 pathway (Wettstein *et al.*, 1995). This pathway utilizes glutamyl-tRNA synthetase, glutamyl-tRNA hydrogenase, and glutamate-1-semi-aldehyde aminotransferase to carry out three sequential enzymatic reactions that produce ALA from glutamate (Beal & Castelfranco, 1974). In the four-carbon (C4) pathway, which is present in animals and microorganisms, ALA is formed by the enzyme 5-aminolevulinic acid synthetase (ALAS), which catalyzes the pyridoxal phosphate-dependent condensation of succinyl-coenzyme A (succinyl-CoA) (Avissar *et al.*, 1989). A few microorganisms have both C4 and C5 pathways, as is distinct in Euglena gracilis (Weinstein & Beale, 1983). ALA is very expensive because it is usually synthesized chemically via complex processes. Therefore, biological production using microorganisms has been suggested as an inexpensive way to produce ALA. It has been well known that photosynthetic Rhodospirillaceae such as mainly Rhodospirillum, Rhodobacter, and Rhodopseudo-monas species can extracellularly excrete ALA (Sasaki *et al*, 1990).

The biosynthesis of porphyrin is tightly regulated at several levels to coordinate apoprotein synthesis with cofactor availability and to avoid the accumulation of the intermediates, protoporphyrin IX (Proto IX) and protochlorophyllide (Pchlide), which are photosensitive to light, generating reactive oxygen species, at the stage preceding chlorophyll(Chl) biosynthesis (Papenbrock & Grimm, 2001). Plants suffer severe photodynamic damage if these control mechanisms are circumvented, e.g., by feeding early intermediates like ALA or by the action of protoporphyrinogen IX oxidase (Protox)-inhibiting herbicides, producing an accumulation of excess Proto IX (Menon *et al.*, 1989; Böger & Wakabayashi,1999; Mock *et al.*, 2002). The damage is accompanied by the destruction of photosynthetic reactions and is irreversible. When ALA-treated plants are exposed to sunlight, excess tetrapyrroles absorb the energy that is normally used for photochemical reactions and use it instead to photosensitize the production of $^1O_2$ (Tripathy & Chakraborty, 1991). $^1O_2$ oxidizes unsaturated membrane lipids, generating free radicals, which damage the membrane system and lead to the death of the plant. Therefore, ALA has been proposed as a selective and biodegradable herbicide and insecticide (Rebeiz *et al.*, 1984 and 1988a).

ALA was proposed as a tetrapyrrole-dependent photody-

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namic herbicide (TDPH) that force green plants to accumulate undesirable amount of metabolic intermediates (protoporphyrin IX) of the chlorophyll and heme metabolic pathway in darkness, namely tetrapyrrole (Rebeiz *et al.*, 1990) or as a 'laser' herbicide that is photodynamic (Rebeiz *et al.*, 1984). Under the light, the accumulated tetrapyrroles photosensitize the formation of singlet oxygen that kills the treated plants by oxidation of their cellular membranes as like diphenyl ether (DPE) herbicides. Rebeiz *et al.* (1984) showed that the extent of photodynamic damage was increased with increasing of light intensity, when applied to cucumber seedlings at ALA concentrations of 5 to 20 mM. A variety of DPE herbicides such as acifluorfen-methyl, oxadiazone, and oxyfluorfen cause rapid peroxidative photobleaching and desiccation of green plant tissues (Duke *et al.*, 1991; Scalla & Matringe, 1994). The target site of action of these herbicides has been well known to be protoporphyrinogen oxidase (Protox), which catalyzes the oxidation of protoporphyrinogen IX (Protgen IX) to protoporphyrin IX (Proto IX), in the biosynthesis of hemes and chlorophylls (Duke *et al.*, 1991; Beale & Weinstein, 1990).

