Two P$_{1B-1}$-ATPases of *Amanita strobiliformis* With Distinct Properties in Cu/Ag Transport

Vojtěch Beneš, Tereza Leonhardt, Jan Sácký and Pavel Kotrba*

Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague, Prague, Czechia

As we have shown previously, the Cu and Ag concentrations in the sporocarps of Ag-hyperaccumulating *Amanita strobiliformis* are correlated, and both metals share the same uptake system and are sequestered by the same metallothioneins intracellularly. To further improve our knowledge of the Cu and Ag handling in *A. strobiliformis* cells, we searched its transcriptome for the P$_{1B-1}$-ATPases, recognizing Cu$^+$ and Ag$^+$ for transport. We identified transcripts encoding 1097-amino acid (AA) AsCRD1 and 978-AA AsCCC2, which were further subjected to functional studies in metal sensitive *Saccharomyces cerevisiae*. The expression of AsCRD1 conferred highly increased Cu and Ag tolerance to metal sensitive yeasts in which the functional AsCRD1:GFP (green fluorescent protein) fusion localized exclusively to the tonoplast, indicating that the AsCRD1-mediated Cu and Ag tolerance was a result of vacuolar sequestration of the metals. Increased accumulation of AsCRD1 transcripts observed in *A. strobiliformis* mycelium upon the treatments with Cu and Ag (8.7- and 4.5-fold in the presence of 5 µM metal, respectively) supported the notion that AsCRD1 can be involved in protection of the *A. strobiliformis* cells against the toxicity of both metals. Neither Cu nor Ag affected the levels of AsCCC2 transcripts. Heterologous expression of AsCCC2 in mutant yeasts did not contribute to Cu tolerance, but complemented the mutant genotype of the *S. cerevisiae ccc2* strain. Consistent with the role of the yeast Ccc2 in the trafficking of Cu from cytoplasm to nascent proteins via post-Golgi, the GFP fluorescence in AsCCC2-expressing *ccc2* yeasts localized among Golgi-like punctate foci within the cells. The AsCRD1- and AsCCC2-associated phenotypes were lost in yeasts expressing mutant transporter variants in which a conserved phosphorylation/dephosphorylation site was altered. Altogether, the data support the roles of AsCRD1 and AsCCC2 as genuine P$_{1B-1}$-ATPases, and indicate their important functions in the removal of toxic excess of Cu and Ag from the cytoplasm and charging the endomembrane system with Cu, respectively.

**Keywords:** ectomycorrhizal fungi, P$_{1}$-type ATPase, copper transporter, silver transporter, metal homeostasis, *Amanita strobiliformis*

**INTRODUCTION**

Studies have revealed that ectomycorrhizal (EM) fungi effectively mobilize heavy metals from soils and minerals (Gadd et al., 2012) and that ectomycorrhizae improve plant fitness in metal polluted environments also because metal tolerant mycobionts function as a barrier for the entry of metals into plant tissues (Colpaert et al., 2011; Reddy et al., 2016). High concentrations of heavy metals and
metaloids accumulated in the sporocarps further support the notion that EM fungi substantially contribute to the environmental cycling of these elements, including Cu and Ag (Falandysz and Borovička, 2013). It is noteworthy that studies indicate that macrofungi could be considered the most effective Ag accumulators among eukaryotes with two known outstanding EM species, Amanita strobiliformis and Amanita solitaria (Borovička et al., 2007, 2010). The concentrations of Ag in their sporocarps collected from unpolluted sites range from 200 to 1200 mg kg⁻¹. We have documented that the intracellular detoxification of Cu and Ag in A. strobiliformis largely relies upon binding with cysteinyl-rich, cystolic metallothionein (MT) peptides, AsMT1a, 1b, and 1c (Osobová et al., 2011; Beneš et al., 2016; Hložková et al., 2016), and that two peptides, AsMT1a, 1b, and 1c (Osobová et al., 2011; Beneš et al., 2016; Hložková et al., 2016), and that two A. strobiliformis transporters of the copper transporter family (CTR; specifically AsCTR2 and AsCTR3) can recognize not only Cu, but also Ag (Falandysz and Borovička, 2013). It is noteworthy that several P1B-type ATPases have been shown to also contribute to the homeostasis and redistribution of essential metal ions (s). The transporters highly specific for monovalent Cu ions (the dominant intracellular Cu species in eukaryotes; Nevitt et al., 2012) comprise P1B−1 subgroup, while P1B−2, P1B−3, and P1B−4 subgroups are known for distinct preferences for their substrate heavy metal ions(s). The transporters highly specific for monovalent Cu ions (the dominant intracellular Cu species in eukaryotes; Nevitt et al., 2012) comprise P1B−1 subgroup, while P1B−2, P1B−3, and P1B−4 subgroups are known for distinct preferences for their substrate heavy metal ions(s). The transporters highly specific for monovalent Cu ions (the dominant intracellular Cu species in eukaryotes; Nevitt et al., 2012)

