Substrate-Dependent Activation of Latent Potato Leaf Polyphenol Oxidase by Anionic Surfactants

Alvaro Sánchez-Ferrer, Francisco Laveda, and Francisco García-Carmona*

Departamento de Bioquímica y Biología Molecular (A), Facultad de Biología, Universidad de Murcia, E-30071 Murcia, Spain

Potato leaf polyphenol oxidase was purified with a 5-fold increase in specific activity and a recovery of 65% by using two sequential phase partitionings in Triton X-114. The second phase separation, which has never been used previously, increased both the purification rate and the removal of phenolic compounds until they were reduced to only 3% of the original concentration with minimal loss in activity. The enzyme obtained was latent and was only activated by anionic surfactants (sodium dodecyl sulfate and dodecanesulfonic acid). The degree of activation depended on the pH and hydrophobicity of the substrate used and was highest for *tert*-butyl catechol (4-fold) and at pH 4.0, less for 4-methylcatechol (1.7-fold), and zero in the most hydrophilic substrate (chlorogenic acid). This activation also affected the kinetic constants, K_M decreasing and V_{max} increasing, thus resulting in a 4-fold increase in the catalytic efficiency for *tert*-butylcatechol and 1.5-fold increase for 4-methylcatechol.

INTRODUCTION

Potato tuber polyphenol oxidase (EC 1.14.18.1) (PPO) has been extensively characterized in both its particulate and soluble forms [for a review see Matheis (1987a,b)]. However, the PPO of aerial tissues and that of the plant tubers differ greatly (Satô, 1982). In fact, plants that have both organs have been studied in an attempt to differentiate these two forms of PPO (Satô, 1976). In general terms, the difference stems from the presence of latent PPO in plant leaves, whereas the enzyme found in tubers is fully active. Latent PPO forms have been characterized in leaves from spinach beet (*Beta vulgaris* L.) (Parish, 1972), broad bean (*Vicia faba*) (Satô, 1976; Lieberi and Biehl, 1978; Golbeck and Camarata, 1981; Sánchez-Ferrer et al., 1989a), and spinach (*Spinacea oleracea*) (Satô, 1976; Lieberi and Biehl, 1978; Golbeck and Camarata, 1981; Sánchez-Ferrer et al., 1989b), but surprisingly no studies have been carried out on potato leaf PPO. A study of these different latent enzymes might clarify the physiological role of this complex enzyme (Vaughn and Duke, 1984) and identify the real *in vitro* triggers which produce the huge increase in activity (up to 50–120 times) such as described for grape berry chloroplast PPO (Sánchez-Ferrer et al., 1989b) and broad bean leaf PPO (King and Flurkey, 1987; Sánchez-Ferrer et al., 1990). This activation can be provoked by several agents and treatments, the most effective of which are proteolytic attack (Tolbert, 1973; King and Flurkey, 1987; Sánchez-Ferrer et al., 1989b), changes in pH (Valero and García-Carmona, 1992), and the release of phospholipids (surfactants) to occur *in vivo* (Kenten, 1958; King and Flurkey, 1987; Sánchez-Ferrer et al., 1990).

This paper shows, for the first time, that PPO in latent form can be purified from potato leaves by using a mild extraction method involving Triton X-114 (TX-114). It also shows how its activation by anionic detergents can be affected by the type of substrate catalyzed by the enzyme.

MATERIALS AND METHODS

Reagents. Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Triton X-114 was obtained from Fluka AG (Bucks, Switzerland) and condensed three times as described by Bordier (1981) but using 100 mM sodium phosphate buffer, pH 7.3, containing 0.33 M sorbitol, 2 mM EDTA, and 1 mM $MgCl_2$. The detergent phase of the third condensation had a concentration of 25% TX-114 (w/v).

