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Identification, evolution and functional characterization of two Zn CDF-family transporters of the ectomycorrhizal fungus Suillus luteus Peer-reviewed author version

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1	Identification,	evolution	and	functional	characterization	of	two	Zn	CDF-family
2	transporters of	the ectomy	corrl	nizal fungus	Suillus luteus				

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22 Summary

23 Two genes, SlZnT1 and SlZnT2, encoding Cation Diffusion Facilitator (CDF) family transporters were isolated from Suillus luteus mycelium by genome walking. Both gene 24 25 models are very similar and phylogenetic analysis indicates that they are most likely the result 26 of a recent gene duplication event. Comparative sequence analysis of the deduced proteins 27 predicts them to be Zn transporters. This function was confirmed by functional analysis in 28 yeast for SlZnT1. SlZnT1 was able to restore growth of the highly Zn sensitive yeast mutant 29 $\Delta zrc1$ and localized to the vacuolar membrane. Transformation of $\Delta zrc1$ yeast cells with 30 SlZnT1 resulted in an increased accumulation of Zn compared to empty vector transformed 31 $\Delta zrc1$ yeast cells and equals Zn accumulation in wild type yeast cells. We were not able to express functional SIZnT2 in yeast. In S. luteus, both SlZnT genes are constitutively expressed 32 33 whatever the external Zn concentrations. A labile Zn pool was detected in the vacuoles of S. luteus free-living mycelium. Therefore we conclude that SlZnT1 is indispensable for 34 35 maintenance of Zn homeostasis by transporting excess Zn into the vacuole.

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38 Keywords

39 Zinc transporter, *Suillus luteus*, Zinc detoxification, Zinc storage, Cation Diffusion Facilitator

40 Introduction

41 Zinc (Zn) is an essential micronutrient as it is involved as co-factor, structural or signalling 42 element in a wide range of cellular processes (Eide, 2009). Nevertheless, it becomes toxic 43 when present in excess. The cellular Zn concentration of healthy, well-functioning cells ranges from 0.1 - 0.5 mM. Most of the cellular Zn is bound to proteins and the labile/free 44 45 fraction is only in the nano to picomolar range (Eide, 2006; Simm et al., 2007). To assure 46 cellular homeostasis in situations of Zn limitation as well as Zn surplus, all organisms require 47 a system to fine-tune Zn availability in the cell. This system is well studied in yeast and 48 mammals (Sekler et al., 2007; North et al., 2012) and is mainly relying on transporters. In all 49 eukaryotic cells, ZIP (Zrt-, Irt-like proteins) and CDF (cation diffusion facilitator) families of 50 transporters account for most of the Zn transport across membranes. ZIP transporters mediate 51 Zn transport towards the cytoplasm. They are involved in Zn uptake from the extracellular 52 space (environment) and remobilization from organelles (Kambe et al., 2006). CDF 53 transporters remove Zn from the cytoplasm. Members of this family of transporters move Zn 54 to the extracellular space or into cellular compartments and therefore are involved in Zn 55 export and storage (Montanini et al., 2007). However, ZIP and CDF family transporters are 56 not restricted to the transport of Zn. Both families enclose Zn, Fe and Mn transporters and several of them are able to transport Cd in an unspecific way (Guerinot, 2000; Montanini et 57 58 al., 2007). Substrate specificity of CDF family transporters can be predicted by phylogenetic 59 analysis that classifies CDF family transporters into three major groups, of which the characterized members share the same metal specificity. Metal specificity of a newly 60 61 identified member can be inferred by its phylogenetic position in one of the three major groups (Montanini et al., 2007). Until now, metal specificity of ZIP transporters cannot be 62 63 predicted unambiguously from protein sequence only.

64 Mycorrhizal fungi are mutualists living in symbiosis with plant roots. They provide their host 65 plant with essential low-bioavailable nutrients as nitrogen and phosphorus in exchange for photosynthesis-derived sugar (Smith & Read, 2008). Besides, this mutualism results in other 66 67 benefits for the host plant including protection from heavy metal stress. Mitigation of toxic effects in plants by mycorrhizal fungi when grown in Zn-contaminated soils is well-68 69 documented (Adriaenssen et al., 2004; Ferrol et al., 2016). Nevertheless, molecular 70 mechanisms of cellular Zn homeostasis in mycorrhizal fungi are not well-characterized and 71 their impact on plant nutrient balances is poorly understood. Detoxification of excess Zn in 72 mycorrhizal fungi includes storage in subcellular compartments. The ectomycorrhizal (ECM) 73 fungus Suillus bovinus stores excess Zn in vacuoles (Ruytinx et al., 2013); Hebeloma 74 cylindrosporum, another ECM fungus in ER-derived vesicles (Blaudez & Chalot, 2011). In H. 75 cylindrosporum a CDF family transporter HcZnT1, localized at the ER-membrane, is most 76 likely involved in the transport of cytoplasmic Zn towards the ER. A similar transporter was 77 characterized in the ericoid mycorrhizal (ERM) fungus Oidiodendron maius (Khouja et al., 78 2013). RaCDF1 of Russula atropurpurea (ECM) clusters in phylogenetic analysis close to 79 HcZnT1 and OmZnT1, confers Zn tolerance to Zn sensitive yeast mutants but localizes on the 80 tonoplast and is likely involved in vacuolar Zn storage. A second transporter of the same 81 family, RaCDF2 was identified in this Zn-accumulating ectomycorrhizal fungus. RaCDF2 is 82 closely related to Mn transporting CDF's, localizes to the plasma membrane when 83 heterologous expressed in yeast, does not confer Mn tolerance to Mn sensitive yeast mutants 84 and likely acts as a bidirectional transporter of Zn, Cd and Co (Sacky et al., 2016). In 85 arbuscular mycorrhizal (AM) fungi GintZnT1 of Rhizophagus intraradices was identified and predicted to be a vacuolar Zn transporter of the CDF-family (Gonzalez-Guerrero et al., 2005). 86 87 Here we localize the labile Zn pool of S. luteus and report the functional characterization of 88 two CDF-family transporters. S. luteus is a cosmopolitan ectomycorrhizal fungus, associated 89 with pine trees. In particular, in primary successions of pines this species is abundant and involved in seedling establishment (Hayward et al., 2015). On severely metal-contaminated 90 91 sites, Zn-tolerant S. luteus populations evolved and protect their host tree effectively from Zn 92 toxicity (Adriaensen et al., 2004; Colpaert et al., 2011). The Suillus-Pinus association has a high potential for use in bio-stabilisation and restoration of metal-disturbed sites. However, 93 94 fundamental knowledge on the molecular mechanisms involved in metal homeostasis in the 95 plant and fungal partner is required to select most suited ecotypes and to fully exploit this 96 potential.

