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Extra- and Intracellular Laccases of the Chestnut Blight Fungus, *Cryphonectria parasitica*

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A double-stranded RNA virus of the chestnut blight pathogen, *Cryphonectria parasitica*, has been shown previously to reduce accumulation of mRNAs of extracellular laccase (laccase A) produced by this fungus. Both extra- and intracellular laccases have been detected after growth of the fungus in liquid culture. In addition to cellular localization, the two laccases are distinguishable by time of appearance during growth and electrophoretic mobility. Laccase A was purified from the culture filtrate by standard protein purification procedures. The enzyme was characterized as a glycoprotein with a molecular mass of approximately 77 kDa. Both laccase A and laccase B activities were significantly reduced in the hypovirulent (double-stranded RNA-infected) strain UEP1 compared with the isogenic virulent (double-stranded RNA-free) strain EP155/2.

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) is an enzyme which oxidizes a large variety of organic substrates. The enzyme is widely distributed in fungi; however, its biological function is still not clear (26). In *Aspergillus nidulans*, laccase is involved in conidial pigmentation (10). In other fungi, suggested roles for laccase include degradation of lignin (2), pathogenesis (3), and formation of fruiting bodies (23). Interest in laccase has been increasing recently because of its potential use in detoxification of environmental pollutants (6).

The laccase of the chestnut blight fungus, *Cryphonectria parasitica* (Murr.) Barr (4), recently has attracted interest because it is one of a number of fungal gene products which are specifically suppressed in the presence of a double-stranded (ds) RNA virus (8, 9, 32, 33, 30). This virus is a biological control agent of chestnut blight, an extremely destructive disease of chestnut trees in North America and Europe (for reviews, see references 1, 16, and 34). Infection by the virus results in a phenotype which is viral strain specific but which can include reduced virulence (hypovirulence), reduced sporulation, and reduced pigmentation. The molecular basis of the phenotypic effects of the virus appear to be the result of viral suppression of specific gene products of the fungus. These products include laccase (9, 33), a cell surface hydrophobin with properties of a lectin (8), and genes which are involved in sporulation (36). Part of the complex of phenotypes caused by the virus could be attributed to the suppression of laccase. Consequently, we thought it was important to clarify the nature of laccase production by this fungus. We report here that two different laccases are produced by *C. parasitica* and that both are suppressed by the virus; we also describe the purification and properties of the extracellular laccase.

MATERIALS AND METHODS

Fungal strains and culture conditions. The dsRNA-free strain EP155/2 and the isogenic dsRNA-infected strain UEP1 of *C. parasitica* (30) were used in this study. The strains were grown in a complete liquid medium (11) at pH 5.6. To produce inoculum, the fungus was grown on agar plates of potato dextrose agar supplemented with L-methionine (100 mg/liter) and biotin (1 mg/liter) (PDAmb) in the dark at 25°C until the mycelial growth almost covered the plates (6 to 7 days). The total contents of the plates were then homogenized in distilled water (100 ml per PDAmb culture plate) with a Waring blender, and the resulting slurry was used as inoculum (1/10 of the medium volume). The fungus was grown in Fernbach flasks containing 1 liter of medium for production of large amounts of extracellular laccase. Alternatively, 500-ml Erlenmeyer flasks containing 100 ml of medium were used for time course comparisons of strains EP155/2 and UEP1. The cultures were incubated under constant light at 26°C on a rotary shaker (105 rpm). The culture fluid was separated from the mycelium by filtration through Miracloth (Calbiochem). The mycelium was washed with distilled water and lyophilized to determine dry weight.

Laccase assay. Laccase activity was determined with 2,6-dimethyoxyphenol as substrate (32). One unit was defined as an increase of A_{468} of 1.0 per min at 25°C. Extracellular laccase activity was determined with filtered culture fluid. For determination of intracellular laccase activity, 0.5 g of lyophilized mycelium ground in liquid nitrogen was added to 10 ml of cold 0.1 M sodium phosphate (pH 6.0), and the mixture was homogenized at 8,000 rpm for 1 min in a Polytron homogenizer (Kinematica). The homogenate was centrifuged for 5 min at 9,000 × *g*, and laccase activity was determined in the supernatant.