Enzyme Purification. Chloroplasts were isolated from nonsenescent leaves of potato (*Solanum tuberosum* var. Desirée) as described by Smith (1988), in a modified buffer, pH 7.3, 0.33 M sorbitol, 2 mM EDTA, and 1 mM $MgCl_2$. Fifty grams of leaves was blended for 30 s, and the slurry was filtered through 10 layers of gauze and centrifuged at 800*g* for 2 min. The pellet was discarded, and the supernatant was centrifuged at 60000*g* for 10 min. The resultant pellet was resuspended in a solution containing 100 mL of 10 mM sodium phosphate buffer, pH 7.3, and kept at 4 °C for 20 min. The solution was then centrifuged at 40000*g* for 10 min, thus pelleting the chloroplast membranes. These membranes were resuspended in 20 mL of 1.5% (w/v) TX-114 in 100 mM sodium phosphate buffer, pH 7.3, for 30 min at 4 °C and centrifuged at 60000*g* for 15 min. The slightly green supernatant with PPO activity (supernatant of 1.5% w/v TX-114, Table I), was subjected to temperature phase partitioning by adding TX-114 at 4 °C, so that the final detergent concentration was 8% (w/v). The mixture was kept at 4 °C for 15 min. It became spontaneously turbid due to the formation, aggregation, and precipitation of large mixed micelles composed of detergent, hydrophobic proteins, and the remaining chlorophylls. This solution was centrifuged at 5000*g* for 10 min at room temperature. The clear supernatant of 8% (w/v) TX-114 (Table I) was subjected to additional phase partitioning to remove the remaining phenols. For this, more TX-114 was added to a concentration of 4% (w/v) and the mixture brought to 37 °C. After centrifugation at room temperature at the same speed as described above, the supernatant of 4% (w/v) TX-114 (Table I) was used as enzyme source.

Enzyme Assay. PPO activity was determined spectrophotometrically at 400 nm (Sánchez-Ferrer et al., 1988). One unit of enzyme was defined as the amount of the enzyme that produced 1 μmol of *o*-benzoquinone/min. Unless otherwise stated, the reaction medium (1.0 mL) at 25 °C contained 75 μg/mL protein, 50 mM acetate buffer, pH 4.0, and 3 mM *tert*-butylcatechol (TBC) with or without 130 μM SDS. In the proteolytic activation assays, the sample was preincubated with trypsin (1000 units/mL) for 5 min.

Other Methods. Protein content was determined according to the dye binding method of Bradford using bovine serum albumin as a standard (Bradford, 1976). Chlorophylls were measured in 80% acetone (Arnon, 1949), and total phenolic

* Author to whom correspondence should be addressed (fax +34 68 305101).

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Table I. Purification of Leaf Potato PPO

	total act.,[a] units/mL		sp act., units/mg of protein	purification,	yield,	Chl,[b]	phenols,
	-SDS	+SDS	+SDS	x-fold	%	mg	mg
supernatant of TX-114 1.5% (w/v)	0.57	1.78	0.22	1.0	100	1.1	121
supernatant of TX-114 8% (w/v)	0.69	1.53	0.28	1.3	70	ND[c]	32
supernatant of TX-114 4% (w/v)	0.33	1.53	0.98	4.5	63	ND	4

[a] Assayed with 3 mM *tert*-butylcatechol in 50 mM acetate buffer, pH 4.0, with or without 130 μM SDS. [b] Chlorophylls. [c] ND, not detected.

Table II. Activation of Latent Potato Leaf PPO by Trypsin and Detergents[a]

	tert-butylcatechol[b]		4-methylcatechol[c]		chlorogenic acid[d]	
	act., μM/min	activation, x-fold	act., μM/min	activation, x-fold	act., μM/min	activation, x-fold
none	82	1.0	104	1.0	157	1.0
trypsin (10 μg/mL)	65	0.8	83	0.8	125	0.8
SDS	330	4.0	177	1.7	162	1.0
DS	320	3.9	125	1.2	159	1.0
CTAB	75	0.9	96	0.9	151	1.0
Brij 96	80	1.0	101	1.0	154	1.0
$C_{12}E_9$	81	1.0	103	1.0	155	1.0

[a] Optimal detergent concentration is used for each substrate. See text for details. [b] Assayed in the standard reaction media. [c] Assayed in the standard reaction media with 30 mM 4MC. [d] Assayed in the standard reaction media with 0.5 mM chlorogenic acid.

compounds were determined in 80% ethanol by the Folin–Denis method (Kidron et al., 1978).