97 Results and discussion

98 Localization of labile Zn pool in S. luteus

99 All fungi store excess Zn in a specific organelle where it is no longer able to harm the cell and 100 from where it can be remobilised in case of deficiency. For most fungi the vacuole is the main 101 site for Zn storage (Gonzalez-Guerrero et al., 2008; Ott et al., 2002; Simm et al., 2007). On 102 the other hand, some fungi have special ER related vesicles (or zincosomes) for Zn storage (Clemens et al., 2002; Blaudez & Chalot 2011). Subcellular labeling of Zn in S. luteus 103 mycelium was performed with a fluorescent marker for free Zn^{2+} , FluoZin3 (Molecular 104 Probes, Invitrogen), which is able to detect free Zn^{2+} in the 1-100 nM range. A fluorescence 105 106 pattern, clearly indicating vacuoles, was observed (Fig. 1). Hyphae containing vacuoles with 107 labile Zn were distributed all over the mycelium (Fig. 1 a-c). External Zn concentration did 108 not change the observed fluorescence pattern, only intensity of the fluorescence changed. S. 109 luteus clearly stores Zn into the vacuole. No other accumulation pattern was detected despite 110 of different external Zn concentrations. Therefore vacuolar Zn storage is expected to be one of 111 the mechanisms to detoxify Zn and to maintain homeostasis in case of excess Zn in S. luteus. 112 Identification and evolutionary origin of two S. luteus transporters of the CDF family

113 CDF family transporters are often involved in Zn storage in vacuoles or ER related vesicles in 114 fungi. These transporters are key elements of the Zn homeostatic network of eukaryotes 115 (Montanini et al., 2007; Kambe et al., 2008; Gustin et al., 2011). By removing Zn from the 116 cytosol they are particularly important in the prevention from Zn toxicity (Gaither & Eide, 117 2001). Using a genome walking approach targeting vacuolar Zn transporters of the CDF 118 family we picked up two S. luteus gene fragments. Further analysis by genome walking and 119 RACE protocols revealed that those fragments belong to the genes encoding proteins with 120 protein ID 807028 and 814105 in the JGI S. luteus genome database. The genes were named 121 SlZnT1 and SlZnT2 and both gene models are very alike (Supplemental figure S1). They 122 consist of 9 exons interspersed with introns of +/- 50 nucleotides. In silico translations of full 123 length cDNA's (1623 and 1516 bp) identified open reading frames of 1320 and 1362 bp 124 encoding a 440 and 453 amino acid-long polypeptide respectively (Fig. 2). A high percentage 125 of sequence identity (85%) between both predicted proteins is observed. The predicted 126 proteins show sequence and structural features typical of CDF family transporters.

127 CDF transporters are characterized by six transmembrane domains and a histidine rich motif 128 $(HX)_n$ in the cytosolic loop between transmembrane helices IV and V. For most proteins of 129 this transporter family the histidine rich motif is located directly after helix IV and contains 130 three to six HX repeats (Gaither & Eide, 2001). The topology prediction program TMHMM 131 predicted 6 transmembrane domains for both deduced S. luteus proteins. The deduced proteins 132 are very similar but show a considerable level of sequence diversification in the cytosolic loop 133 between helix IV and V (Fig. 2). SlZnT1 is with its predicted six transmembrane domains and 134 (HX)₃ domain a typical CDF family member. SIZnT2 is somewhat aberrant since the normal 135 $(HX)_n$ motif shows seven repeats and an extra, second $(HX)_n$ motif (n=5) is present just before 136 helix V. The exact function of the $(HX)_n$ motif is unclear but it is expected to have a role in 137 metal recruitment (Gaither & Eide, 2001). In plants the histidine-rich loop is hypothesized to

138 play a role as a Zn chaperone to determine the identity of the transported ions (Podar et al., 139 2012). The atypical sequence of SIZnT2 with the presence of an additional $(HX)_n$ motif might 140 therefore have some implications for metal selectivity and specificity. However, Lin et al. 141 (2009) showed that metal specificity is determined by a cooperation between transmembrane 142 domain II and V. Several single amino acid substitutions within transmembrane helices II and 143 V of the S. *cerevisiae* vacuolar Zn transporter ZRC1 resulted in an Fe and Mn transporting 144 protein. Some of these proteinscreated by site directed mutagenesis retained the ability to 145 transport Zn, others not (Lin et al., 2008; Lin et al., 2009). In particular the amino acid 146 located four residues before the highly conserved aspartate (D) in transmembrane domain II 147 and V is very important in metal selectivity (Montanini et al., 2007). Both identified S. luteus 148 transporters have a HXXXD motif in transmembrane helices II and V, a feature specific for 149 the group of Zn transporting CDFs (Fig. 2).

