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The full length *PtSRP* (*Pisolithus tinctorius* symbiosis related protein) fungal mRNA encodes a potential marker of ectomycorrhiza formation*

Helder Elísio E. Vieira[1], Cláudia E. P. Lima[1], Carlos E. Calzavara-Silva[2], Bartolomeu Acioli-Santos[1,3#], Elaine Malosso[1]

[1]Departamento de Micologia, Universidade Federal de Pernambuco, Recife, Brazil
[2]Laboratório de Imunologia Celular e Molecular, Centro de Pesquisas René Rachou, Belo Horizonte, Brazil
[3]Centro de Pesquisas Aggeu Magalhães—Fundação Oswaldo Cruz, Laboratório de Virologia e Terapia Experimental, Universidade Federal de Pernambuco, Recife, Brazil
Email: #bartacioli@cpqam.fiocruz.br

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ABSTRACT

The *Pisolithus tinctorius* symbiosis related protein expressed sequence tag (EST *PtSRP*) was previously identified in the first hours of the interaction between the fungus *Pisolithus tinctorius* and sweet chestnut *Castanea sativa*, and partially characterized as a fungal marker gene of ectomycorrhizal symbiosis formation. We used the 5' rapid amplification of cDNA ends (RACE) to obtain the *PtSRP* mRNA 5' region, and together with our previously reported 3' mRNA region, the full mRNA sequence was assembled by use of bioinformatics tools and deposited to GenBank (Accession: GU733439). The full-length mRNA sequence (636 bp) revealed the locations of the 5' and 3' untranslated regions (UTRs) and contained the Kozak sequence (ccc aag ATG A) in the 5' UTR. The *in silico* translated *PtSRP* open reading frame (ORF) codes for a 127 amino acid protein and contained four putative post-translational modification sites (two N-glycosylation and two phosphorylation). The protein secondary structure is postulated to be composed of one N-terminal hydrophobic transmembrane alpha helix and at least six hydrophilic beta-strands spread across the protein. Sub-cellular localization prediction suggests that the protein is involved in cellular secretory pathway, supported by the presence of a cleavage site motif close to the membrane anchor. The data presented herein indicate the role of *PtSRP* as a fungal membrane secreted protein involved in early stages of ectomycorrhizal formation, with application as a possible marker for nascent ectomycorrhiza fungal development.

*Competing interests: The authors declare that they have no competing interests.
#Corresponding author.

Keywords: Ectomycorrhiza; Symbiosis Related Genes/Proteins; RACE; EST

1. BACKGROUND

The establishment of ectomycorrhiza involves controlled, intense gene expression in both partners that leads to drastic morphological and physiological changes, crucial to the development of mutualism and symbiotic harmony [1-3]. The comparison of protein extracts from mycorrhizal and non-mycorrhizal mycelia in previous studies has shown differences that suggest specific gene activation during the symbiosis process [4-6]. These findings highlighted a new class of biomolecules thought to control the ectomycorrhiza symbiosis process: the ectomycorrhizins [7]. However, recent studies evaluating the fungal transcript pattern during symbiosis formation have demonstrated that mycorrhization also induces changes in the expression of genes normally expressed in free organisms [3,8-10].

Among the ectomycorrhizins, SRAPs (Symbiosis Related Acid Proteins) and hydrophobins are the most investigated and discussed classes of proteins. However, these proteins were generally isolated from fully established mycorrhiza or those developing associations after several days of interaction [8,9]. The identification of new early stage ectomycorrhizal molecules could bring new insight to the molecular and functional understanding of the ectomycorrhiza formation process. The fungal *PtSRP* mRNA (previously called *Pisolithus tinctorius* symbiosis related receptor 1, accession number EL563703) was isolated [3] and partialy characterized [11] as a possible fungal membrane protein probably secreted in the first hours of fungus-root interaction. In this paper, we present the full-length *PtSRP* fungal mRNA sequence, supported by sequencing of the 5' region and our pre-

viously reported 3' region, followed by *in silico* characterization of the most probable ORF and its relationship with early stages of ectomycorrhiza.