Studies in eukaryotes have revealed that while CTors transport Cu ions into the cytoplasm, the members of P1B−1 subgroup of P1B−type ATPases (also called heavy metal ATPases, HMA) contribute to the homeostasis and redistribution of essential Cu by exporting the metal ion from the cytoplasm into the subcellular compartments or out of the cell (Nevitt et al., 2012; Bashir et al., 2016). The homology of P1B-ATPases and their characteristic sequence features suggest a division into seven subgroups (Smith et al., 2014). While the roles of the members of the P1B−5 to P1B−7 subgroups (predicted so far only in prokaryotes) remain elusive, the transporters belonging to P1B−1, P1B−2, prokaryote P1B−3, and P1B−4 subgroups are known for their ability to excrete heavy metal ions.(Riggle and Kumamoto, 2000; Weissman et al., 2000). Recently, the P1B−1-ATPase CrpA that also localizes to the plasma membrane has been shown to confer substantial Cu- but not Ag-tolerance in filamentous fungus Aspergillus nidulans (Antsotegi-Uskola et al., 2017).

Since our previous studies revealed certain overlap in the cell biology of Ag and Cu in A. strobiliformis, we investigated whether or not this species may employ P1B−1-ATPases in the intracellular handling of both Cu and Ag. We searched its transcriptome for the homologs of P1B−1-ATPases and describe here the isolation and functional characterization of cDNA coding the Cu- and Ag-inducible AsCRD1 that can protect metal-sensitive yeasts against the toxicity of both metals. We also describe the second isolated P1B−1-ATPase of A. strobiliformis, the homolog of yeast Ccc2 named AsCcc2. To our knowledge, these are the first P1B−1-ATPases characterized in mycorrhizal fungi.