150 Comparisons with the NCBI nr protein sequences (BLASTx) or fungal protein models at jgi 151 MycoCosm resulted in the same hits for SlZnT1 and SlZnT2. However, ranking of the hits is 152 different. Previously characterized CDF family transporters with the highest sequence identity 153 are the RaCDF1 protein of Russula atropurpurea (53%) and the GintZnT1 protein of 154 Rhizophagus intraradices (44%) for SIZnT1 and SIZnT2, respectively. All hits are protein 155 models corresponding to CDF family transporters. Remarkable is that only species from the 156 suborder Suillineae and Coniophora puteana occur twice in the list of BLASTx hits. To 157 elucidate the origin and relationship of SIZnT1 and SIZnT2 a neighbour-joining (NJ) tree was built using previously characterized fungal CDF family transporters and BLASTx hits. In this 158 159 tree (Fig. 3) both SIZnT1 and SIZnT2 cluster to the Zrc1/Cot1-like Zn-CDFs (Montanini et al. 160 2007). Within the cluster of Zrc1/Cot1-like CDFs, SlZnT1 clusters with the majority of the 161 BLASTx hits while SIZnT2 divaricates earlier and groups in a cluster that only contains 162 sequences of species that had two BLASTx hits. Reconciliation of the tree with an ITS

163 phylogeny of the considered taxa supports a gene duplication in the common ancestor of 164 Suillineae and the Coniophora/Serpula clade (supplemental figure S2). Gene expansion and 165 loss are common events in fungal genome evolution and may result in phenotypic alterations 166 (Floudas et al., 2012, Kohler et al., 2015). Interestingly, S. luteus and some other species 167 within the Suillineae clade are known to evolve Zn-tolerant phenotypes on severely metal-168 contaminated soils (Colpaert et al., 2004). Members of the SIZnT2 cluster could therefore be 169 candidate genes to study in adaptive Zn tolerance of Suilloid fungi. Five additional S. luteus 170 genes predicted to encode CDF transporters were identified and cluster within different 171 clusters of the phylogenetic tree (Fig.3 and Supplemental figure S3).

172 Functional characterization of *SlZnT1* in yeast

173 SlZnT1 was expressed in yeast to confirm the functionality predicted by comparative 174 sequence analysis. Heterologous expression in the eukaryotic model system S. cerevisiae is a 175 common strategy to get insight into gene function (Osborn & Miller, 2007; Mokdad-Gargouri 176 et al., 2012). By comparative gene analysis, SlZnT1 is predicted to encode a vacuolar Zn 177 transporter. SlZnT1 gene product was able to partly restore growth of $\Delta zrc1$, a yeast mutant 178 defective in vacuolar Zn storage and highly sensitive for Zn, on Zn enriched medium (Fig. 4). 179 The highly sensitive phenotype of $\Delta \cot 1$ (defective vacuolar CDF transporter) on cobalt (Co) 180 containing medium could not be restored by the identified S. luteus gene product. Also, the 181 defective vacuolar ATP (adenosine triphosphate) binding cassette of $\Delta ycf1$ (Cd sensitive) 182 yeast and the defective golgi P-type ATPase of $\Delta pmr1$ (Mn sensitive) yeast could not be 183 complemented by SIZnT1 (Supplementary figure S4).

To better understand the role of SlZnT1 in Zn homeostasis the cellular metal content of wild type yeast (+ empty vector (EV)), $\Delta zrc1$ (+EV) and $\Delta zrc1$ carrying SlZnT1 was determined after exposure to Zn. All yeast cultures showed an increased Zn content after growing in Zn enriched medium (Supplemental fig. S6). Wild type yeast and $\Delta zrc1$ yeast containing SlZnT1 188 accumulated a comparable amount of Zn. This amount is significantly higher than the amount 189 measured in $\Delta zrc1$. No significant differences in Fe and Mn content were observed among the 190 yeast mutants when exposed to Zn (Supplementary figure S7), indicating the Zn specificity of 191 the transporter. Translational fusion of SIZnT1 to GFP confirmed its vacuolar localization in 192 yeast (Fig. 5). Yeast cells containing the SIZnT1::EGFP fusion construct showed a bright 193 green GFP fluorescent ring at the level of the vacuolar membrane (Fig. 5a-d). Clear colocalisation of the GFP fluorescence with the red fluorescence of the tonoplast specific 194 195 staining FM4-64 was observed for the SIZnT1::EGFP fusion construct.

All together our observations support a ZRC1-like function for SIZnT1. ZRC1 is involved in vacuolar Zn storage and largely determines yeast's ability to detoxify excess Zn (Kamizono et al., 1989). Most likely, SIZnT1 has a role in cellular Zn homeostasis in *S. luteus* by transporting excess Zn towards the vacuolar stock.