Protein determination. Protein concentrations were determined by measuring the A_{280} or by the method of Bradford (7), using ovalbumin (Sigma) as the standard.

Purification of extracellular laccase. Extracellular laccase of *C. parasitica* was purified from the culture filtrate collected 4 days after inoculation. All purification steps were done in the cold. Culture fluid collected from 8 liters of cell broth was concentrated to 200 ml by ultrafiltration with a PM10 membrane (Amicon). The concentrate was centrifuged for 15 min at 10,000 × *g*, dialyzed overnight against distilled water, and centrifuged again as described above. The supernatant was adjusted to 10 mM sodium phosphate (pH 7.5) and applied to a DEAE-Sepharose column (2.5 by

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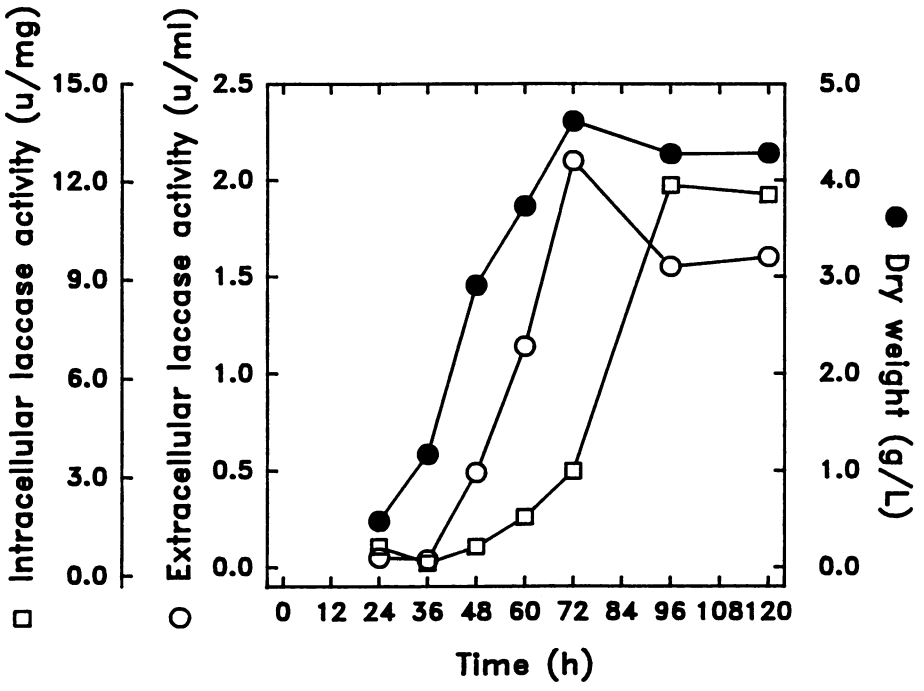

FIG. 1. Biomass production and intra- and extracellular laccase activities of *C. parasitica*. Biomass (dry weight) and laccase activities were followed as a function of time after inoculation of liquid media. These are results of a representative experiment. All data from each time point were from a single flask.

10 cm) equilibrated in the same buffer. The column was washed with 150 ml of 10 mM sodium phosphate (pH 7.5), and then the laccase was eluted with 0.1 M NaCl in the buffer (flow rate, 50 ml/h). Fractions containing laccase activity were pooled, concentrated to about 4 ml in a Centriprep 30 unit (Amicon), and then applied to a Bio-Gel P-100 (Bio-Rad) gel filtration column (1.6 by 80 cm) equilibrated in 50 mM sodium citrate, pH 3.4 (flow rate, 5 ml/h). Fractions with laccase activity were pooled and applied to an SP-Sephadex (Pharmacia) ion-exchange column (1.5 by 15 cm) equilibrated in 50 mM sodium citrate, pH 3.4. The column was washed with 30 ml of buffer and then eluted with a linear 0 to 0.5 M sodium chloride gradient in 120 ml of buffer (flow rate, 7 ml/h). The laccase fractions were pooled and concentrated with a Centriprep 30 unit to about 3 ml. The concentrated enzyme was dialyzed against 50 mM sodium phosphate (pH 6.0) and stored at −20°C for several months without loss of activity.

Carbohydrate determination. The amount of carbohydrate present was determined by the phenol-sulfuric acid method (13), with D-glucose as the standard.