The present study was conducted to determine herbicidal effect of ALA on seedling growth of barnyardgrass (*Echinochloa crus-galli* Beauv. var. *oryzicola* Ohwi.), corn, and soybean by post-emergence application, and to examine the accumulation of metabolic intermediates in darkness, and subsequently chlorophyll content of the chlorophyll and heme metabolic pathway. The fundamental assessment would be useful for development of ALA as a new biodegradable herbicide that is environmentally sound and safe to human, animals and crops.

MATERIALS AND METHODS

Chemicals

ALA produced by overexpressing the *hemA* gene isolated from *Bradyrhizobium japonicum* (Choi *et al.*, 1999) was provided by Environgen Co., Korea (Bio-ALA). Extracellular accumulation of ALA by an *E. coli* overexpressing ALA synthase was achieved by inserting a *hemA* gene from *Bradyhizobium japonicum* and expressed under the control of T7 promoter (Choi *et al.*, 1999). ALA amount produced by this method was 30 mM. To compare the biological activity with Bio-ALA, synthetically-produced ALA (synthetic-ALA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Herbicidal activity of Bio- and Synthetic ALA

Two different types of ALA, bio-ALA and synthetic-ALA, were assayed to determine the difference in their herbicidal effects on seedling growth of barnyardgrass (*Echinochloa crus-galli*), corn (*Zea mays*), and soybean (*Glycine max*). Imbibed seeds of three plant species were planted in small horticulture pot (10x10x5cm) filled with silt-loam soil, and grown for 12 days under greenhouse conditions (28/21°C day/night temperature) from July to September 2004. Then ALA at 0, 2, 4, 6, and 8 mM mixed with Tween 80 was foliar applied to test plants. The concentrations were suggested in the previous work (Chon, 2003). A 15 ml of ALA solution was applied by handy sprayer 6:00 PM. At the time of application, leaf stage of test plants was about 2-leaves. After application, post-spray dark incubation period was kept for 14 hrs, and next morning exposed under the natural sunlight ranged from 1000 to 1500 μmol photons m^{-2} s^{-1} to elicit photodynamic damage. Shoot length was measured on all seedlings 7 days after exposure to sunlight. When the F-test was significant ($P<0.05$), means were separated on the basis of least significant difference (LSD) (SAS Institute 2000).

Response of seedling ages to ALA

ALA at 0, 2, 4, 6, and 8 mM mixed with Tween 80 was foliar applied at three different growth stages; 10, 20, and 30 day-old seedling (DOS) of three plant species. A 15 ml of ALA solution was applied with handy sprayer at 6:00 PM. At the time of application, leaf stages of test plants were about 2, 3, and 4 leaves for 10, 20, and 30 DOS, respectively. After application, post-spray dark incubation period was kept for 14 hrs, and next morning exposed to the natural sunlight ranged from 1000 to 1500 μmol photons m^{-2} s^{-1} to elicit photodynamic damage. Shoot length, and fresh and dry weights were measured on all seedlings 7 days after exposure to sunlight. Growth conditions and statistical method were the same as those described in the previous section.

Chlorophyll synthesis

Growth conditions and statistical method were the same as those described in the previous section. ALA at 0, 2, 4, 6, and 8 mM mixed with Tween 80 was foliar applied to 10 day-old seedlings (2-leaf stage) of three plants. Shoot parts of whole plant from each species were harvested 7 days after ALA foliar application. Chlorophyll concentration was measured spectrophotometrically by the method of Lichtenthaler (1987).

Determination of porphyrins

ALA at 0 and 8 mM mixed with Tween 80 was foliar applied to 10 day-old seedlings (2-leaf stage) of barnyardgrass, corn, and soybean. After application, post-spray dark incubation period (PSDIP) was kept for 14 hrs. During PSDIP, the first leaves of each plant were collected in darkness for porphyrin analysis. The plant tissue (0.1g) was ground in 2 ml of methanol : acetone : 0.1 N NaOH (9 : 10 : 1, v/v) and the homogenate was centrifuged at 10,000 g for 10 min to remove cell debris and proteins. Porphyrins were separated by HPLC on a Novapak C18 column (4 μm, 4.6 mm × 250 mm, Waters, Milford, MA, USA) at a flow of 1 ml/min. Porphyrins were eluted with a solvent system of 0.1 M ammonium phosphate (pH 5.8) and methanol. The column eluate was monitored by a fluorescence detector (Waters, Milford, MA) at excitation and emission wavelengths of 400 and 630 nm, respectively). Porphyrins were identified and quantified using authentic standards. Growth conditions were the same as those described in the previous section.