200 Functional characterization of *SlZnT2* in yeast

201 Although *SlZnT1* and *SlZnT2* are very similar, we were not able to express functional *SlZnT2* 202 in S. cerevisiae. None of metal sensitive phenotypes of the tested yeast mutants defective in 203 metal transport could be complemented by expression of SlZnT2 (Fig. 4 and supplemental 204 figure S4). Though, the gene is clearly expressed since SlZnT2 transcript could be detected in 205 the transformed yeast cells by PCR (Supplementary figure S5). Translational fusion to GFP 206 resulted in accumulation of GFP inside the vacuole (Fig. 5). Fusion of the EGFP protein to 207 SIZnT2 resulted in a green fluorescent vacuolar content when expressed in yeast for both N-208 terminal and C-terminal fusion. Figure 5 (e-g) shows that EGFP fluorescence is nicely 209 surrounded by FM4-64 fluorescence. Zn content of $\Delta zrc1$ containing SlZnT2 was similar to 210 that of $\Delta zrc1$ containing the empty vector and is significantly lower than in WT yeast cells 211 exposed to the same external Zn concentration (Supplemental fig. S6).

212 Heterologous expression is a powerful way to study gene function but has some limitations 213 because of differences in e.g. codon usage, posttranscriptional regulation, posttranslational 214 modifications and protein targeting signals (Yin et al., 2007; Mattanovich et al., 2012). These 215 differences might be at the basis of the non-functioning of SIZnT2 in yeast. The functional 216 characterization of the R. intraradices CDF transporter GintZnT1 in yeast resulted in similar 217 problems. Although, GintZnT1 could be detected by western blotting in transformed cells, it 218 was not able to complement any metal sensitive yeast mutants. This transporter could not be 219 affiliated to a specific membrane; the expressed protein seemed to accumulate all over the 220 cytoplasm (Gonzalez-Guerrero et al., 2005). However, the exact reason of non-functioning is 221 probably different for both proteins since they accumulate in different cellular compartments 222 in yeast. Regulation of posttranslational modifications and protein targeting are only little 223 explored in mycorrhizal fungi and deserve further attention.

224 SlZnT1 and SlZnT2 gene expression in S. luteus

225 Gene expression levels of SlZnT1 and SlZnT2 were determined in S. luteus after 48h exposure 226 to different concentrations of Zn, including concentrations inducing cellular Zn deficiency 227 and toxicity. Figure 6a and 6b show that both SlZnT1 and SlZnT2 expression were 228 constitutive. Neither exposure to excess Zn, nor limiting Zn changed the expression level of 229 the transporters when compared to the control condition ($20\mu M Zn$). On average SlZnT1 and 230 SlZnT2 expression level differ by at least a factor five, with SlZnT2 showing the lowest 231 transcript abundance (Fig. 6a and 6b). Insensitivity of gene expression for high external Zn 232 concentrations was demonstrated previously for the Zn CDF transporter ZRC1 of S. cerevisiae 233 (MacDiarmid et al., 2003) and HcZnT1 of Hebeloma cylindrosporum (Blaudez & Chalot, 234 2011). However, in *R. intraradices* gene expression of the *SlZnT* homologous gene *GintZnT1* 235 is transiently induced by elevated external Zn concentrations (Gonzalez-Guerrero et al., 236 2005). S. cerevisiae cells show a proactive strategy of homeostatic regulation of free cellular Zn content by an induction of *ZRC1* gene expression in Zn limited cells (MacDiarmid *et al.*,
2003). Being proactive guarantees a rapid resistance in case of repletion. In *S. luteus* no
change in SlZnT gene expression level was detected after growth without Zn for 48h (Fig. 6).
This might imply that both SlZnT's are not regulated proactive neither reactive by external Zn
concentration at the transcriptional level.

242 Conclusion

243 SlZnT1 probably has a key role in vacuolar Zn storage in S. luteus considering the results 244 obtained by heterologous expression in yeast. Based on the phylogenetic analysis it is likely 245 that SlZnT2 is involved in vacuolar Zn storage as well. However, redundancy caused by gene 246 duplication might lead to diversification and neo-functionalization (Assis & Bachtrog, 2013). 247 Subcellular targeting of SIZnT2 is unclear and vacuolar localisation was not confirmed. This 248 protein might have evolved to transport Zn out of the cell by localisation to the plasma 249 membrane or ER. Since we could never observe a zincosomes related accumulation pattern in 250 S. luteus, a role for SlZnT2 in Zn detoxification by storage or secretion via zincosomes is 251 rather unlikely unless Zn is tightly bound to a chelator, preventing its detection by the 252 fluorescent marker. Also, Zn specificity of SIZnT2 was not confirmed and comparative 253 sequence analyses are not always conclusive. RaCDF2 of Russula atropurpurea is a Zn 254 exporting plasma membrane transporter nested within a cluster of Mn transporters and 255 without the Zn-specific HXXXD motif in transmembrane helices II and V (Sacky et al., 256 2016). CDFs are conserved proteins and this kind of changes in metal specificity seem rather 257 exceptional since all other previously characterized CDFs of Bacteria, Plants and Animals 258 cluster in phylogenetic trees according to the metal they are transporting (Montanini et al., 259 2007; Cubillas et al., 2013). A role of SlZnT2 in Zn transport and homeostasis of S. luteus is 260 likely. Other S. luteus genes are predicted to function in Fe and Mn transport. Five additional CDF-encoding genes were identified in the S. luteus genome (Fig. 3 and Supplemental figure 261

S3). These genes need to be further characterized to confirm their putative role andunderstand their contribution in Zn, Fe and Mn homeostasis of *S. luteus*.