RESULTS

Enzyme Purification. Enzyme purification in plant extracts is difficult because of the presence of a large variety and quantity of secondary products which can bind tightly to the enzymes, thus changing their characteristics (Loomis, 1974). To overcome this problem, different methods have been developed such as acetone powders, ammonium sulfate fractionation, salts, insoluble polymers, and detergents (Weselake and Jain, 1992). Of this last group, Triton X-114 shows the special feature of forming clear solutions in buffers at 4 °C, while separating into two phases at 20 °C due to the formation of large micellar aggregates (Bordier, 1981). This characteristic has been used to separate integral proteins from hydrophilic proteins, since the former remain in the detergent-rich phase (Bordier, 1981; Pryde and Phillips, 1986).

Recently, TX-114 has found another use in plant biochemistry (Weselake and Jain, 1992) in the removal of phenolic compounds from grape berries (Sánchez-Ferrer et al., 1989b) and broad bean leaves (Sánchez-Ferrer et al., 1990).

When this detergent was used to extract PPO from the potato leaf thylakoid membranes obtained from osmotically shocked chloroplasts, the detergent failed to maintain all of the components in solution. After a few minutes, a dark green precipitate which contained membrane proteins, phospholipids, and chlorophylls could be recovered by high-speed centrifugation. The slightly green supernatant of 1.5% (w/v) TX-114 (Table I) was subjected to temperature-induced phase separation by addition of TX-114 to a final concentration of 8% (w/v), thus removing all of the chlorophylls [supernatant of 8% (w/v) TX-114, Table I]. However, 26% of phenolic compounds remained in solution, perhaps due to the saturation of TX-114 by the relatively high phenolic concentration compared with that found in broad bean leaves (Sánchez-Ferrer et al., 1990) or grape berries (Sánchez-Ferrer et al., 1989b). This saturation may alter the phase behavior of TX-114 (Werck-Reichhart et al., 1991). To check this hypothesis, an additional phase-partitioning step was carried out with a final concentration of 4% TX-114 [supernatant of 4% (w/v) TX-114, Table I], which reduced the phenolic content to 3% with no drastic loss in activity. The PPO thus obtained was in its inactive form (latent) and could be activated by SDS (4.5-fold), as has been described for other latent thylakoid-bound PPO (Sánchez-Ferrer et al., 1989b, 1990). This degree of activation decreases during plant senescence and reaches half the original level 2 weeks after normal potato tuber harvest time.

The purification was 5 times, and the recovery was 63%. The degree of purification and recovery cannot be compared to any published data on potato leaf PPO but it is similar to that found when using TX-114 to purify thylakoid-bound PPO (Sánchez-Ferrer et al., 1989a,b, 1990). The additional step in TX-114 (4%) not only removes phenols but also removes other hydrophobic proteins, thus increasing the specific activity of the enzyme.

Activation of Latent Enzyme. The activation processes of potato thylakoid-bound PPO were studied with three substrates (*tert*-butylcatechol, 4-methylcatechol, and chlorogenic acid) using the two agents used normally for activating plant latent PPO, proteases and detergents (Table II) (Tolbert, 1973; King and Flurkey, 1986; Sánchez-Ferrer et al., 1989b, 1990). The former had no enhancing effect on the activity of the latent form and even produced a slight inhibition. With regard to the detergents used, only the anionic detergent, SDS, and dodecanesulfonic acid were effective, whereas cationic (CTAB) and nonionic ($C_{12}E_9$, Brij 96) detergents produced no effect or inhibition (Table II).

Activation by anionic detergents depended on the surfactant concentration, pH, and substrate used to follow the enzymatic activity. When chlorogenic acid (CGA) acted as substrate, there was no activation with SDS in the reaction medium and a similar pH profile with and without SDS was obtained (Figure 1). On the other hand, *tert*-butylcatechol (TBC) showed a 4-fold increase in activity at 130 μM SDS (Figure 2) with a clear increase in activity at pH 4.0 (Figure 3). In addition, 4-methylcatechol (4MC) produced only a 1.7-fold increase in activation at 50 μM and pH 4.0 (Table II). These results seem to be related to the degree of hydrophobicity of the substrate used (TBC > 4MC > CGA), the conformational changes produced by the surfactant favoring the access of the hydrophobic substrates to the active center.