**MATERIALS AND METHODS**

**Amplification of AsCRD1 and AsCcc2 Genes and Sequence Analyses**

Partial sequences of AsCRD1 and AsCcc2 transcripts were obtained from tBLASTn analysis (Altschul et al., 1990) of the transcriptome of A. strobiliformis (Paulet ex Vittad.) isolate PRM 857486 (Hložková et al., 2016) by using characterized fungal P1B−1-type ATPases as queries. The entire coding sequence information was established by 5\’ and 3\’ RACE, using a SMARTer RACE cDNA Amplification Kit (Clontech Labs) with 1 µg of total RNA to produce the population of the first cDNA strand; the Q5 High-Fidelity DNA polymerase (New England Biolabs) was used to obtain double-stranded cDNAs. The total RNA was isolated by using an RNaseasy Plant Mini Kit and RNase free DNase set (Qiagen) from 50 mg of freeze-dried tissue of the A. strobiliformis PRM 857486 sporocarp. Transcript-specific primers were 5rCRD1_R1 to R5 for AsCRD1 5\’ RACE, and 5rCRD2_R1 to R3 or 3rCRD2R1 and R2 for AsCcc2 5\’ or 3\’ RACE, respectively (for primer sequences see Supplementary Table S1), and the amplicons were subjected to 3\’-A tailing with GoTaq DNA polymerase (Promega). Genomic fragments harboring AsCRD1 and AsCcc2 genes were amplified from 200 ng of chromosomal DNA template by PCR using Q5 DNA polymerase and pairs of gene-specific primers designed based on 5\’ and 3\’ untranslated regions of the corresponding cDNAs; the primers were CRD1_F/R for AsCRD1 and CRD2_F/R for AsCcc2 (Supplementary Table S1). The chromosomal DNA was isolated from 50 mg of freeze-dried tissue of A. strobiliformis PRM 857486 by using a NucleoSpin Plant II Kit (Macherey-Nagel). The amplicons were inserted into a pGEM-T vector (Promega) and then amplified in E. coli DH5α according to standard protocols. The recombinant DNAs were subjected to custom DNA sequencing on both strands with the vector-specific primers. The sequences of AsCRD1 and AsCcc2 cDNAs were deposited in GenBank under the accession numbers MF317930 and MF317931, respectively.

**Sequence Analyses**

The protein sequences deduced from the cDNAs were subjected to a transmembrane domain and signal peptide predictions.

**Genes and Sequence Analyses**

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in silico at the CCTOP web server (Dobson et al., 2015). The signal peptide prediction was also done by submitting the sequences to SignalP 4.1 server (Pettersen et al., 2004). The homology modeling of transporter 3D structure used the Phyre2 protein homology/analogy recognition engine (Kelley et al., 2015), the Modeller (Webb and Sali, 2014), and UCSF Chimera (Pettersen et al., 2004) programs. The closest AsCRD1 and AsCCC2 homologs among the RCSB Protein Data Bank (PDB) entries used for comparative modeling were 2EW9 (N-terminal domain of ATP7B, 23% and 40% identity, respectively) and 3J09 (P1B model) were 2EW9 (N-terminal domain of ATP7B, 23% and 40% identity, respectively) and 3J09 (P1B model). The signal peptide prediction was also done by submitting the sequences to SignalP 4.1 server (Pettersen et al., 2004). The signal peptide prediction was also done by submitting the sequences to SignalP 4.1 server (Pettersen et al., 2004).

Functional Complementation in Yeasts
The S. cerevisiae strains used in complementation assays were cup1Δ strain DTY113 (MATa trp1-1 leu2-3,112 gal1 ura3-50 cup1Δ61; Tamai et al., 1993) and the Euroscarf Y00569 (yap1Δ; YML007w:kanMX4) and Y03629 (cdc2Δ; YDR270w:kanMX4) mutants strains of BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). To constitutively express AsCRD1 and AsCCC2 in yeasts, the entire coding sequences produced by Q5 DNA polymerase from cDNA using primer pairs eifCRD1_F/R (AsCRD1) and eifCRD2_F/R (AsCCC2) were inserted into the HindIII-treated and EcoRI-treated yeast expression vector p416GPD (Mumberg et al., 1995), respectively, by using an In-Fusion HD Cloning Kit (Clontech Labs) according to manufacturer’s instructions. Site-directed mutagenesis of AsCRD1 and AsCCC2 in p416GPD was performed by the inverse PCR method (Füzik et al., 2014) with Phusion High-Fidelity DNA Polymerase (Thermo Scientific); the overlapping primers used were mCRD1_F plus mCRD1_R and mCRD2_F plus mCRD2_R, respectively. Primer sequences are listed in Supplementary Table S1. For complementation plate assays, the mid-log cultures of transformed S. cerevisiae were adjusted to an optical density at 590 nm (OD590) of 0.1, and 5 µl of serial dilutions were spotted on agar medium. The mycelium isolate from the PRM 857486 pileus (Osobová et al., 2011) was grown at 25°C and routinely maintained on potato dextrose (PD) agar containing 4 g l−1 potato extract (Sigma-Aldrich) and 10 g l−1 glucose (0.5× PD). The metal dose-dependent growth was observed with mycelia grown for 8 weeks on 0.5× PD agar with CuCl2 or AgNO3 supplements. The expression of target genes was assessed in the mycelium propagated in liquid PD medium (basal Cu, Ag and Cd concentrations below the atomic absorption spectrometry detection limit of 0.21, 0.04, and 0.09 µg ml−1, respectively) for 16 weeks and then subjected to metal (added as CuCl2, AgNO3, or CdCl2) exposures for 24 h. The gene expression analysis was performed on independent biological samples from three replicate experiments in two technical replicates. The RNA extraction from freeze-dried mycelia and quantitative reverse-transcribed PCR measurements including the quality/specificity controls were conducted essentially as described previously (Hložková et al., 2016). Briefly, the population of transcripts present in 1 µg of total RNA was reverse transcribed in a 20 µl reaction and 1.5 µl of the resulting cDNA product was used in a 12 µl quantitative PCR (qPCR) reaction for the measurements