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268 Competing interest

269 The authors declare that they have no competing interest

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377 Supplementary Table 1. Degenerative primers used in the genome walking protocol.

_	name	sequence
	DG1aS	GTNGCNGAYAGYTTYCAYATGCT
	DG1bS	ATYGCNGAYTCATTYCAYATG
	DG2aS	CAMCGWGCRGARATTCTNGCNGC
	DG2bS	AAMGNGCRGARATTTTRGGTGCT
	DG3aS	CTTGCSCTNTGYNTCTCNAT
	DG3bS	ATTGCCYTNTGYNTSTYNATT
	DG4aAS	AGNASASCAYGCATRTTCAT
	DG4bAS	AASACACCATGCATATATTYAA
	DG5aAS	CCNACRTTNCCNAGRGCRTC
	DG5bAS	RCCRATRTTRCCNAGAGCATC
	DG6aAS	TCNAARTARTAYTTCCARCTCCA
	DG6bAS	RTARTANYKCCAAGAATAKTCRGT

name	sequence	target	protocol
ZnT1L	GGCATCACGAAACCATGACTTCAT	ZnT1	walking/RACE/gene expression
ZnT1R	GGTACTCGGGTTGAATAGTACTAGAATGT	ZnT1	walking/RACE/gene expression
ZnT1a	TCGGAGGAGTCTTATGGTCG	ZnT1	walking/RACE
ZnT1b	TGCGGGGGCGTATAATGAGA	ZnT1	walking
ZnT1c	CCTGGAGTAAAGAAGCGCTCT	ZnT1	walking
ZnT1d	CCTGCAATGAGCTCAATGAAGAAGAAGAA	ZnT1	walking
ZnT1Ra	ACATTCTAGTACTATTCAACCCGAGTACC	ZnT1	walking/RACE
ZnT2L	CGACGGTAAGGTGGAAATAAAC	ZnT2	walking/RACE/gene expression
ZnT2R	TGGTGAGCCAGATGACAAGA	ZnT2	walking/RACE/gene expression
ZnT2a	CCATGCGAATGAGAGTGACCA	ZnT2	walking
ZnT2b	CCAGCCGTAGGAGTAACGA	ZnT2	walking
ZnT2c	GATGCGAGCTGAACGAGATAA	ZnT2	walking/RACE
ZnT2d	CGTCATTACATCTTCCAGAACATTCCAT	ZnT2	walking

378 Supplementary Table 2. Gene specific primers used in the different protocols.

Figure 1. Labile Zn pool, marked with FluoZin 3, in *S. luteus*. (a-c) Overview of
peripheral hyphae of the mycelium; (d-f) detailed view of hyphae showing vacuoles. (a,d)
Differential interference contrast image, (b,e) green fluorescence of labile Zn bound to
FluoZin3, (c,f) merged image showing fluorescence in vacuoles. Scale bars: (a-c) 10 μm; (d-f)
5 μm.

Figure 2. Alignment of SIZnT1 and SIZnT2 encoded proteins. Residues are Rasmol coloured. The six transmembrane domains predicted by topology prediction program TMHMM are indicated by arrows; the histidine rich motifs (HX)_n between transmembrane helix IV and V are indicated by braces. The Zn specific HXXXD domains are framed by a dotted box.

Figure 3. Neighbour-joining (NJ) tree of the Cation Diffusion Facilitator (CDF) family proteins from selected fungi. Sequences were aligned by the MAFFT algorithm. Bootstrap values (1000 replicates) are indicated and branch lengths are proportional to phylogenetic distances. Localization and substrate (metal) are indicated for functionally characterized proteins. Mn and Fe clusters are collapsed. *S. luteus* sequences are framed, SlZnT1 and SlZnT2 are indicated by an arrow. V = vacuole; ER = endoplasmic reticulum, G = Golgi apparatus.

Figure 4. Functional complementation of the Zn sensitive yeast mutant Δ **zrc1.** Cultures of wild type and mutant yeast were tenfold serial diluted and spotted on control and Znsupplemented SD medium. The wild type strain was transformed with the empty vector, the mutant strain with either the empty vector or the vector containing *SlZnT1* or *SlZnT2*. The experiment was carried out twice for three independent clones and pictures were taken after 4 days of growth.

Figure 5. Localisation of SlZnT1:EGFP (a-d) and SlZnT2:EGFP (e-h) fusion proteins in
yeast. (a,e) bright field image, (b,f) EGFP fusion protein, (c,g) FM4-64 vacuolar membrane

staining, (d, h) merged images. SIZnT1:GFP and FM4-64 tonoplast staining co-localize and
SIZnT2:GFP is detected inside the vacuole.

407 Figure 6. Relative gene expression level of (a) SIZnT1 and (b) SIZnT2 in *S. luteus*408 mycelium after 48h exposure to different concentrations of Zn as measured by qPCR.
409 Data are the average +/- SE of seven biological replicates. Both genes were constitutively
410 expressed, no significant differences as compared to the control were detected.

Supplemental Figure S1. Gene model for the two newly identified *S. luteus* transporters,
(a) *SlZnT1* and (b) *SlZnT2*. Untranslated regions (UTRs) are coloured green, exons red and
introns are represented by a line. Length (amount of nucleic acids) of each individual part is
indicated above (UTR and exon) or beneath (intron) the corresponding region.