2. RESULTS

2.1. The Full-Length *PtSRP* mRNA

The 5' RACE technique generated a partial 355 bp fragment corresponding to the 5' portion of *PtSRP* mature mRNA (deposited at NCBI as nucleotide record GU733-439). The complete sequence, assembled by contig construction between the 5' RACE fragment and a 3' previously reported sequence (EL563703, [11]) resulted in a 636 bp sequence (**Figure 1**). Additionally, a unique 604 bp product was obtained by direct PCR of fungal cDNA samples. The nearly perfect alignment of the 604 bp consensus fragment to the 636 bp contig supported the reliability of the contig; only two nucleotide differences (291 C/T and 306 A/C, **Figure 2**) were observed and they were in the putative ORF region, with no changes to the amino acid (synonymous mutations). The *PtSRP* mRNA putative ORF is 384 bp long and codes for a protein of 127 amino acids (a.a.) (**Figure 1**), with untranslated regions (UTRs) upstream and downstream of the ORF. The Kozak motif sequence (ccc aag ATG A) was present in the 5' UTR, albeit slightly variable from the original Kozak sequence (gcc Rcc AUG G) for three of the nucleotides: –6 (C), –2 (A), –1 (G) and 4 (A) (**Figure 1**).

2.2. *In Silico* Analysis of *PtSRP*

In silico analysis of *PtSRP* primary structure indicated theoretical molecular weight of 13,969 kDa and an iso-

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                                                                -6-5-4
1    ACC TCT CTC TCG AAC ACC TCC ACA TCC TTT ACT TAC TAC CCT CCC   45
                -3-2-1 +1+2+3 +4
46   AAG ATG ATG TTC ACG AAG TCT CTC ATG TCC TCA TTC TTC GCT CTC   90
 1   ←— Met Met Phe Thr Lys Ser Leu Met Ser Ser Phe Phe Ala Leu   14

91   ATG TCC CTC CTT GCA CTG GCG ATG TCC GTG CCT CTC CAG AAA CGA  135
15   Met Ser Leu Leu Ala Leu Ala Met Ser Val Pro Leu Gln Lys Arg   29

136  GAT GTC TTC GTC CCC CCG GTC CTC GAT CCT CAC CAG GGG ACC GTC  180
30   Asp Val Phe Val Pro Pro Val Leu Asp Pro His Gln Gly Thr Val   44

181  TGG TAC GTT GGG CAA CAA GCA GAA GTC GTC TGG GAC ACG AGT AAC  225
45   Trp Tyr Val Gly Gln Gln Ala Glu Val Val Trp Asp Thr Ser Asn   59

226  GCC CCT GCA CAA ATC ACA AAT TCA GAG GGA CAG ATT TAT CTC GTC  270
60   Ala Pro Ala Gln Ile Thr Asn Ser Glu Gly Gln Ile Tyr Leu Val   74

271  GTA AAC AAC CTC ATC GAT TTC GAC TAC TTG TTG GCA AAT GAT TTC  315
75   Val Asn Asn Leu Ile Asp Phe Asp Tyr Leu Leu Ala Asn Asp Phe   89

316  AAT ATT CTC GAT GGG AGT GTG ATG GTC ACA GTA CCG GAC GTG CCG  360
 90  Asn Ile Leu Asp Gly Ser Val Met Val Thr Val Pro Asp Val Pro  104

361  ACT GGC ATT TAT GCC ATC GTC TTG TTT GGT GAT TCT GGT AAC TTT  405
105  Thr Gly Ile Tyr Ala Ile Val Leu Phe Gly Asp Ser Gly Asn Phe  119

406  AGC CAG AAC TTC ACC ATC ATA GCG TGA TCC CAT CAC GTC CTT GCA  450
120  Ser Gln Asn Phe Thr Ile Ile Ala End —→                      127

451  ACT TTA TCT CTC TGA ACG ATT TCA TGA ACA ATG ATG AAG GAC TTC  495

496  TGT TTC GTT TAC CAC TCA GGA CTT GGT TTC ATA CAT TAG GAC GAC  540

541  AAA TAC AAT GCA TCC GGA ACA TTT AGC AAT GGA CTT GTA ACC CCC  585

586  TTT CGC ATT CTG CTG TAC GTA TAT GGA CTA GGA TCC GGG ACC ATT  630

631  CTA CTA   636
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Figure 1. The *PtSRP* mRNA. Complete nucleotide sequence (636 bp) and its probable ORF (the codons and corresponding 127 amino acids). The ORF represents the largest translation region for the sequence. Stop codon is represented by the word "End". Full line = new fragment obtained by RACE 5'; Interrupted line = 3' previously reported sequence (EL563703, Acioli-Santos *et al.*, 2009); ← = 5' UTR; → = 3' UTR; □ = Kozak sequence with variation and respective base positions.