Fluorescence Microscopy of AsCRD1:GFP and AsCCC2:GFP-Expressing Yeasts
To construct the translational AsCRD1:GFP and AsCCC2:GFP fusions, the coding sequences without the termination codons were amplified from cDNA by using primer pairs gifCRD1_F plus gifCRD1_R for AsCRD1 and gifCRD2_F plus gifCRD2_R for AsCCC2 (Supplementary Table S1). The amplicons were amplified into a BamHI-digested plasmid p416GFP. The plasmid p416GFP is a p416GPD derivative, harboring GFP from plasmid pEGFP-C1 (Clontech Labs) inserted as a BamHI/HindIII DNA fragment (Hložková et al., 2016). The cells of AsCRD1:GFP-expressing cup1Δ and AsCCC2:GFP-expressing ccc2Δ yeasts were obtained from mid-log cultures grown in SD medium supplemented with 0.5 µg ml−1 DAPI (Invitrogen) when needed. Vacuoles were labeled at 30°C for 4 h in SD medium with 400 µg ml−1 of the tonoplast-specific FM4-64 dye (Molecular Probes). The fluorescence microscopy was performed by using a BioSystems Imaging station Cell®R with a MT20 illumination and a DSU semi-confocal unit on a IX-81 microscope (Olympus BioSystems) equipped with the model C9100 EM-CCD camera (Hamamatsu Photonix). A GFP-deriving fluorescence was observed with the U-DM-DA-Tx2 FITC filter (excitation band: 495/15 nm, emission band: 530/30 nm; Olympus) and nuclei stained with DAPI were visualized with the U-DM-DA-Tx2 DAPI filter (excitation band: 400/15 nm, emission band: 460/20 nm). Vacuoles were observed with the U-DM-Cy5 filter (excitation band: 590–650 nm, emission band: 665–740 nm). The recorded black and white images were processed using the ImageJ software.

Gene Expression Analysis in A. strobiliformis
The mycelium isolate from the PRM 857486 pileus (Osobová et al., 2011) was grown at 25°C and routinely maintained on potato dextrose (PD) agar containing 4 g l−1 potato extract (Sigma-Aldrich) and 10 g l−1 glucose (0.5× PD). The metal dose-dependent growth was observed with mycelia grown for 8 weeks on 0.5× PD agar with CuCl2 or AgNO3 supplements. The expression of target genes was assessed in the mycelium propagated in liquid PD medium (basal Cu, Ag and Cd concentrations below the atomic absorption spectrometry detection limit of 0.21, 0.04, and 0.09 µM, respectively) for 16 weeks and then subjected to metal (added as CuCl2, AgNO3, or CdCl2) exposures for 24 h. The gene expression analysis was performed on independent biological samples from three replicate experiments in two technical replicates. The RNA extraction from freeze-dried mycelia and quantitative reverse-transcribed PCR measurements including the quality/specificity controls were conducted essentially as described previously (Hložková et al., 2016). Briefly, the population of transcripts present in 1 µg of total RNA was reverse transcribed in a 20 µl reaction and 1.5 µl of the resulting cDNA product was used in a 12 µl quantitative PCR (qPCR) reaction for the measurements