RESULTS AND DISCUSSION

Herbicidal activity of Bio- and Synthetic ALA

Biological production using microorganisms has been suggested as a less expensive way to produce ALA. For this study, we used several concentrations from 30 mM of ALA accumulated by overexpressing the *hemA* gene isolated from *Bradyrhizobium japonicum* (Choi *et al.*, 1999). The results showed that no significant difference in herbicidal activity of two types of ALA on plant heights of barnyardgrass, corn, and soybean was observed (Fig. 1). Kuk *et al.* (2003), as a similar report, found that no significant difference in biological activity between bio-ALA and synthetic ALA on barley, wheat, rice, and weed, *Ixeris dentate* tested was observed. Even thought, we did not confirm the variations in stability and activity persistence of ALA, ALA produced by microorganisms has the potential to become an industrial process, provided appropriate technologies are developed for making industrial production technically feasible. In addition, barnyardgrass was more sensitive to ALA ranged from 2 to 8 mM than was corn or soybean, showing less phytotoxicity on crop plants. ALA at 8 mM significantly reduced plant height of barnyardgrass by 70% in dose-dependent manner, whereas it did not affect those of corn and soybean plants. On the other hand, fresh and dry weight of barnyardgrass was affected by ALA with increasing of the concentration. However, no significant difference in herbicidal activity between Bio- and Synthetic-ALA exhibited at all the concentrations (Fig. 2). Recently, ALA has received great attention as a new biodegradable herbicide at given concentrations (Rebeiz *et al.*, 1984; Rebeiz *et al.*, 1988a and b). ALA is known to be not harmful to crops, animals, and humans, and used as a prodrug for photodynamic diagnosis and therapy of cancer (Schuimaker *et al.*, 1999). However, ALA is very expensive because it is usually synthesized chemically via complex processes.

