Supplementary information

Expression of a fungal sterol desaturase improves tomato drought tolerance, pathogen resistance and nutritional quality

Ayushi Kamthan[1], Mohammad Azam[2], Niranjan Chakraborty[1], Subhra Chakraborty[1] and Asis Datta[1]

[1] National Institute of Plant Genome Research, New Delhi, India.,

[2] Division of Pathology & Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center and University of Cincinnati, Cincinnati, OH 45229-3039, USA

To whom correspondence should be addressed: Asis Datta, National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi- 110067, India, Tel: 0091-11-26735119, 0091-11-26742750; Fax: 0091-11-26741658; email: asis_datta@rediffmail.com

Figure legends

Figure S1 | Nucleotide and deduced amino acid sequences of the Fv*C5SD* gene. An open reading frame of 891 bp and the predicted 296 amino acids residues are shown. The exons are indicated by upper case letters, the introns and non coding regions are indicated by lower case letters. Initiation and termination codons ATG and TAG, respectively, are in bold letters. The intron junctions GT/AG are highlighted.

Figure S2 | Phylogenetic tree showing the relationship between Fv*C5SD* and other C-5 sterol desaturase. Tree was constructed by neighborhood-joining method using MEGA4 software[1]. Bar 0.2 amino acid substitutions per site. The sequences used were from *F. velutipes* (JN696291), *H. sapiens* (BAA33729), *A. thaliana* (CAA62079), *C. reinhardtii* (XP_001701457), *S. pombe (*NP_593135), *S. cerevisiae* (EDN59602), *N. crassa* (XP_962923), *N. tabacum* (AAD20458), *M. musculus* (O88822),*C. albicans* (XP_713612), *O. sativa (*NP_001041944),*T. brucei* (XP_844558), *Z. elegans* (BAE16981), *C. neoformans* (XP_566786), *L. major* (XP_001685044), *P. haloplanktis* (YP_341714), *A. macleodii* (YP_002126091), *A. fumigates* (XP_747563), *M. tuberculosis* (NP_336321), *R. solanacearum* (ZP_00946648), *M. loti* (NP_107904) , *L. interrogans* (YP_001329), *C. immitis* (XP_001248839, *E. gossypii* (Q754B9)

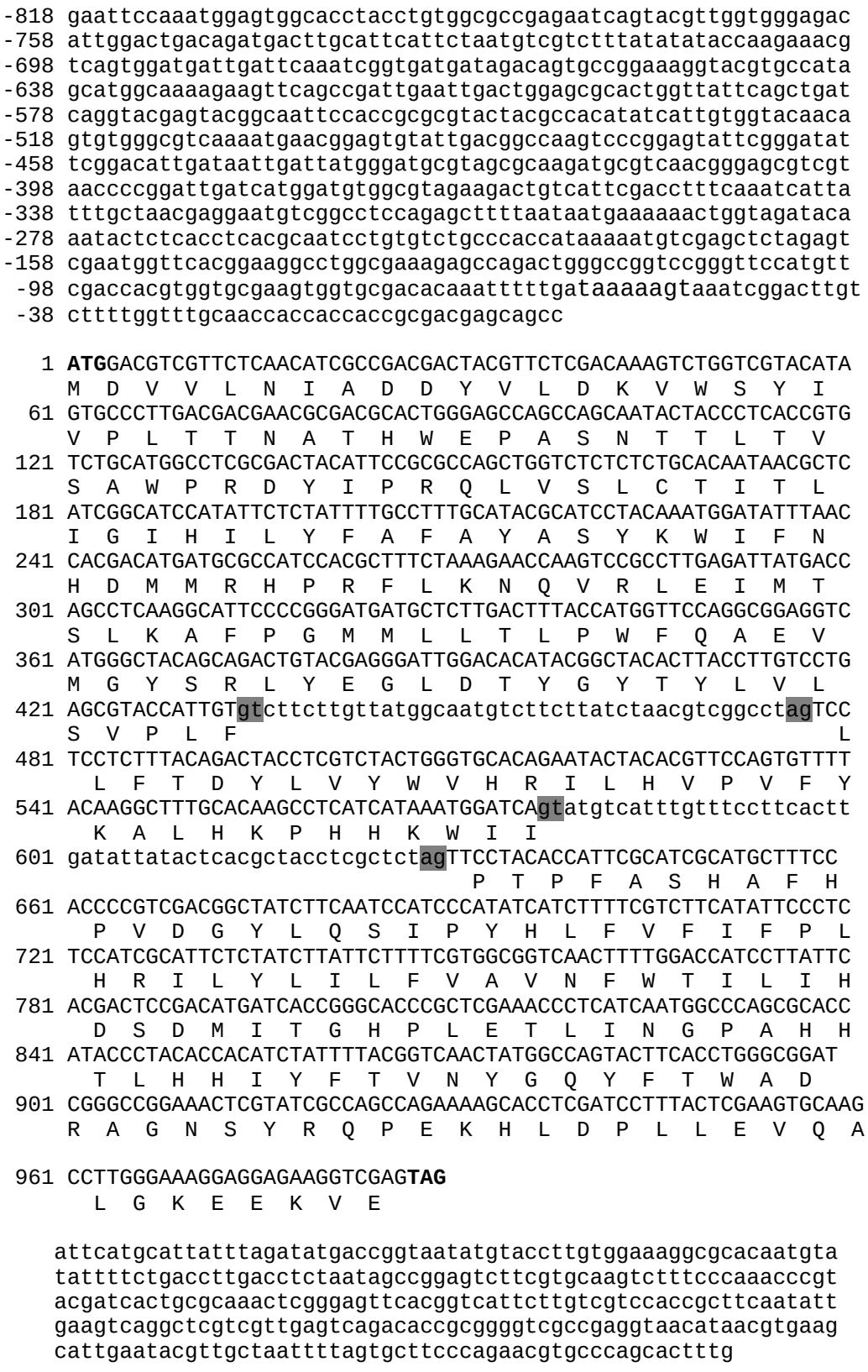
Figure S3 | Expression of FvC5sdp in *E. coli*. (a) 12.5% SDS PAGE showing purified FvC5sdp protein (≈34.8 kDa). (b) Purified FvC5sdp protein on 6% non-denaturing native PAGE.