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electric point of 3.92. Further, four targets of post-translation modifications were predicted: two N-glycosylation sites (residues 118 to 121 (NFSQ) and 122 to 125 (NFTI)) and two casein kinase II phosphorylation sites (residues 65 to 68 (TNSE) and 99 to 102 (TVPD), **Figure 3**).

The predicted secondary structure was an N-terminal hydrophobic transmembrane alpha-helix, from residues 10 to 20, followed by six beta-sheets interspersed by short loops (**Figure 3**). A signal peptide cleavage site was predicted close to the membrane, between the 23rd (serine) and 24th (valine) residues. Sub-cellular localization prediction indicated strong probability that the PtSRP protein is involved in cellular secretory pathway, which is also supported by the presence of the N-terminal signal peptide cleavage site. Hydrophobicity analysis demonstrated that the *PtSRP* initial region is strongly hydrophobic (**Figure 4**) supporting the secondary structure prediction analysis (*i.e.* an initial alpha-helix region).

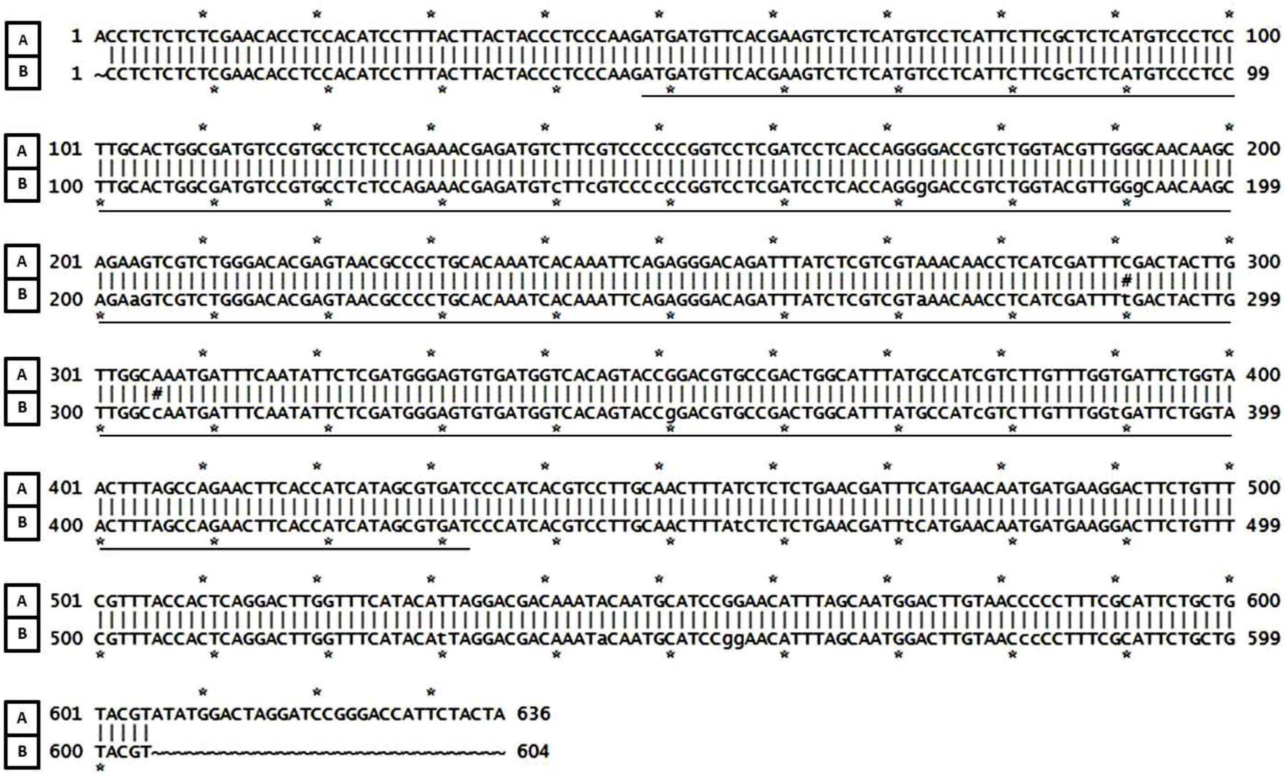

Figure 2. Alignment between the RACE assembly (636 bp; A) and the directly amplified and cloned sequence (604 bp; B) indicating only two nucleotide changes in 291C/T and 306 A/C positions (#). ▬: *PtSRP* ORF.

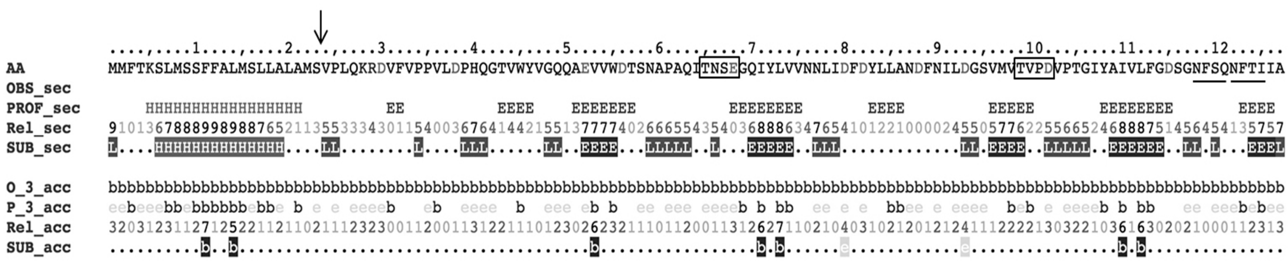

Figure 3. Secondary structure prediction and transmembrane domain of the PtSRP. Legend: *AA* = amino acid sequence, *PHD_htm* = transmembrane helix prediction (M = transmembrane helix, blank spaces = non-membrane regions), *Rel_htm* = PHD_htm prediction reliability index (Reliable predictions are marked "*"), *PROF_sec* = secondary structure prediction (H = Helix, E = sheet, blank: "loop"), *Rel_sec* = PROF_sec prediction reliability index (0 = low to 9 = high), *SUB_sec* = all PROF_sec predictions subset, for all residues with an expected average accuracy > 82% (L = "loop", "." = no prediction for the residue), *O_3_acc* = observed relative solvent accessibility (b = 0% - 9%, i = 9% - 36%, e = 36% - 100%). *P_3_acc* = predicted relative solvent accessibility, *Rel_acc*: P_3_acc prediction reliability index, *SUB_acc*: P_3_acc prediction subset, for all residues with an average correlation > 0.69. Glycosylation sites NFSQ and NFTI = (—); Phosphorylation sites TNSE and TVPD = ( ). The cleavage site near to membrane is represented by (↓). Analyses were carried out in http://www.predictprotein.org.

GLOBE prediction indicated that *PtSRP* is not a globular protein.

3. DISCUSSION

The studies of unknown ectomycorrhizal genes need to be carried out in several stages, as there are as yet none or few elements for comparison. Hydrophobins are fungal proteins usually found during the early stages of *P. tinctorius-E. globulus* interaction [12]. However, its expression is dubious in some cases, and its use as a symbiotic development marker is debatable [9]. SRAP (Symbiosis Related Acid Proteins) genes have been considered as robust ectomycorrhiza development marker after two days of contact [8,9,13]. To the best of our knowledge, there is no report of SRAPs expression during shorter intervals, such as within early hours of mycorrhizal establishment. *PtSRP* QRT-PCR data [11] confirmed the cDNA microarray analysis [3] of its high relative transcription at 12 h of ectomycorrhizal stimulus. Transcription of this gene apparently does not occur until 6 h of contact, suggesting that the period between 6 and 12 h can be crucial for the *PtSRP* expression [11]. The complete *PtSRP* mRNA sequence showed 78% identity with a *Pisolithus microcarpus* sequence (CB010071; [14]) in ectomycorrhizal association with *Eucalyptus globulus*. Further, 78% identity was detected when compared with a fungus cDNA from a four-day-old *Pisolithus tinctorius-Eucalyptus globulus* association (BF942-674; [8]).