If this assumption is true, the enzyme activated by SDS should modify its kinetic parameters, as occurs when SDS is used to activate other enzymes, such as pyruvate oxidase (Schrock and Gennis, 1977).

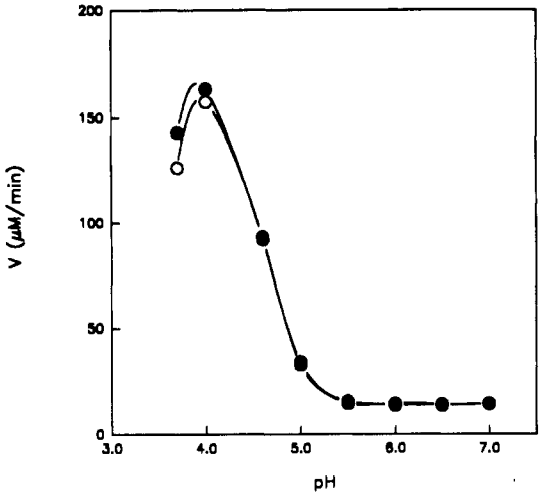

Figure 1. Effect of pH on the catecholase activity of potato leaf PPO toward chlorogenic acid with (●) and without (○) SDS. The reaction medium at 25 °C included 75 μg/mL protein, 0.5 mM chlorogenic acid in 50 mM acetate (pH 3.5–5.5), and phosphate (pH 5.5–7.0) buffers. The SDS concentration used was 25 μM.

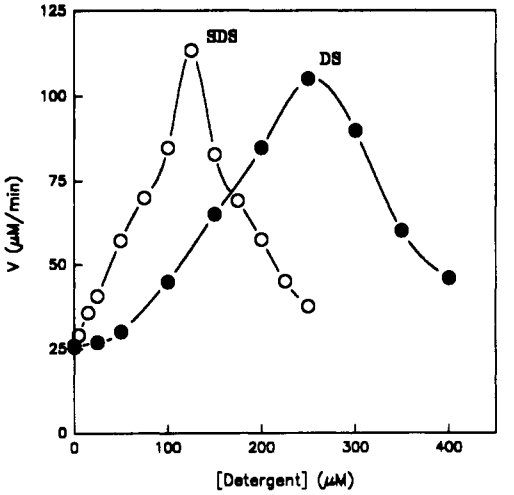

Figure 2. Activation of latent potato leaf PPO by the anionic detergents SDS and dodecanesulfonic acid (DS). The reaction medium at 25 °C included 25 μg/mL protein and 3 mM TBC in 50 mM acetate buffer, pH 4.0.

The effect of SDS on the kinetic parametes is shown in Table III. The presence of SDS decreased the K_M for TBC and increased the V_{max}, resulting in a 4-fold increase in the catalytic efficiency of the activated enzyme, whereas only a 1.5-fold increase was found for 4MC. These increases in catalytic efficiency are lower than those described for broad bean PPO and grape PPO activated by SDS (Sánchez-Ferrer et al., 1989b, 1990).

This substrate-dependent phenomenon with SDS has been explained for pyruvate oxidase (Schrock and Gennis, 1977), where, in the presence of the substrate and cofactor, the enzyme is more susceptible to surface denaturation, thus favoring the binding of SDS molecules. These changes in the structure of latent leaf potato PPO increased the V_{max} in the three substrates used, indicating that the active center catalyzed the substrates more efficiently.

Similar results were found with dodecanesulfonic acid, which showed maximum activation (4 times) at 250 μM using TBC (Figure 2) as substrate and a 1.2-fold increase at 500 μM, using 4MC as substrate (Table II).