Figure S 4 | Predicted topology model of FvC5sdp in the membrane environment. According to the model, FvC5sdp consist of five transmembrane helices (span the region from 49-78, 99-119,136-160 and 184-205 and 209-228 a. a) with N terminus oriented toward the ER lumen and C terminus facing the cytosolic side. Cytosolic loops house three conserved histidine rich motifs and probably from the catalytic site for the enzyme. Topology prediction and orientation was checked by MEMSAT-SVM Topology analysis [2].

Figure S 5 | Molecular analysis of transgenics tomato lines expressing Fv*C5SD*. Northern blot analysis (upper panel) of tomato transgenic lines. 20μg of total RNA isolated from leaves was resolved on 1.5% agarose gel and P^{32} labeled Fv*C5SD* cDNA was used as probe.
Western blot analysis (bottom panel) of total protein isolated from tomato transgenic lines. Total protein was separated on 12.5% PAGE and after transfer blot was probed with peptide based polyclonal antibody raised in rabbit against FvC5sdp (in 1:10,000 dilution) and alkaline phosphatase conjugated anti-rabbit secondary antibody (1:20,000). C, wild type control.

Figure S 6 | Multiple sequence alignment of Fv*C5SD* with various wax biosynthesis enzymes in plants. Wax2 (Q8H1Z0) and Cer1 from *Arabidopsis* (NP_171723), Glossy1 from maize (NP_001105247) and g1-1 from rice (AAB87722) were used in the alignment. Highlighted in yellow is the position of conserved histidine rich motifs.

Figure S1


-818 gaattccaaatggagtggcacctacctgtggcgccgagaatcagtacgttggtgggagac
-758 attggactgacagatgacttgcattcattctaatgtcgtctttatatataccaagaaacg
-698 tcagtggatgattgattcaaatcggtgatgatagacagtgccggaaaggtacgtgccata
-638 gcatggcaaaagaagttcagccgattgaattgactggagcgcactggttattcagctgat
-578 caggtacgagtacggcaattccaccgcgcgtactacgccacatatcattgtggtacaaca
-518 gtgtgggcgtcaaaatgaacggagtgtattgacggccaagtcccggagtattcgggatat
-458 tcggacattgataattgattatgggatgcgtagcgcaagatgcgtcaacgggagcgtcgt
-398 aaccccggattgatcatggatgtggcgtagaagactgtcattcgacctttcaaatcatta
-338 tttgctaacgaggaatgtcggcctccagagcttttaataatgaaaaaactggtagataca
-278 aatactctcacctcacgcaatcctgtgtctgcccaccataaaaatgtcgagctctagagt
-158 cgaatggttcacggaaggcctggcgaaagagccagactgggccggtccgggttccatgtt
 -98 cgaccacgtggtgcgaagtggtgcgacacaaattttgataaaaagtaaatcggacttgt
 -38 cttttggtttgcaaccaccaccaccgcgacgagcagcc