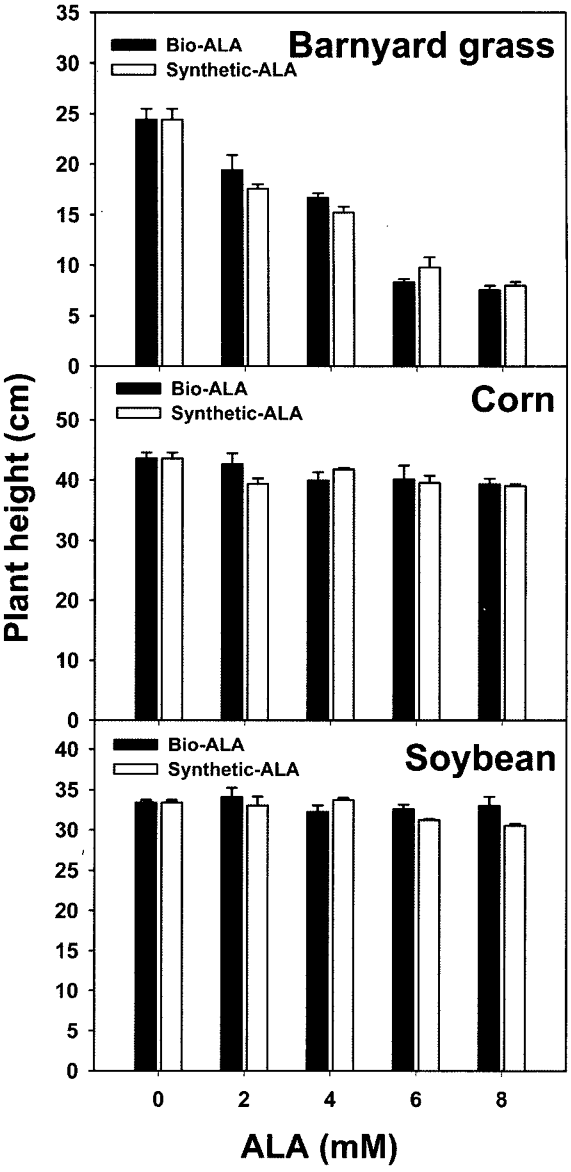

Fig. 1. Effects of Bio- and Synthetic-ALA on shoot growth of barnyardgrass, corn, and soybean (C) 7 days after application. Within an extract concentration, means followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

Response of seedling age to ALA

Selectivity among seedling ages to ALA was examined in greenhouse experiment using barnyardgrass, corn, and soybean. Barnyardgrass was the most sensitive to ALA and fol-

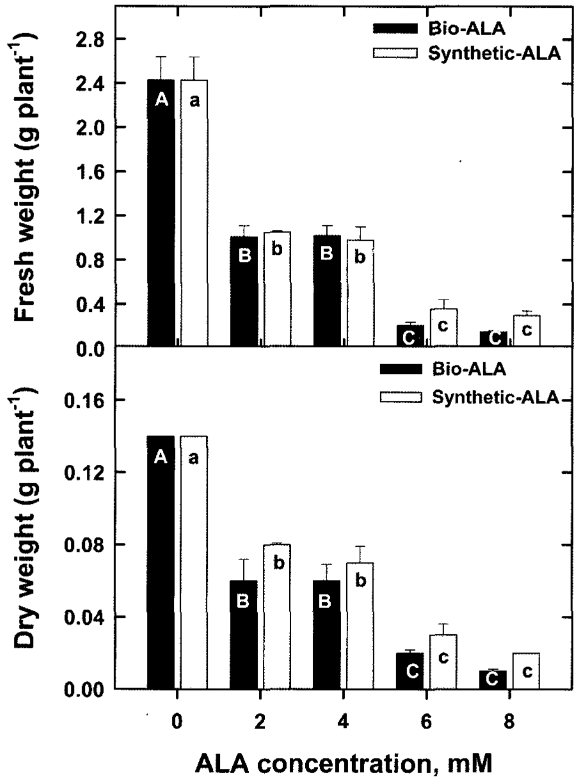

Fig. 2. Effects of Bio- and Synthetic-ALA on shoot fresh and dry weights of barnyardgrass 7 days after application. Within an extract concentration, means followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