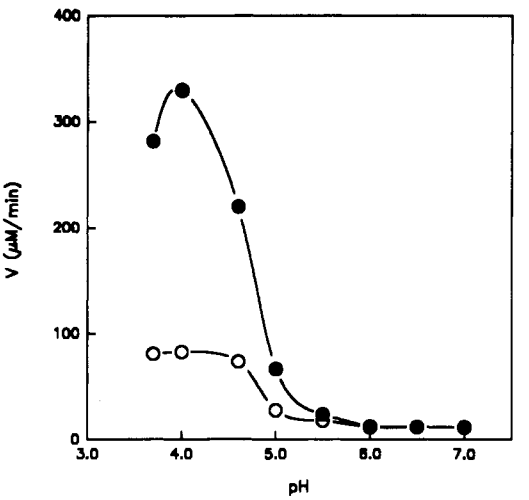

Figure 3. Effect of pH on the catecholase activity of potato leaf PPO toward TBC with (●) and without (○) SDS. The reaction medium at 25 °C included 75 μg/mL protein, 3 mM TBC in 50 mM acetate (pH 3.5–5.5), and phosphate (pH 5.5–7.0) buffers. The SDS concentration used was 130 μM.

Table III. Effect of SDS in the Kinetic Parameters of Latent Potato Leaf PPO

	tert-butylcatechol		4-methylcatechol		chlorogenic acid	
	-SDS	+SDS[a]	-SDS	+SDS[b]	-SDS	+SDS[c]
V_{max}, μM/min	22	60	185	280	303	312
K_M, mM	0.5	0.4	27	25	0.37	0.35

[a] Assayed in the standard reaction media containing 130 μM SDS. [b] Assayed in the standard reaction media containing 50 μM SDS. [c] Assayed in the standard reaction media containing 50 μM SDS.

DISCUSSION

The results presented in this paper show for the first time that chloroplast-bound potato leaf PPO is present in a latent form and is activated mainly by anionic surfactants (SDS and DS). This latency is common in thylakoid-bound PPO and has been described for spinach (Satô, 1976; Lieberi and Biehl, 1978; Golbeck and Camarata, 1981; Sánchez-Ferrer et al., 1989a), broad bean (Satô, 1976; Lieberi and Biehl, 1978; Golbeck and Camarata, 1981; Sánchez-Ferrer et al., 1989a), spinach beet (Parish, 1972), and grape berries (Sánchez-Ferrer et al., 1989b). The degree of activation depends on the plant material and the extraction method used and is highest when a mild extraction method, such as TX-114, is used. In particular, broad bean PPO purified by TX-114 is activated 10 times more than when purified by acetone powders (King and Flurkey, 1987). In addition, when latent leaf PPO purified by TX-114 is compared in different plant leaf sources, its activation increases 4-fold in potato, 4.5-fold in spinach, and 129-fold in broad bean (Sánchez-Ferrer et al., 1990). The same trend is found when the total activity extracted from the tissue is compared.

In addition, it should be noted that the method developed for potato leaf PPO includes an additional phase separation in TX-114 to remove the high level of phenolic compounds present in the extract. This means that the phenol binding capacity of TX-114 reaches saturation, and additional cycles of phase partitioning are necessary to remove the remaining phenols. These additional phase separations do not cause a great decrease in enzyme recovery (Table I).

The substrate-dependent activation of latent PPO has not been described for any latent plant PPO, indicating that higher degrees of activation can be achieved for previously described latent enzymes. The importance of this effect on the physiological role or activation mech-

anism of PPO *in vivo* is not clear and needs further clarification.

ABBREVIATIONS USED

PPO, polyphenol oxidase; TBC, *tert*-butylcatechol; CGA, chlorogenic acid; 4MC, 4-methylcatechol; TX-114, Triton X-114; CTAB, cetyltrimethylammonium bromide; $C_{12}E_9$, poly-(9)oxyethylene lauryl ether; SDS, sodium dodecyl sulfate; DS, dodecanesulfonic acid.

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LITERATURE CITED

Arnon, D. I. Cooper Enzymes in Isolated chloroplast, Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* **1949**, *24*, 1–15.

Bordier, C. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **1981**, *256*, 1604–1607.

Bradford, M. M. A rapid and sensitive method for the quantification of microgrâm quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Golbeck, J. H.; Cammarata, K. V. Spinach thylakoid polyphenol oxidase. Isolation, activation and properties of native chloroplast enzyme. *Plant Physiol.* **1981**, *67*, 977–984.

Kenten, R. H. Latent phenolase in extracts of broad bean (*Vicia faba* L.) leaves. 2. Activation by anionic wetting agents. *Biochem. J.* **1958**, *68*, 244–251.