The Kozak consensus sequence was found in the 5' UTR of the *PtSRP* mRNA. This regulatory element plays an important role during early processes of gene translation [15] through recognition by the ribosome, resulting in higher or lower protein synthesis [16]. Typical Kozak sequences are followed by the start codon and a guanine base, gccRccAUGG, where R is a purine base three positions before the AUG start codon [17]. In *PtSRP* mRNA, a variant Kozak sequence is observed, but these differences are common, as variation between species [18-20]. In *Saccharomyces cerevisiae*, for example, adenine is commonly observed to precede the initial methionine codon [19].

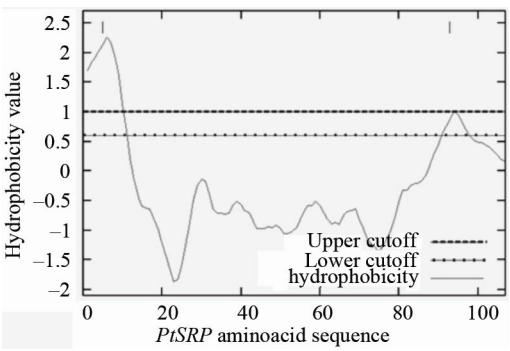

Figure 4. *PtSRP* hydrophobicity analysis. A strong hydrophobic initial region is observed in the protein (Scaled by the Kyte and Doolittle, 1982).

The computationally translated protein ORF showed significant local sequence alignment with two previously reported proteins. The first (48% identity and 65% similarity) was isolated from the mycelia of the ectomycorrhizal *Laccaria bicolor* (accession XP_001876100.1). This protein is associated with a small-secreted protein (SSP) of unknown function, up regulated in symbiotic tissues [10]. The other homologous protein (45% identity and 61% similarity) was isolated from the non-ectomycorrhizal *Schizophyllum commune* (accession AF335537) and is highly expressed when the mycelium is growing under low nitrogen availability, an important environment aspect to mycorrhizal formation.

The presence of the integrin binding RGD domain (arginine-glycine-aspartic acid) in a protein has been related to cell adhesion [21], a key feature in the initial stages of ectomycorrhiza formation, as has been observed in some SRAPs [13]. The RGD domain and its variants [21] have not been found in the PtSRP protein. This, however, is not an isolated event. Other genes from ectomycorrhizal systems do not present this motif (e.g. SC13 and SC25 in [22] and Lbras in [23]), suggesting it is not a critical ectomycorrhizal domain.

The *PtSRP* post-translation modification sites previously indicated [11] were confirmed after obtention of the full-length mRNA. In addition, the prediction of a transmembrane region (composed of an alpha-helix between the 10^{th} and the 20^{th} a.a. residues) in the protein with a probable cleavage site close to the membrane, and an external portion composed of beta-sheets interspersed with loops were observed. These data suggest that the PtSRP protein could act as a signalling secreted protein during early stages of symbiosis. Further studies of the *PtSRP* gene and its protein are required to confirm its function as a potential controller/marker of fungal development in ectomycorrhiza symbiosis.

4. MATERIALS AND METHODS

4.1. Biological Material and Culture Conditions

The fungal strain, culturing and ectomycorrhizal induction were done as described in [24]. *P. tinctorius* (isolate 289/Marx from the University of Tübingen) was maintained on modified Melin-Norkrans agar MNM [25]. Liquid cultures were obtained by transferring mycelia discs from solid cultures to 250 ml liquid MNM contained in Erlenmeyer flasks and kept in the dark at 25˚C until a dense mycelium was observed. Fungal biomass was washed in sterile water, immediately frozen in liquid

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nitrogen and stored at –80˚C.