  1 ATGGACGTCGTTCTCAACATCGCCGACGACTACGTTCTCGACAAAGTCTGGTCGTACATA
    M D V V L N I A D D Y V L D K V W S Y I
 61 GTGCCCTTGACGACGAACGCGACGCACTGGGAGCCAGCCAGCAATACTACCCTCACCGTG
    V P L T T N A T H W E P A S N T T L T V
121 TCTGCATGGCCTCGCGACTACATTCCGCGCCAGCTGGTCTCTCTCTGCACAATAACGCTC
    S A W P R D Y I P R Q L V S L C T I T L
181 ATCGGCATCCATATTCTCTATTTTGCCTTTGCATACGCATCCTACAAATGGATATTTAAC
    I G I H I L Y F A F A Y A S Y K W I F N
241 CACGACATGATGCGCCATCCACGCTTTCTAAAGAACCAAGTCCGCCTTGAGATTATGACC
    H D M M R H P R F L K N Q V R L E I M T
301 AGCCTCAAGGCATTCCCCGGGATGATGCTCTTGACTTTACCATGGTTCCAGGCGGAGGTC
    S L K A F P G M M L L T L P W F Q A E V
361 ATGGGCTACAGCAGACTGTACGAGGGATTGGACACATACGGCTACACTTACCTTGTCCTG
    M G Y S R L Y E G L D T Y G Y T Y L V L
421 AGCGTACCATTGTgtcttcttgttatggcaatgtcttcttatctaacgtcggcctagTCC
    S V P L F   L
481 TCCTCTTTACAGACTACCTCGTCTACTGGGTGCACAGAATACTACACGTTCCAGTGTTTT
      L F T D Y L V Y W V H R I L H V P V F Y
541 ACAAGGCTTTGCACAAGCCTCATCATAAATGGATCAgtatgtcatttgtttccttcactt
      K A L H K P H H K W I I
601 gatattatactcacgctacctcgctctagTTCCTACACCATTCGCATCGCATGCTTTCC
                                  P T P F A S H A F H
661 ACCCCGTCGACGGCTATCTTCAATCCATCCCATATCATCTTTTCGTCTTCATATTCCCTC
      P V D G Y L Q S I P Y H L F V F I F P L
721 TCCATCGCATTCTCTATCTTATTCTTTTCGTGGCGGTCAACTTTTGGACCATCCTTATTC
      H R I L Y L I L F V A V N F W T I L I H
781 ACGACTCCGACATGATCACCGGGCACCCGCTCGAAACCCTCATCAATGGCCCAGCGCACC
      D S D M I T G H P L E T L I N G P A H H
841 ATACCCTACACCACATCTATTTTACGGTCAACTATGGCCAGTACTTCACCTGGGCGGAT
      T L H H I Y F T V N Y G Q Y F T W A D
901 CGGGCCGGAAACTCGTATCGCCAGCCAGAAAAGCACCTCGATCCTTTACTCGAAGTGCAAG
    R A G N S Y R Q P E K H L D P L L E V Q A

961 CCTTGGGAAAGGAGGAGAAGGTCGAGTAG
      L G K E E K V E

   attcatgcattatttagatatgaccggtaatatgtaccttgtggaaaggcgcacaatgta
   tattttctgaccttgacctctaatagccggagtcttcgtgcaagtctttcccaaacccgt
   acgatcactgcgcaaactcgggagttcacggtcattcttgtcgtccaccgcttcaatatt
   gaagtcaggctcgtcgttgagtcagacaccgcggggtcgccgaggtaacataacgtgaag
   cattgaatacgttgctaattttagtgcttcccagaacgtgcccagcactttg