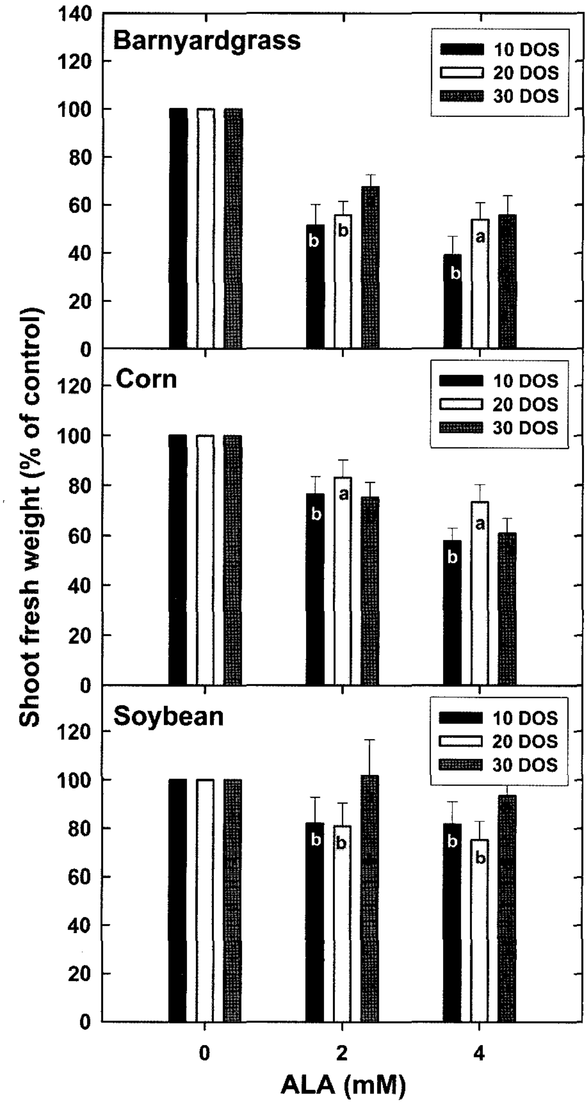

Fig. 3. Effects of ALA on shoot fresh weight of barnyardgrass, corn, and soybean as affected by different seedling ages 10 days after application. Within an extract concentration, means followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

lowed by corn and soybean. Earlier seedlings of barnyardgrass were more affected by ALA than older seedlings (Fig. 3). ALA at 4mM reduced shoot fresh weight of 10-day, 20-day, and 30 day-old barnyardgrass seedlings by 60, 45, and 40%, respectively. Both crop plants were less affected by ALA concentration as well as different growth stages of barnyardgrass, showing less injury. Rebeiz *et al.* (1983) suggested that photodynamic herbicides exhibit a very pronounced organ, age, and species-dependent selectivity. Our result contrasts with the conclusion that dicotyledonous weeds such as mustard, red root pigweed, common purslane and lamsquarter are more susceptible than monocotyledonous plants such as corn, wheat, barley and oats (Rebeiz *et al.*, 1983). Chon (2003) reported that, among different application methods, post-emergence application with ALA exhibited greatest photodynamic activity against test plants, and that alfalfa was the most tolerant to ALA. The physiological actions of ALA at high concentrations suggests that ALA increases the levels of porphyrin intermediate such as protochlorophyllide, protoporphyrin IX, and Mg-protoporphyrin IX abnormally, and the accumulated tetrapyrroles act as a photosensitizer for the formation of singlet oxygen triggering photodynamic damage (Askira *et al.*, 1991; Rebeiz *et al.*, 1984). Thus, in our study the selectivity among plant species would be based on tetrapyrrole accumulating capability and the tetrapyrrole metabolism in various plant species (Rebeiz *et al.*, 1988).

Chlorophyll synthesis

Symptoms of photodynamic injury within the first 1 hour after exposure to light after post-spray dark incubation period for 15 hrs become apparent. Initial symptoms appeared on green foliage of susceptible plants as isolated

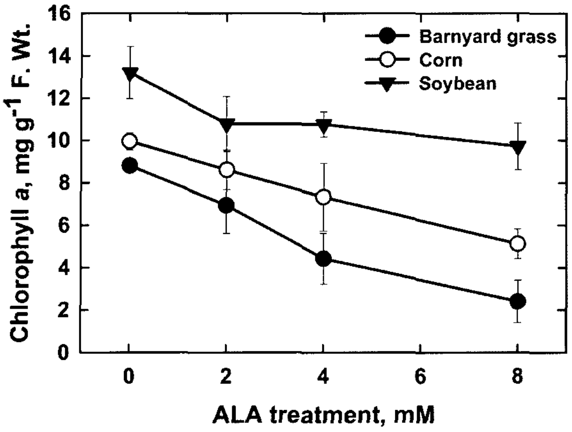

Fig. 4. Effects of ALA on chlorophyll a content of barnyardgrass, corn, and soybean leaves 7 days after application. Within an extract concentration, means followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

bleached spots contiguous. Bleaching is accompanied by severe loss of turgidity followed by desiccation. Within 24 hrs the green plant tissue turns into a brownish desiccated mass of dead tissue (Data not shown). Greatly reduced chlorophyll contents from leaves of three plant species were observed with increasing of ALA concentration, especially chlorophyll content of barnyardgrass was more reduced than that of two crop plants. The chlorophyll synthesis was inhibited by ALA in order of barnyardgrass > corn > soybean (most tolerant).