1http://web.uni-frankfurt.de/fb15/mikro/euroscarf/

2http://imagej.nih.gov/ij/
with 0.35 μM gene-specific primers (Supplementary Table S1). The measurements used a DyNAmo Flash SYBR Green 2-Step qPCR Kit (Life Technologies) and a MiniOpticon Real Time PCR System (Bio-Rad). The primers were qF- plus qR-CRD1 for AsCRD1, qF- plus qR-CRD2 for AsCRD1, and qPtub-b plus qRtub-b for β-tubulin AsTUB-b gene (GenBank: JX463743), which was used for normalization of the qPCR data as internal reference, stably expressed under Ag and Cu exposures (Hložková et al., 2016). A Bio-Rad CFX Manager was used to calculate the baseline range and the experiment threshold cycle (Ct) values recorded during the elongation period of the qPCR. The levels of gene transcription as relative to the controls (unexposed mycelium) were calculated by using the \( 2^{-\Delta\Delta Ct} \) method (Livak and Schmittgen, 2001), where \( \Delta Ct = Ct_1 - Ct_2 \times [\log(1+E)/\log2] \). The amplification efficiency values (E) were calculated using the equation \( E = [10^{(1/slope)}] - 1 \); the slopes were determined from the standard quantification curves obtained with serial dilutions of first strand cDNA templates. The obtained E values for AsCRD1, AsCCC2 and AsTUBb genes were 102%, 98%, and 108%, respectively.

RESULTS

Identification and Sequence Analysis of AsCRD1 and AsCCC2

To obtain information about the sequences coding for P\(_{1B-1}\)-ATPases in A. strobiliformis, the sporocarp transcriptome of A. strobiliformis was screened by using tBLASTn search with known P\(_{1B-1}\)-ATPases as queries. The screening retrieved two partial transcript sequences: one 822 nucleotides long in which a termination codon was included (a part of mRNA named AsCCC2) and another 528 nucleotides long without a termination codon (a part of mRNA named AsCRD2). As the deduced protein fragments showed a substantial identity with the C-terminal sequences of known P\(_{1B-1}\)-ATPases, the corresponding full-length coding sequences were established via the RACE method.

The predicted 1097-AA AsCRD1 and 978-AA AsCCC2 proteins showed the characteristic sequence features of P\(_{1B-1}\)-ATPases described in other organisms (Argüello et al., 2007; Smith et al., 2014). These involve putative N-terminal Cu/Ag-binding CxxC motifs (three in AsCRD1, two in AsCCC2) and two P\(_{1B-1}\) subgroup signature sequences in predicted transmembrane domains (TMD), Nx6YNx4P (x represents any AA residue), and Px6MxxSx3S, which are in P\(_{1B-1}\)-ATPases conserved in TMD7 and TMD8, respectively (Figure 1 and Supplementary Figure S1). Like other P\(_{1B}\)-type ATPases, AsCRD1 and AsCCC2 contained eight predicted TMDs with CPCx6P sequence in TMD6 and HP locus between TMD6 and TMD7. In addition, both predicted proteins possess features typical for all the members of the P-ATPase superfamily (Figure 1), particularly the DKTGTxT motif in the predicted large cytoplasmic loop with an aspartyl residue whose phosphorylation from ATP and dephosphorylation is prerequisite for active metal ion transport (Palmgren and Nissen, 2011). Despite the identified regions of conservancy at the protein level, the corresponding genes showed different structure and appeared dissimilar. The cDNA and genomic sequences of AsCRD1 and AsCCC2 were clearly distinct, with coding sequences interrupted with nine and three introns, respectively (Figure 1).