Gel electrophoresis. Native gel electrophoresis was performed in 1% agarose gels, using a Tris-citrate buffer at pH 5.2 (12). Samples were loaded in 15% glycerol containing 0.05% bromophenol blue and run at 8 V/cm until the dye was about 1 cm from the edge of the gel. The gels were incubated in the same laccase detection solution as used for the laccase assay. This stain gives an orange band within a short time when laccase is present in the gel. Overnight inoculation results in formation of a dark red precipitate, which is easier to photograph. Sodium dodecyl sulfate (SDS)-polyacrylamide gels (21) were stained with Coomassie blue.

RESULTS

Production of extra- and intracellular laccases. Biomass production and intra- and extracellular laccase activities were monitored as a function of time during growth in liquid culture (Fig. 1). Liquid cultures which were started with macerated mycelium from agar culture plates exhibited a lag phase of about 24 h, after which the fungus grew rapidly and reached stationary growth phase at about 72 h after inoculation. Extracellular laccase activity was first detected 48 h after inoculation. The activity reached a maximum at 72 h and decreased slowly thereafter. As shown in Fig. 1, the time course of intracellular laccase activity lagged behind the

TABLE 1. Purification of extracellular laccase (laccase A) from *C. parasitica*

Purification step	Vol (ml)	Total activity (U)	Total A_{280}	Sp act (U/A_{280})	A_{280}/A_{250} ratio	Yield (%)	Purification (fold)
Culture filtrate	7,530	15,800	10,500	1.5	0.81	100	1
Ultrafiltration	180	17,800	1,230	14.5	0.97	112	10
DEAE-Sepharose CL-6B	4	12,700	65	193	1.32	80	129
Bio-Gel P-100 gel filtration	4	9,480	25	374	1.41	60	249
Sp-Sephadex C-50 chromatography	2.8	4,590	7	665	2.21	29	443

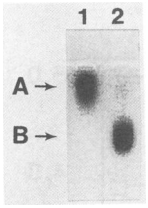

FIG. 2. Electrophoretic separation of extracellular (A) and intracellular (B) laccases of *C. parasitica*. Crude extracellular (lane 1) and intracellular (lane 2) protein extracts of a 4-day-old culture were run in an agarose gel at pH 5.2 and stained for laccase activity. The origin is at the top.

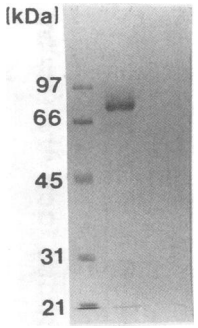

FIG. 4. SDS-PAGE of the purified extracellular laccase A of *C. parasitica*. Molecular mass markers are shown in the left lane.

extracellular one. Intracellular laccase activity was also first detected after 48 h but reached its maximum at 96 h after inoculation. As with the extracellular laccase, intracellular laccase activity decreased slowly after reaching the maximum level. The maximum amount of laccase activity produced by a 1-liter culture of the fungus was 2,300 U for the extracellular laccase and 2,400 U for the intracellular laccase.

Extra- and intracellular laccases were distinguished further by their different electrophoretic mobilities in a nondenaturing activity gel (Fig. 2). At pH 5.2, both laccases migrated towards the anode in broad multicomponent or diffuse bands but with different mobilities. We conclude that the extra- and intracellular laccases are different enzymes, which we have named laccase A and laccase B, respectively (Fig. 2).

Purification and characterization of the extracellular laccase (laccase A). The purification procedure for laccase A of *C. parasitica* is summarized in Table 1. Evidence of purity of the enzyme was based on SDS-polyacrylamide gel electrophoresis (PAGE) analysis and the ratio of A_{280} to A_{250} (15). Four purification steps were required to separate the laccase from several other extracellular proteins, from pigments, and from extracellular polysaccharides (EPS). SDS-PAGE analysis indicated that laccase A was basically purified from other proteins after three purification steps. The low ratio of A_{280} to A_{250} indicated, however, that laccase was still contaminated with large amounts of EPS and pigments (15). These contaminants were removed from laccase A by cationic ion-exchange chromatography (Fig. 3). Two peaks detected by A_{280} were separated with this method. The first peak, which did not bind to the column, was found to contain a large amount of carbohydrate and had an A_{280}/A_{250} ratio of 1.0. We assume that this peak consisted mainly of EPS. The laccase A activity was eluted in a single peak at approximately 0.2 M in the NaCl gradient.