Kidron, M.; Harel, M.; Mayer, A. M. Catechol oxidase activity in grapes and wine. *Am. J. Enol. Vitic.* **1978**, *219*, 30–35.

King, R. S.; Flurkey, W. H. Effects of limited proteolysis on broad bean polyphenol oxidase. *J. Sci. Food Agric.* **1987**, *41*, 231–240.

Lieberi, R.; Biehl, B. Activation of latent phenolase from spinach chloroplast by aging and by frost. *Phytochemistry* **1978**, *17*, 1427–1429.

Loomis, W. D. Overcoming problems of phenolics and quinones in the Isolation of Plant Enzymes and Organelles. *Methods Enzymol.* **1974**, *31*, 528–540.

Matheis, G. Polyphenol Oxidase and Enzymatic Browning of Potatoes (*Solanum tuberosum*) II. Enzymatic browning and Potato constituents. *Chem. Mikrobiol. Technol. Lebensm.* **1987a**, *11*, 33–41.

Matheis, G. Polyphenol Oxidase and Enzymatic Browning of Potatoes (*Solanum tuberosum*) I. Properties of Potato polyphenol oxidase. *Chem. Mikrobiol. Technol. Lebensm.* **1987b**, *11*, 5–12.

Parish, R. W. The intracellular location of phenol oxidases, peroxidase and phosphatases in the leaves of spinach beet (*Beta vulgaris* L subsp vulgaris). *Eur. J. Biochem.* **1972**, *31*, 446–455.

Pryde, J. G.; Phillips, J. H. Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114—application to subcellular fractions of the adrenal medulla. *Biochem. J.* **1986**, *233*, 525–533.

Sánchez-Ferrer, A.; Bru, R.; Cabanes, J.; García-Carmona, F. Characterization of catecholase and cresolase activities of Monastrell grape polyphenol oxidase. *Phytochemistry* **1988**, *27*, 319–321.

Sánchez-Ferrer, A.; Bru, R.; García-Carmona, F. Novel procedure for extraction of a latent grape polyphenol oxidase using temperature-induced phase separation in Triton X-114. *Plant Physiol.* **1989a**, *91*, 1481–1487.

Sánchez-Ferrer, A.; Villalba, J.; García-Carmona, F. Triton X-114 as a tool for purifying spinach polyphenol oxidase. *Phytochemistry* **1989b**, *28*, 1321–1325.

Sánchez-Ferrer, A.; Bru, R.; García-Carmona, F. Partial purification of a thylakoid-bound enzyme using temperature-induced phase partitioning. *Anal. Biochem.* **1990**, *184*, 279–282.

Satô, M. Multiplicity of Spinach root phenolase and its monophenolase activity. *Phytochemistry* **1982**, *21*, 1229–1231.

Satô, M.; Hasegawa, M. The latency of spinach chloroplast phenolase. *Phytochemistry* **1976**, *15*, 61–65.

Schrock, H. L.; Gennis, R. B. High Affinity lipid binding sites on the Peripheral membrane enzyme Pyruvate Oxidase. *J. Biol. Chem.* **1977**, *252*, 5990–5995.

Smith, H. O. The structure and function of grana-free thylakoid membranes in gerontoplasts of senescent leaves of *Vicia faba* L. *Z. Naturforsch.* **1988**, *43*, 149–159.

Tolbert, N. E. Activation of polyphenol oxidase of chloroplast. *Plant Physiol.* **1973**, *51*, 234–244.

Valero, E.; García-Carmona, F. Hysteresis and cooperative Behavior of Latent Plant Polyphenol oxidase. *Plant Physiol.* **1992**, *98*, 774–776.

Vaughn, K. C.; Duke, S. O. Polyphenol oxidase: The chloroplast oxidase with no established function. *Physiol. Plant.* **1988**, *72*, 659–665.

Werck-Reichhart, D.; Benveniste, I.; Teutsch, H.; Durst, F.; Gabriae, B. Glycerol allows low-temperature phase separation of membrane proteins sulubilized in Triton X-114: Application to the purification of plant cytochromes P-450 and b_5. *Anal. Biochem.* **1991**, *197*, 125–131.

Weselake, R. J.; Jain, J. C. Strategies in the purification of plant proteins. *Physiol. Plant.* **1992**, *84*, 301–309.

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