The comparison of the predicted AsCRD1 and AsCCC2 proteins revealed that along the sequence, they show lower identity and similarity with each other (25% and 38%, respectively) than they individually showed to P\(_{1B-1}\)-ATPases characterized from other species. Predicted AsCRD1 shares 38%, 36%, and 31% identity (54%, 50%, and 48% similarity) with A. nidulans CrpA, C. albicans CaCRD1, and cucumber (Cucumis sativus) CsHMA5.2, respectively, while AsCCC2 shows 35% identity and 51% similarity with both the S. cerevisiae Ccc2 and A. thaliana AtHMA5. As further indicated in the Neighbor-joining tree (Supplementary Figure S2), AsCRD1...
and AsCCC2 sort into two distinct clusters. The AsCRD1-containing cluster comprised the characterized CaCRD1 and a clade of predicted agaricomycete P1B S. cerevisiae AsCCC2 in the clade of predicted agaricomycete containing cluster comprised the characterized CaCRD1 and a clade of predicted agaricomycete S. cerevisiae AsCCC2 (Bouknight et al., 2010) and Colletotrichum lindemuthianum (Parisot et al., 2002), and human pathogen Cryptococcus neoformans (Walton et al., 2005).

**Functional Expression of AsCRD1 and AsCCC2 in S. cerevisiae**

The homology to known fungal P1B ATPases suggested that AsCRD1 and AsCCC2 are P1B ATPases, which could be involved in metal tolerance and delivery of Cu to metalloproteins, respectively. In order to gain information regarding the function of AsCRD1 and AsCCC2 in handling Cu and Ag, the corresponding mutant AsCRD1D742A and AsCCC2D555A variants were constructed, in which the codons for aspartyl 742 (in AsCRD1) and aspartyl 555 (in AsCCC2) were changed to encode alanyl residues.

The Cu tolerance assays were conducted in the cup1Δ strain carrying a deletion of its single-copy MT gene cup1, which renders the cells hypersensitive to Cu. Heterologous expression in yeasts grown on SD medium containing 50 or 100 µM Cu2+ revealed that only AsCRD1, but not AsCCC2, protected the yeasts from Cu toxicity (Figure 2A). The protective effect of AsCRD1 became weaker when the cells were subjected to 200 µM Cu2+ (Figure 2A). Considering that Ag+, particularly in respiratory conditions, acts as a potent inducer of oxidative stress (Mijnendonckx et al., 2013), and yeasts with defects in oxidative stress response proved useful in attributing Ag-detoxification functions to heterologous proteins (Sácky et al., 2011; Migocka et al., 2015), the yap1Δ strain, deficient in a transcription factor upregulating genes involved in oxidative stress response (Rodrigues-Pousada et al., 2010), was used in Ag toxicity assays. As documented in Figure 2B, the yap1Δ cells grown on non-fermentable YPEG medium and expressing AsCRD1 grew much better in the presence of 5–30 µM Ag+ than did the controls. The observation that the expression of AsCRD1D742A did not confer increased resistance against either Cu (Figure 2A) or Ag (Figure 2B) suggested that the Cu- and Ag-tolerance phenotypes associated in the model yeasts with wild-type AsCRD1 were indeed due to the metal-transport ability of the encoded protein.

The apparent lack of the Ag/Cu toxicity-related phenotype of AsCCC2 in cup1Δ and yap1Δ yeasts was congruent with the expected function of AsCCC2 as the transporter involved in handling of physiological Cu inside the cell. The properties of AsCCC2 were thus further tested in the ccc2Δ strain in which the absence of Ccc2 causes a severe growth defect on non-fermentable media because of the lack of sufficient mitochondrial iron (Fu et al., 1995; Yuan et al., 1997); note that high affinity iron uptake pathway in S. cerevisiae involves Fet1 permease that works together with Cu-dependent, plasma membrane ferroxidase Fet3 that receives its Cu ions (supplied by Ccc2) in Golgi. The growth tests on YPEG medium revealed that AsCCC2 was able to fully complement the respiratory deficiency of the ccc2Δ cells, whilst the control cells transformed with empty p416GPD and those expressing AsCCC2D555A (and AsCRD1; not shown) failed to grow under the same conditions (Figure 2C). The
controls, AsCRD1 (not shown), and AsCCC2<sup>DSSSA</sup> cells showed full growth on the YPEG medium supplemented with 1 mM Cu<sup>2+</sup>, respectively.