SDS-PAGE analysis showed that the purified laccase A migrated as a polypeptide with a molecular mass of approximately 77 kDa (Fig. 4). The ratio of A_{280} to A_{250} of the purified laccase was 2.2. The purified laccase was blue, with a ratio of A_{280} to A_{600} of 33 (average of three different purifications). The laccase was found to be a glycoprotein with an estimated carbohydrate content of 24%. SDS-PAGE gels treated with Coomassie blue and Schiff's base or dansyl hydrazine (14) stained the laccase A band by each of these three methods, confirming the glycoprotein nature of laccase

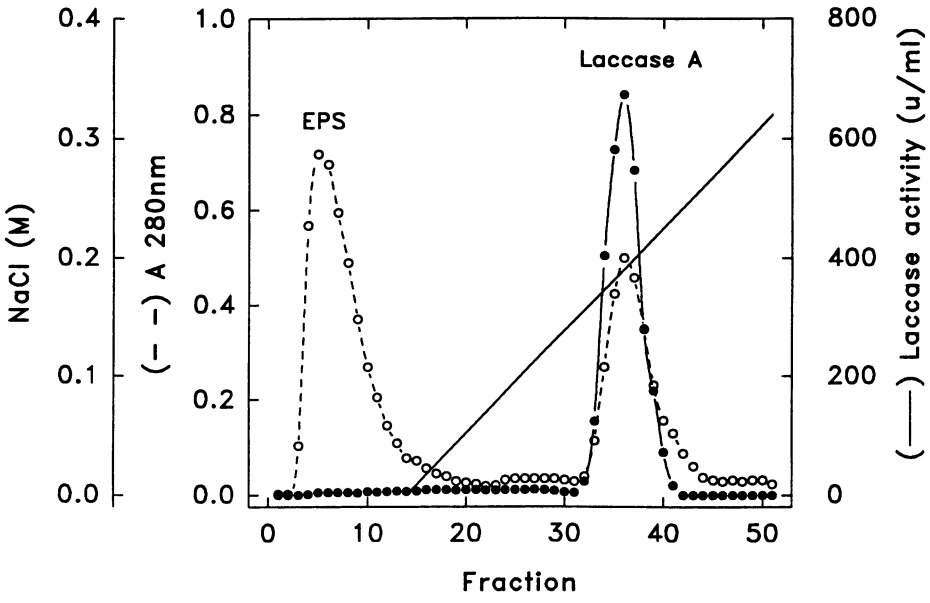

FIG. 3. Separation of laccase A from EPS by SP-Sephadex C-50 chromatography.

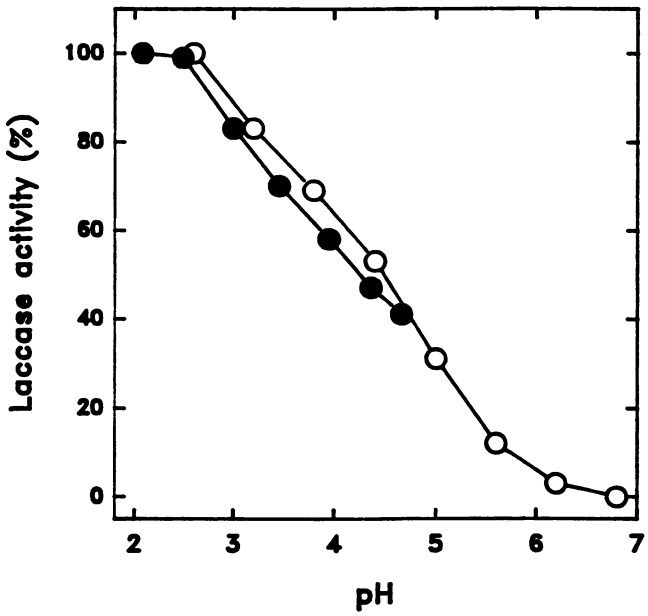

FIG. 5. Effect of pH on laccase A activity of *C. parasitica*. The reaction mixtures (1 ml) contained 2 mM 2,6-dimethoxyphenol and 0.06 U of purified laccase A in 64 mM Na-tartrate (closed circles) or 50 mM Na-citrate (open circles) buffer.