Supplemental Figure S2. Evolution of fungal CDF transporters of the zrc1/cot1 cluster.
Reconciled tree of the zrc1/cot1 cluster of CDF transporters using a maximum likelihood ITS
phylogeny of selected fungal species supporting five independent gene duplication events
(indicated in red). Seven gene loss events were predicted (represented in grey). *SlZnT1* and *SlZnT2* originate from a duplication event (indicated by an arrow) in the common ancestor of
Suillineae and Coniophora/Serpula clade.

Supplemental Figure S3. Neighbour-joining (NJ) tree of the Cation Diffusion Facilitator
(CDF) family proteins from selected fungi. Sequences were aligned by the MAFFT
algorithm. Bootstrap values (1000 replicates) are indicated and branch lengths are
proportional to phylogenetic distances. Localization and substrate (metal) are indicated for
functionally characterized proteins. *S. luteus* sequences are framed. Zn clusters are collapsed.
V = vacuole; ER = endoplasmic reticulum, G = Golgi apparatus.

427 Supplemental Figure S4. Heterologous expression of *SlZnT1* and *SlZnT2* in yeast 428 mutants. Cultures of wild type and mutant yeast were tenfold serial diluted and spotted on 429 control and metal-supplemented SD medium. The wild type strain was transformed with the 430 empty vector, the mutant strains with either the empty vector or the vector containing *SlZnT1* 431 or *SlZnT2*. (a) A Co sensitive mutant Δ cot1, (b) a Cd sensitive mutant Δ ycf and (c) a Mn 432 sensitive strain Δ pmr1 were used. The experiment was carried out twice for three independent 433 clones and pictures were taken after 4 days of growth.

434 Supplemental Figure S5. PCR-product separated on gel-red stained 0.8% agarose gel. 435 (a) PCR using *SlZnT2* targeting primers was run on cDNA samples of transformed yeast cells 436 and plasmid DNA (positive control). SlZnT2 transcript was detected in Δ zrc1 yeast cells 437 transformed with SlZnT2 containing plasmid but not in cells transformed with the empty 438 vector (EV). (b) A PCR using primers targeting the plasmid was performed to control for 439 plasmid contamination of RNA samples. A PCR-product was detected for the plasmid DNA 440 sample only. The PCRs were carried out for three independent clones.

441 Supplemental Figure S6. Zn concentration in transformed yeast cells in control 442 conditions or after exposure to Zn. The wild type strain was transformed with the empty 443 vector, the mutant strain with either the empty vector or the vector containing *SlZnT1* or 444 *SlZnT2*. Data are the average +/- SE of three biological replicates, significant differences (p < 445 0.01; two-way ANOVA followed by Student-Newman-Keuls) are indicated by different 446 letters.

Supplemental Figure S7. Fe and Mn concentration in transformed yeast cells in control conditions or after exposure to Zn. The wild type strain was transformed with the empty vector, the mutant strain with either the empty vector or the vector containing *SlZnT1* or *SlZnT2*. Data are the average +/- SE of three biological replicates, significant differences (p < 0.01; two-way ANOVA followed by Student-Newman-Keuls) are indicated by different letters.

1 **Experimental procedures**

2 Fungal material and growth conditions

3 The isolate UH-Slu-P4 from a Suillus luteus basidiocarp collected in a pine plantation in Paal, 4 Belgium was used. Mycelium was cultured for one week on cellophane-covered solid Fries 5 medium (28 mM glucose, 5.4 mM ammonium tartrate, 1.5 mM KH₂PO₄, 0.4 mM MgSO₄·7H₂O, 6 5 µM CuSO₄·5H₂O, 20 µM ZnSO₄·7H₂O, 0.1 µM biotin, 0.5 µM pyridoxine, 0.3 µM riboflavin, 7 0.8 µM nicotinamide, 0.7 µM p-aminobenzoic acid, 0.3 µM thiamine, 0.2 µM Ca-pantothenate 8 and 0.8% agar; pH-adjusted to 4.8) as described by Colparet et al. (2004). Fungal colonies were 9 used immediately in DNA and RNA extraction protocols or to prepare liquid cultures according 10 to Ruytinx et al. (2016). After one week, 1 g of spherical mycelia grown in liquid culture was 11 transferred to a petri dish containing 30 ml modified liquid Fries medium with a concentration of 12 0, 20, 200 or 1000 µM Zn and incubated shaking for 48h at 23°C. Zinc exposure was performed 13 in triplicate. Spherical mycelia (200 mg) were stored at -70°C for gene expression analyses or 14 used directly for staining of labile Zn pool.

15 Localization of labile Zn pool in *S. luteus*

16 Spherical mycelia obtained from liquid cultures exposed to different concentrations of Zn (20, 17 200, 1000 μ M) were mixed in fresh medium of the same composition and grown for two 18 additional days. Five mg FW mycelium was transferred to a 2 ml eppendorf tube with 1.5 ml 19 TBS (Tris Buffered Saline: 137 mM NaCl, 3 mM KCl, 25 mM Tris; pH 7) containing 5µM 20 FluoZin3. Following an incubation of 30 min (shaking), mycelia were washed twice in TBS for 5 21 min. FluoZin3 fluorescence was visualized with a Zeiss LSM 510 META laser scanning confocal 22 microscope, using a Zeiss 40x NA1.3 oil immersion objective. The 488 nm excitation line of the 23 laser and a BP 500-550 nm emission filter were used. Image processing was carried out with 24 ImageJ (NIH, Bethesda, MD, USA) software.