**Targeting of AsCRD1 and AsCCC2 in S. cerevisiae**

Distinct phenotypes associated with AsCRD1 and AsCCC2 in yeasts suggested that the corresponding proteins localized to different membranes. To obtain information about the cellular localization of AsCRD1 and AsCCC2 using direct fluorescence microscopy, the proteins were translationally fused with GFP at their C-termini, and the recombinant AsCRD1:GFP and AsCCC2:GFP genes were expressed in <i>cup1</i>Δ and <i>ccc2</i>Δ yeasts grown in SD medium. Complementation assays revealed that the phenotypes conferred by the fusions upon the yeasts were essentially the same as those observed with the corresponding transporters without GFP (Figure 2), thereby indicating that AsCRD1 and AsCCC2 tagged with GFP at their C-termini remained functional.

The microscopy of AsCRD1:GFP-expressing <i>cup1</i>Δ yeasts revealed strong GFP fluorescence co-localizing exclusively with the tonoplast stained with the vacuole-specific fluorophore FM4-64 (Figure 3A). The expression of AsCCC2:GFP in the <i>ccc2</i>Δ strain resulted in a strong, punctuated GFP signal in vesicular bodies within the cell (Figure 3B). The absence of GFP fluorescence from the perinuclear region attributable to ER may suggest that AsCCC2:GFP localized to Golgi rather than ER. The localization of GFP fluorescence in AsCRD1:GFP- and AsCCC2:GFP-transformed yeasts was not affected by the presence of subtoxic concentrations of Cu or Ag or the length of culture period (not shown).

**Metal Responsiveness of AsCRD1 and AsCCC2 in A. strobiliformis**

Considering the AsCRD1-associated, metal tolerance-related phenotypes in the model yeasts and the typically induced expression of metal tolerance genes during metal overload, the transcription rates of the studied P<sub>1B-1</sub>-ATPases of <i>A. strobiliformis</i> – both metals can enter the cells via AsCTR2 and AsCTR3 transporters (Ben et al., 2016) and intracellular Cu and Ag are sequestered in the cytoplasm through binding with AsMT1s (Hložková et al., 2016). It is worth noting that MTs have been considered principal in the sequestration of Cu or Ag in many EM fungi, including <i>Pisolithus albus</i> (Reddy et al., 2016), <i>Laccaria bicolor</i> (Reddy et al., 2014), <i>Hebeloma mesophaeum</i> (Sácký et al., 2014), <i>Hebeloma cylindrosporum</i> (Ramesh et al., 2009), <i>Amanita submembranacea</i> (Borovička et al., 2010), and <i>Paxillus involutus</i> (Bellion et al., 2007). The present study aimed to identify P<sub>1B-1</sub>-ATPases of <i>A. strobiliformis</i> and inspect their potential role in the handling of intracellular Cu and Ag in this species. Our search of the sporocarp transcriptome suggested the presence of several putative P<sub>1B-1</sub>-ATPases of which only two showed sequence features characteristic of the P<sub>1B-1</sub> subgroup.

Unlike for Zn or Cd, information about the deposition of the excess of the accumulated Cu in fungal vacuoles is scarce. In <i>Aspergillus niger</i> (Fomina et al., 2007) and in arbuscular mycorrhizal <i>Rhizophagus intraradices</i>...
particular of the P$_{1B}$-ATPases from $S$. cerevisiae mobilization of the vacuolar Cu back into the fungal cytoplasm. While the transporters of the CTR family responsible for the assembly are hypersensitive to Cu (Szczypka et al., 1997).