A. Further examination with a fast protein liquid chromatography (FPLC)-Mono Q column (Pharmacia) revealed the existence of two major and several minor glycoforms of the enzyme. The specific activity of laccase A was 677 U per A_{280} or 925 U/mg of protein (average of three preparations). The laccase exhibited a pH optimum of 2.5, using 2,6-dimethoxyphenol as substrate (Fig. 5).

Suppression of laccase B by dsRNA. We have reported previously that the accumulation of mRNA of laccase A is suppressed by viral dsRNA (33). To determine whether the dsRNA has a similar effect on laccase B activity, strains EP155/2 (dsRNA-free) and UEP1 (dsRNA infected) were grown under identical conditions, and biomass accumulation and laccase A and B activities were followed as a function of time (Fig. 6). As shown in Fig. 6, both laccase A and laccase B activities were significantly reduced in the dsRNA-infected strain UEP1 compared with the dsRNA-free strain EP155/2. The average reductions were found to be 86 and 84% for laccases A and B, respectively. In contrast, no differences could be observed in dry weight accumulation of the two strains. The maximum activities of laccases A and B were at 4 and 5 days, respectively (Fig. 6), i.e., one day later than in the experiment shown in Fig. 1. The use of different types of flasks in each experiment (see Materials and Methods) may be the explanation for the observed shift in laccase accumulation.

In a previous study on oxalate production of *C. parasitica* (18), an inhibitor of the enzyme oxaloacetate acetylhydrolase was found in extracts of hypovirulent strains. To test whether a similar inhibitor is responsible for the reduction of laccase B activity in UEP1, we have mixed extracts from both strains taken at 2, 3, and 4 days after inoculation. No reduction of laccase B activity was detected even after 3 h of incubation of the extract mixtures at room temperature.

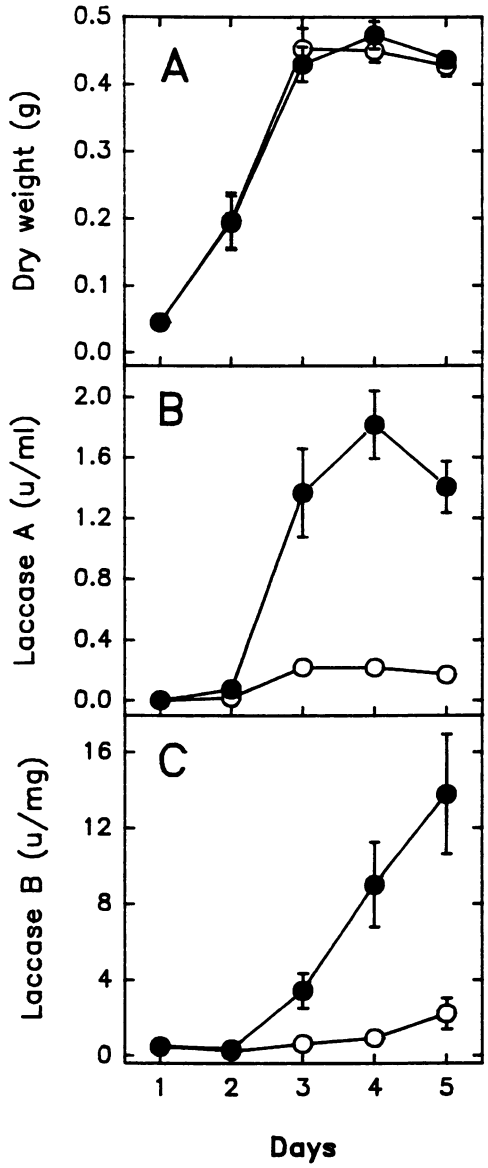

FIG. 6. Accumulation of laccase A and B activities in the dsRNA-free strain EP155/2 (closed circles) and the isogenic dsRNA-infected strain UEP1 (open circles) of *C. parasitica*. Dry weight accumulation (A), extracellular laccase A activity (B), and intracellular laccase B activity (C) were monitored as functions of time after inoculation of liquid media. Cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of complete liquid medium in these experiments. The means (± standard deviations) of three independent experiments are shown.