25 DNA extraction and genome walking

Fungal material (100 mg fresh weight) was thoroughly ground in liquid nitrogen using a mortar 26 27 and pestle. DNA was extracted from the grounded tissue using the DNeasy Plant mini kit 28 (Qiagen). Concentration of the DNA was determined on a NanoDrop ND-1000 29 spectrophotometer and agarose gel analysis was used to control integrity. High quality DNA was 30 used in a genome walking protocol (Genome Walker kit, Clontech). Briefly, DNA was digested 31 by blunt end restriction enzymes, fragments were adaptor ligated and PCR was performed using 32 an adaptor primer and a gene specific primer. Degenerative primers (Supplementary table 1) were 33 designed based on 6 conservative domains of functionally characterized fungal Zn-CDF 34 transporters. Fifty µl reactions containing 10x Advantage 2 PCR Buffer, 0.2 mM dNTP mix, 0.2 35 µM adaptor and gene specific primer, 50x Advantage 2 Polymerase Mix (Clonetech) and 1µl 36 fungal DNA were performed using touchdown cycling conditions (7 cycles of 25s at 94°C, 3 min 37 at 70°C; 32 cycles of 25s at 94°C, 3 min at 67°C and 1 cycle of 7 min at 67°C). Amplicons were 38 visualised on a 1.5% agarose/gelred (Molecular Probes) gel, excised and resolved using the 39 QIAquick Gel Extraction kit (Qiagen). Finally, PCR products were cloned into the pCR4-TOPO 40 vector (Invitrogen) and sequenced. Sequences were assembled using the Staden Package v1.6.0. 41 (www.Staden.SourceForge.net). New gene specific primers (Supplementary table 2) were 42 designed and the protocol was repeated until the whole gene sequence was obtained.

43 RNA isolation, cDNA synthesis and rapid amplification of cDNA ends

Total RNA was extracted from in liquid nitrogen ground fungal colonies (200 mg) using the RNeasy Plant Mini Kit (Qiagen). RNA quality was assessed using the Agilent-2100 Bioanalyser and RNA 6000 NanoChips (Agilent Technologies). $Poly(A)^+$ RNA was isolated from 250 µg samples of total RNA using Oligotex columns (Qiagen). One µg poly(A) ⁺ RNA was converted into double stranded cDNA and adaptor ligated using the Marathon cDNA Amplification Kit

49 (Clontech) following the manufacturer's instructions. cDNA was diluted 50x in Tricine-EDTA 50 buffer. RACE PCR was performed in 50 µl reactions containing 10x Advantage 2 PCR Buffer, 51 0.2 mM dNTP mix, 0.2 µM adaptor and gene specific primer (Supplementary table 2), 50x 52 Advantage 2 Polymerase Mix (Clontech) and 5 µl diluted cDNA. PCR-products were visualised 53 on a 1.5% agarose/gelred (Molecular Probes) gel and excised from the gel. After isolation and 54 clean up of the PCR-products (QIAquick Gel Extraction kit; Qiagen), they were cloned into the 55 pCR4-TOPO vector (Invitrogen) and sequenced. Sequences were assembled and aligned to the 56 gDNA sequences to identify gene structure. In silico translations were performed and 57 transmembrane domains predicted by TMHMM. The identified protein sequences were called 58 ZnT1 and ZnT2. All bio-informatic analyses were performed using CLC Main workbench 6.7 59 and plug-ins unless stated otherwise.

60 **Phylogenetic tree construction and reconciliation**

61 BLASTx against NCBI nr protein sequences and JGI Agaricomycotina gene catalog proteins 62 (using MycoCosm; Grigoriev et al., 2012) was performed using the identified proteins and the 63 CDF-family domain as a query. CDF-family proteins of S. luteus and selected Ascomycota and 64 Basidiomycota species were inventoried using the following criteria: protein length between 350-65 700 amino acids, minimum 5 predicted transmembrane domains, presence of a CDF conserved 66 domain or signature sequence (Montanini et al., 2007). The inventoried protein sequences were 67 aligned together with previously functionally characterized fungal CDF transporters. This 68 alignment was used for phylogenetic tree construction using the neighbour-joining (NJ) method. 69 Bootstrap tests were conducted using 1000 replicates and branch lengths are proportional to 70 phylogenetic distance. A species tree was build using ITS-sequences retrieved from the UNITE 71 database. Phylogenetic trees were built in MEGA v6.06; NOTUNG v2.8.1.7 was used to 72 reconcile both phylogenies.

73 Cloning

74 One µg total RNA was used in a Quantiscript Reverse Transcription reaction (Qiagen), which 75 includes a genomic DNA elimination step and makes use of random hexamer priming. Specific 76 designed to amplify full-length coding sequences of SIZnT1 (left: primers were 77 attcactcaacactcagcactcg; right: aacgcctgagacgggcgga) and SIZnT2 (left: gtgccaaccacaatggcat; 78 right: tagtatcacagtggtcgg). PCR reactions were performed in a total volume of 25 µl, containing 79 10x High Fidelity PCR buffer, 0.2 mM dNTP-mixture, 2 mM MgSO4, 0.2 µM specific forward 80 and reverse primer, 1 µl cDNA and 0.5 U Platinum Taq High Fidelity DNA polymerase 81 (Invitrogen) using general cycling conditions (2 min at 95°C, 35 cycles of 30s at 95°C, 30s at 82 60°C, 1 min at 68°C, 1 cycle of 2 min at 68°C). Amplicons were purified using QIAquick PCR 83 purification Kit (Qiagen) according to manufactures instructions. Purified PCR-products were 84 cloned into the gateway entry vector pCR8/GW/TOPO (Invitrogen) and subsequentially 85 transferred by LR-clonase (Invitrogen) to pYES-DEST52 (Invitrogen), pAG306GAL-ccdB-86 EGFP or pAG306GAL-EGFP-ccdB (Alberti et al., 2007) for complementation, Zn content 87 analysis and localisation by GFP fluorescence in yeast. Finally, the insert was sequenced in both 88 directions to assure correct fusion.