Determination of porphyrins

Accumulation of porphyrins in 8mM ALA-treated barnyardgrass, corn, and soybean was analyzed by HPLC. Three plants were kept to accumulate tetrapyrroles in darkness for 14hr after ALA application. Compared with untreated controls, higher amounts of three tetrapyrroles were detected from three crop plants, indicating more accumulation in ALA-treated plants. Among terapyrroles Proto IV was much more detected than others, Proto IX-ME and Pchlide, when applied with ALA 8-mM. Protox IX was detected as the highest amount in barnyardgrass plant (238.5 nmol g^{-1} fresh wt.) and followed by Pchlide (10.95) and Proto IX-ME (0.44). However, much less amounts of Proto IX and Pchlide in corn and soybean was present. Especially, Pchlide in soybean were more detected than Proto IX. Originally photodynamic herbicides have been known to be nonselective in their mode of action. Rebeiz *et al.* (1988a) showed that various ALA treatments exhibited a significant degree of photodynamic herbicidal selectivity among test plants. The selectivity among plant species would be based on the differences in tetrapyrrole accumulating capabilities, the susceptibility of various greening groups of plant species to the accumulation of various tetrapyrroles, and the tetrapyrrole metabolism in various plant tissues (Rebeiz *et al.*, 1988a; Chon *et al.*, 2004). Also, our result indicated that post-emergence application of ALA exhibited complete photodynamic phytotoxicity.

In conclusion, ALA effect on early plant growth was greatly concentration dependant, suggesting that it promotes plant growth at very low concentration and inhibits at high concentration. No significant difference in herbicidal activity of two types of ALA on plant height and weight of test plants was observed. Even thought the variations in stability and activity persistence of ALA were not confirmed, ALA produced by microorganisms would be developed for making industrial production technically feasible. Barnyardgrass was the most sensitive to ALA and followed by corn and soybean. Both crop plants were less affected by ALA concentration as well as different growth stages of barnyardgrass. Greatly reduced chlorophyll contents from leaves of three plant species were observed with increasing of ALA concentration. Compared with untreated controls, higher

Table 1. Accumulation of porphyrins in 8mM ALA-treated barnyardgrass, corn, and soybean.

Plant species	Treatments	Porphyrins		
		Proto IX	Proto IX-ME	PChlide
		Concentration (nmol g^{-1} fresh wt.)		
Barnyardgrass	Control	0.34 ± 0.07	0.31 ± 0.04	0.86 ± 0.31
	ALA	238.50 ± 62.20	0.44 ± 0.04	10.96 ± 1.28
Corn	Control	0.90 ± 0.45	0.52 ± 0.06	0.48 ± 0.09
	ALA	1.38 ± 0.24	0.55 ± 0.02	0.93 ± 0.02
Soybean	Control	0.37 ± 0.12	0.93 ± 0.03	1.51 ± 0.42
	ALA	2.58 ± 0.70	0.95 ± 0.07	4.39 ± 0.55

Values are mean ± SE of three replications.

amounts of three tetrapyrroles were detected from three crop plants, indicating more accumulation in ALA-treated plants. The differential selectivity among plant species would be explained with the differences in tetrapyrrole accumulating capabilities, the susceptibility of various greening groups of plant species to the accumulation of various tetrapyrroles, and the tetrapyrrole metabolism in various plant tissues. We suggest that negative biological potential of ALA exhibited differently on plant species, and that photodynamic herbicidal activity of ALA against susceptible plants highly correlated with the extent of tetrapyrrole accumulation by the species.

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