DISCUSSION

Chestnut blight is being controlled naturally in some locations by a dsRNA virus that limits virulence and sporulation of the pathogen responsible for this disease. The virus specifically affects these developmental processes without limiting the ability of the fungus to grow in culture. We have demonstrated previously that a small number of fungal proteins are produced in lower quantities in virus-infected strains than in noninfected strains of the fungus (31). We hypothesize that the virus is perturbing regulation of specific

genes important to the developmental processes of sporulation and virulence. Little is known of the basis of virulence of *C. parasitica*, but the high tannin concentrations in chestnut bark suggest that to be successful as a pathogen of chestnut, this fungus must tolerate relatively high tannin concentrations. The production of an extracellular laccase could play a role in such a postulated tolerance to host polyphenolics. Evidence in support of such a role is the discovery (32) that an extracellular laccase of the fungus is produced in much lower quantities by virus-infected strains than by normal strains. This observation was confirmed when a gene for an extracellular laccase (*lacA*) was cloned and it was shown that in a virus-infected strain the mRNA levels of this gene are much reduced compared with an isogenic noninfected strain (9, 33). The mechanism involved in this viral regulation of laccase A is not known, but it is the subject of current investigations. Recent studies suggest that the virus perturbs cellular signaling processes which normally result in laccase A induction (22).

Purification of laccase A was by standard protein purification procedures. The primary difficulty was the removal of the fungal EPS. Laccase A is a glycoprotein that appears to have multiple glyco forms when analyzed by FPLC. The molecular mass estimate of 77 kDa by SDS-PAGE must then be considered to be an average. Laccase A is blue, which is an indication of the presence of copper binding to the molecule. Fungal laccases are a heterogeneous group of copper-binding glycoproteins with molecular weights ranging from about 56,000 to 85,000 (27). In most cases, the enzyme is thought to consist of a single polypeptide chain, although laccase I of *Podospora anserina* is apparently a tetrameric protein (29). Many fungal laccases bind four copper atoms per molecule, giving them a blue color (19, 24, 35). The blue color of laccase A, its size, and its glycoprotein nature are characteristics similar to those reported for a number of other fungal laccases.

The pH optimum of laccase A is 2.5. This is lower than reported for most other extracellular fungal laccases (5, 20). The normal substrate for this fungus is the living bark and vascular tissue of chestnut trees. During the invasion of chestnut bark by *C. parasitica*, it is reported that the pH of the bark decreases from 5.5 to 2.8 (28). Laccase A is obviously well suited for optimal activity in this acidic environment created during the growth of the fungus in its host.

While purifying this extracellular laccase, we found that in liquid culture *C. parasitica* produces two laccases which are distinguishable by cellular localization, time of appearance during growth, and electrophoretic mobility. The newly discovered laccase B is intracellular and possibly a precursor of laccase A. In *Neurospora crassa*, extracellular laccase is synthesized first as an intracellular precursor which is then secreted into the culture medium upon cleavage of a specific leader sequence (17). Consequently, the intracellular form appears first in a time course and is then followed by increasing amounts of extracellular laccase (25). In *C. parasitica*, the extracellular laccase activity temporally precedes the intracellular one (Fig. 1). Recently, we have obtained genetic evidence that laccase A is encoded by a different gene than laccase B. This conclusion was reached by making a laccase A null mutant by site-specific deletion of the *lacA* gene, using marker exchange procedures. This mutant strain lacks laccase A but still produces laccase B (20a). The intracellular form of laccase A is thus present in a form which is not detectable with the laccase assay. We conclude from these results that the extracellular and intracellular laccases of *C. parasitica* described here are enzymes encoded by different genes.

An important observation from this study is that laccase B is down-regulated by the dsRNA along with laccase A. We can assume from this common regulation of both laccases by the dsRNA that these separate genes have common regulatory factors. Although little is known of the biological roles of laccases in fungi, it is likely that an intracellular laccase will have a different biological role than an extracellular one; thus, their common regulation by the virus is of particular interest. It suggests that the functions of these laccases, if not the same, are coordinated during a developmental process.

ACKNOWLEDGMENTS

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