Figure S2

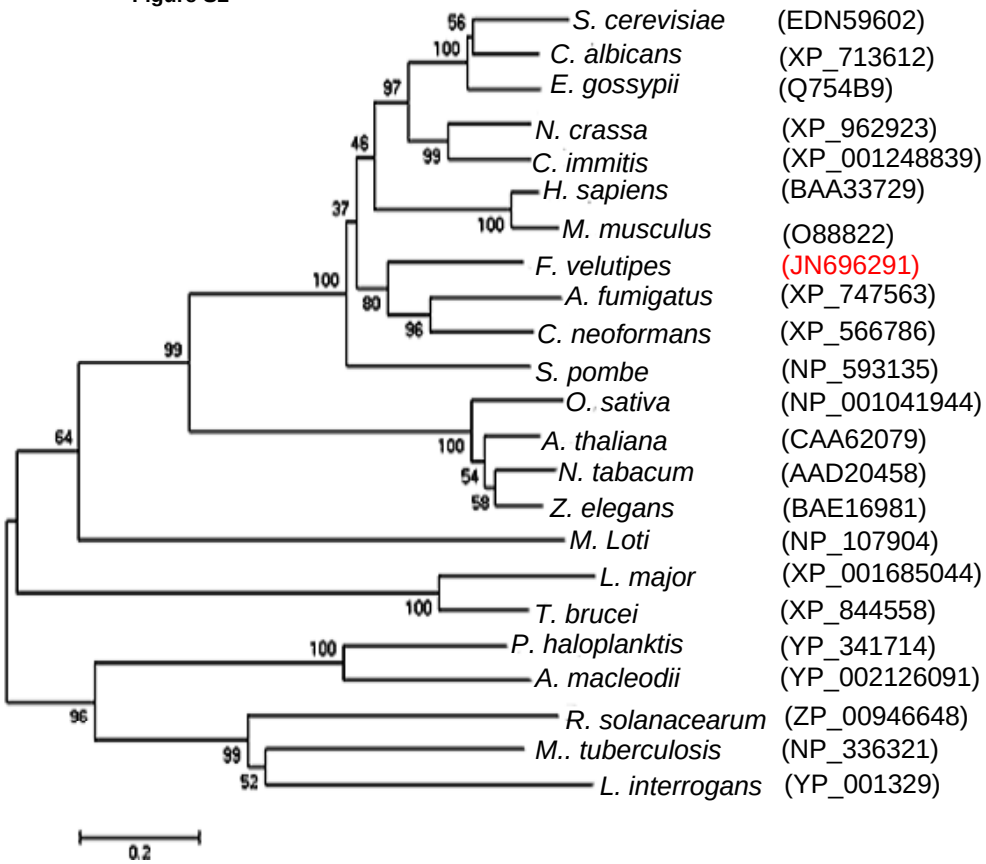

S. cerevisiae (EDN59602)
C. albicans (XP_713612)
E. gossypii (Q754B9)
N. crassa (XP_962923)
C. immitis (XP_001248839)
H. sapiens (BAA33729)
M. musculus (O88822)
F. velutipes (JN696291)
A. fumigatus (XP_747563)
C. neoformans (XP_566786)
S. pombe (NP_593135)
O. sativa (NP_001041944)
A. thaliana (CAA62079)
N. tabacum (AAD20458)
Z. elegans (BAE16981)
M. Loti (NP_107904)
L. major (XP_001685044)
T. brucei (XP_844558)
P. haloplanktis (YP_341714)
A. macleodii (YP_002126091)
R. solanacearum (ZP_00946648)
M.. tuberculosis (NP_336321)
L. interrogans (YP_001329)
56
100
97
46
99
37
100
100
80
96
99
64
100
54
58
100
100
96
99
52
0.2