The predicted AsCCC2 and its homologs from Agaricomycetes appeared phylogenetically associated with the Ccc2 protein from the unicellular basidiomycete $C$. neoformans and to a lesser extent with Ccc2s from ascomycetes $B$. cinerea, $C$. lindemuthianum and $S$. cerevisiae. Congruent with this observation, AsCCC2 functionally complemented the CCC2 gene in $S$. cerevisiae ccc2Δ that is unable to charge its multicopper oxidase Fet3 with Cu in Golgi to establish the Fet3-Ftr1-based iron uptake system (Bleackley and MacGillivray, 2011). The lack of the AsCCC2-associated phenotype resulting from the D-to-A substitution in the DKTGTxT motif of the encoded protein (in the ccc2Δ cells expressing AsCCC2$^{D555A}$), and the GFP fluorescence localizing to the intracellular punctuate bodies resembling Golgi in yeasts expressing AsCCC2-GFP provides further support to the notion that AsCCC2 can mediate active transport of Cu into the Golgi. In $C$. neoformans, $B$. cinerea, and $C$. lindemuthianum, the corresponding functional CCC2 gene appeared critical for the biosynthesis of melanin; the lack of CCC2 in these species lead to a disruption in the delivery of Cu to extracellular multicopper oxidases.

FIGURE 4 | Growth of $A$. strobiliformis and expression of AsCRD1 and AsCCC2 genes in the presence of metal ions as indicated. (A) Mycelia grown on 0.5× PD medium with or without metal supplement for 8 weeks (long-term exposure). (B) Relative transcript levels measured by qRT-PCR in mycelium incubated in liquid 0.5× PD medium with or without (the controls) metal supplement (short term exposure). Expression of β-tubulin gene was used to calculate the relative expression and values plotted are an average of three biological replicates ± standard deviation of the mean (different letters above the bars indicate significant differences as determined by ANOVA followed by Tukey’s test, $p < 0.05$).
(laccases in particular) during their trafficking through Golgi (Parisot et al., 2002; Walton et al., 2005; Saitoh et al., 2010). Multiple copies of laccase genes have been predicted in both saprobic and EM species (Kohler et al., 2015); for example, the genomes of saprobic Amanita thiersii and EM Amanita muscaria contain 15 and 18 putative non-allelic laccase genes, respectively. Recent studies indicate that laccases expressed in EM fungi are, besides the pigmentation, involved in the sporocarp development or nutrient acquisition in extraradical mycelia (Courty et al., 2009; Kües and Rühl, 2011; Elstström et al., 2015; Shah et al., 2016). Considering this and the fact that Fet3-like ferroxidase genes have been found in most sequenced basidiomycetes, including Amanita species (Kües and Rühl, 2011; Kohler et al., 2015), it could be possible that A. strobiliformis benefits from AsCCC2 for both the Fe-uptake complex and laccase(s) assembly via Cu handling.

The results obtained in this study indicate that AsCRD1 and AsCCC2 belong to two separate protein clusters of the P1B-1-ATPase subgroup. The collected data strongly suggest that AsCRD1 is in A. strobiliformis, like AsMT1s and AsCTR1s, involved in the handling of both Ag and Cu, specifically in supporting the detoxification of Ag and Cu, which is, besides efficient transport, the prerequisite for (hyper)accumulation. Our data further indicate that AsCCC2, identified as another efficient transport, the prerequisite for (hyper)accumulation.

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AUTHOR CONTRIBUTIONS

VB conducted the experimental work and analyzed and interpreted data. TL and JS jointly contributed to the conception and design of the study, the bioinformatic analyses, and helped with the interpretation of data. PK was responsible for the conception and design of the work and the interpretation of the results, ensured the scientific issue was appropriately investigated, and commented on the manuscript. All of the authors assisted in writing the manuscript, discussed the results, and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2018.00747/full#supplementary-material

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