4.2. Synthesis of *PtSRP* mRNA 5' Portion

Total fungal RNA was extracted using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen). The 5' *PtSRP* was obtained using 5' RACE technology (GeneRacer—*full-length*, *RNA ligase-mediated rapid amplification of* 5' *cDNA ends*—RLM-RACE, Invitrogen) according to the manufacturer's instructions. The 5' phosphate free ends were linked to GeneRacer™ RNA Oligo and the cDNA was synthesised (Superscript II—Invitrogen). PCR reactions were preformed using the primers supplied in the kit, aiming at the GeneRacer™ RNA Oligo combined with gene specific primers targeted to the 3' sequence (PT-1440 REV: 5'-AAATCGTTCAGAGAGATAAAGTTG-3' and PCR 1R REV: 5'-CGTCCGGTACTGTGACCATC-3'). Cloning of the largest RACE fragment was performed using the pGEM-T Easy Vector System (Promega) and the insertion was confirmed by PCR using Promega's specific primers (SP6 and T7) directed to the cloning vector.

4.3. *PtSRP* 5' Fragment Sequencing and Obtention of Full-Length *PtSRP* mRNA

Cloned plasmids of recombinant bacteria (TG1) were extracted using Mini-prep. ABI PRISM BigDye™ Terminator V3.1 Cycle sequencing Ready Reaction kit (Applied Biosystems) was used for sequencing the 5' RACE fragment in an ABI PRISM 3100 Genetic Analyzer system (Applied Biosystems). Full-length mRNA was obtained by contig formation using SeqMan NGen V1.2 (DNASTAR Lasergene V8.0, Madison-US) after manual edition of sequences using ApE V1.15 (University of California-US) and Chromas Lite V2.01 (Technelysium Pty Ltd., Australia) software. This sequence will be referred in the text as 636 bp.

4.4. *PtSRP* Cloning and Sequencing

To confirm that the above contig sequence was correctly constructed, a primer pair (*PtSRP* FW: 5'-CCTCTCTCTCGAACACCTCCAC-3' and *PtSRP* REV: 5'-ACGTACAGCAGAATGCGAAAG-3'), directed to the flanking regions of the gene ORF were designed (by use of ApE V1.15) for the direct PCR amplification of the gene from cDNA samples. The amplicons were cloned using the CloneJET™ PCR Cloning Kit (Fermentas). Cloned plasmids were extracted from recombinant DH10B using QIAprep® spin Miniprep kit and sequenced on an ABI PRISM 3100 as described above. Twelve experimental sequences were aligned giving a 604 bp consensus gene sequence which was further aligned to the 636 bp sequence described above.

4.5. *PtSRP* mRNA Nucleotide Sequence Analysis and *PtSRP* Protein Prediction

The most probable ORF definition of the 636 bp was achieved using the "Find ORF" routine in the ApE V1.15, with identification of initial methionine and termination codons. Untranslated regions (UTRs) of possible ORFs were compared to the original Kozak sequence [17]. Additionally, online BLASTx searches [26] were carried out to detect significant similarity between the new ORF and those previously deposited in the NCBI Entrez Protein Database.

Structural analysis of the putative ORF was carried out using the Predict Protein Web-server (http://www.predictprotein.org) [27]. Functional databases were searched for conserved domains by use of the Inter Pro Web-server (http://www.ebi.ac.uk/interpro/) [28]. The subcellular location prediction of the PtSRP protein was obtained using the TargetP 1.1 Web-server [29] and [30] and potential cleavage site predicted using ChloroP 1.1 [31] and SignalP 3.0 servers [32]. TargetP (http://www.cbs.dtu.dk/services/TargetP/) incorporates prediction of cleavage sites and signal/non-signal peptide based on combination of artificial neural networks and hidden Markov models. The following cutoff levels were used: 0.780 for mitochondrial targeting peptide (mTP), 0.000 for secretory pathway signal peptide (SP) and 0.730 for the other features. Further, analyses were performed to study the hydrophobicity (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=toppred) and globular shape of the protein (http://www.predictprotein.org; GLOBE function used). Prediction of a three-dimensional protein model based on homology modelling was not performed due to lack of significant similarity crystallized template structures in RCSB Protein Databank (PDB) (http://www.rcsb.org/pdb) [33].

5. ACKNOWLEDGEMENTS

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