89 Yeast mutant complementation

90 The yeast strains used for heterologous expression of SlZnT1 and SlZnT2 are BY4741 (MAT a; 91 his 3Δ 1; leu 2Δ ; met 15Δ 0; ura 3Δ 0), Δ zrc1 (BY4741; MAT a; his 3Δ 1; leu 2Δ ; met 15Δ 0; ura 3Δ 0; 92 YMR243c::kanMX4), (BY4741; his3 Δ 1; $\Delta cot1$ Mat a; leu 2Δ ; met15 $\Delta 0$; ura3 $\Delta 0$; 93 YOR316c::kanMX4), $\Delta ycf1$ (BY4741; Mat a; his3 Δ 1; leu 2Δ ; met15 $\Delta 0$; ura3 $\Delta 0$; YDR135c::kanMX4) and $\Delta pmr1$ (BY4741; Mat a; his3 Δ 1; leu2 Δ ; met15 Δ 0; ura3 Δ 0; 94 95 YGL167c::kanMX4) obtained from Euroscarf (http: www.uni-96 frankfurt.de/fb15/mikro/euroscarf). Yeast cells were transformed according to the LiAc/PEG

97 method described by Gietz & Woods (2002). After transformation, cells were grown at 30°C in 98 synthetic defined (SD) medium without amino acids, containing 2% (w/v) glucose or galactose 99 (induction medium), supplemented with yeast synthetic dropout without uracil, (pH5.3). Positive 100 colonies were PCR tested to confirm transformation. For metal tolerance assays, yeast was grown 101 on induction medium to an OD_{600nm} of one to perform tenfold dilution series. The drop assays 102 were performed for three independent clones on control SD plates (2% w/v galactose) and SD 103 plates supplemented with 8 mM Zn; 30 µM Cd; 1 mM Co or 1 mM Mn. RNA was extracted 104 from colonies growing on control plates and converted in cDNA to verify transcription of the 105 transgene by PCR.

106 Localisation by confocal imaging

107 Yeast cells were transformed, expression was induced by galactose and functionality of the gene 108 product was tested as described previously (see yeast mutant complementation). Cells were 109 grown to an OD_{600nm} of one, vacuolar membranes were selectively stained with the red 110 fluorescence probe FM4-64 (Molecular Probes, Invitrogen) following Vida & Emr (1995). A 3 µl 111 droplet of yeast cells was analyzed at 20°C with a Zeiss LSM 510 META laser scanning confocal 112 microscope, using a Zeiss 63x NA1.4 oil immersion objective and 10x scanning zoom at 113 512x512 pixel resolution (image size: 8 bit, 14,62 µm²). For EGFP fluorescence analysis we used 114 the 488 nm excitation line of the laser and a BP 500-550 nm emission filter. For FM4-64 115 fluorescence analysis we used the 488 nm excitation line of the laser and a LP 560 nm emission 116 filter. Image processing was carried out with ImageJ (NIH, Bethesda, MD, USA) software.

117 Zn content analysis of transformed yeast

118 Yeast cells were transformed and expression was induced as described in "yeast mutant 119 complementation". Wild type yeast containing the empty vector, $\Delta zrc1$ yeast containing the 120 empty vector and $\Delta zrc1$ yeast containing the *SlZnT1* or *SlZnT2* cDNA were grown in liquid SD

medium containing galactose and supplemented with different concentrations of Zn (0 μ M or 500 µM). Zn treatments were performed for three independent clones. Yeast cells were harvested when OD_{600nm} of the cultures equalled one. Cells were washed three times with 20 mM PbNO₃ and milli-Q water. After drying, cells were destructed in concentrated acid (HNO₃/HCl) and Zn content was determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

126 Gene expression analysis

Total RNA extraction and cDNA synthesis occurred as described before. Real-time PCR was 127 128 carried out in 10 µl reactions containing fast SYBR Green Master Mix (Applied Biosystems), 129 300 nM gene-specific forward (ZnT1L or ZnT2L; supplementary table 2) and reverse primer 130 (ZnT1R or ZnT2R; supplementary table 2) and 2.5µl diluted cDNA (fivefold dilution in 1/10 131 Tris-EDTA buffer). An ABI PRISM 7500 sequence detection system (Applied Biosystems) and 132 fast cycling conditions (20s at 95°C, 40 cycles of 3s at 95°C and 30s at 60°C) were used. After cycling, a dissociation stage was added to assure specificity of amplification. Data were 133 expressed relative to the sample with the highest expression $(2^{-(Ct-Ctmax)})$ and normalised against 134 135 four reference genes. GR975621, AM085297, AM085168 and TUB1 were used as reference 136 genes according to Ruytinx et al. (2016). The normalisation factor for each sample was 137 calculated as the geometric mean of the relative expression of the four reference genes. The 138 significance of differences in expression level was examined by 2-way ANOVA and Tukey post-139 test.

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(a)



(b)

Zn (µM)

Zn (µM)

x

ZnT1



ZnT2















Zn μM