Figure S3

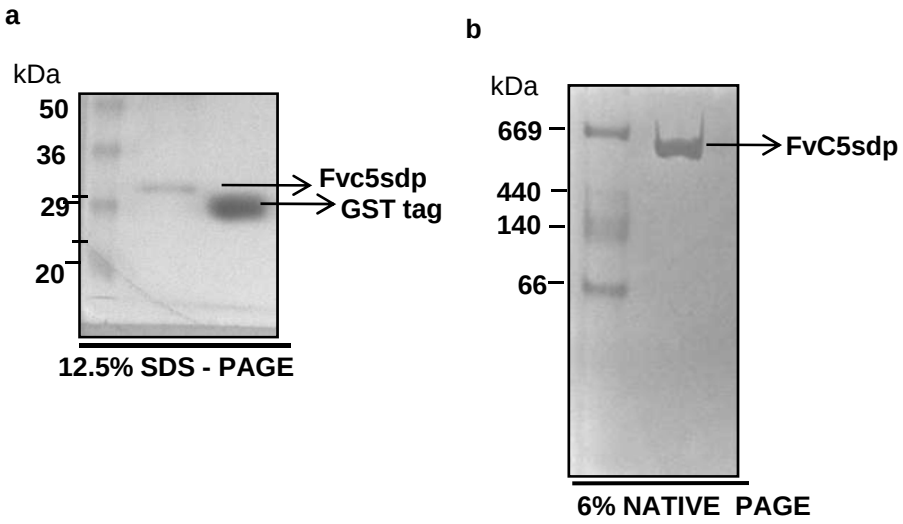

a
kDa
50
36
29
20
Fvc5sdp
GST tag
12.5% SDS - PAGE
b
kDa
669
440
140
66
FvC5sdp
6% NATIVE PAGE

Figure S4

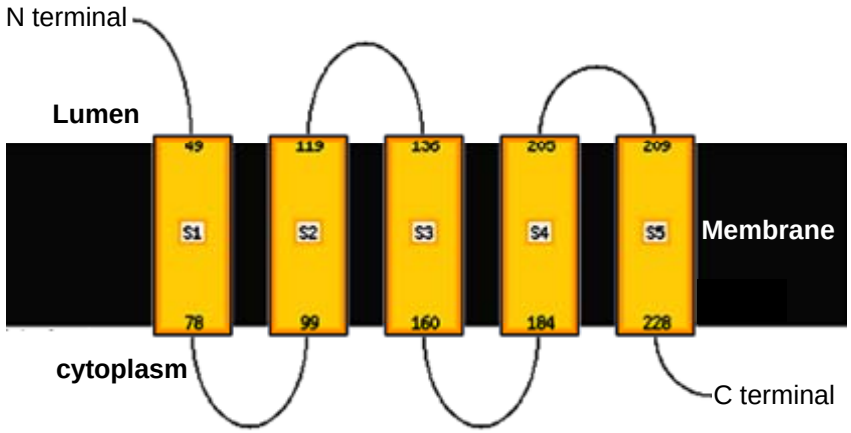
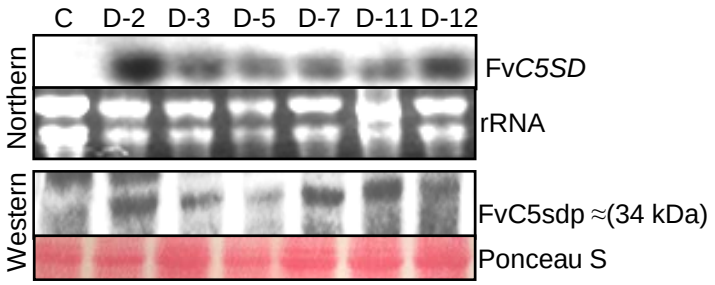

N terminal
Lumen
49
119
136
200
209
S1
S2
S3
S4
S5
Membrane
78
99
160
184
228
cytoplasm
C terminal

Figure S5


C
D-2
D-3
D-5
D-7
D-11
D-12
Northern
Fv*C5SD*
rRNA
Western
FvC5sdp ≈(34 kDa)
Ponceau S

```
Wax2      ---------MVAFLSAWPWENFGNLKYLLYAPLAAQVVYSWVYE-EDISKVLWCIHILIICGLKALVHELWSVFNNMLFV  70
Glossy1   --------MGAALLASWPWDNLGLYKYVLYGPLVGKAVASRAWE-AAS-PDRWILLLLLLFGLRALTYQLWSSFSNMLFA  70
gl-1      ------------------------------------------------------------------------NMLFF   5
Cer1      ------MATKPGVLLTDWPWTPLGSFKYIVIAPWAVHSTYRFVTD-DPEKRDLGYFLVFPFLLFRILHNQVWISLSRYYTS  73
FvC5SD    MDVVLNIADDYVLDKVWSYIVPLTTNATHWEPASNTTLTVSAWPRDYIPRQLVSLCTITLIGIHILYFAFAYASYKWIFN  80

Wax2      TRTLRINPKGIDFKQIDHEWHWDNYIILQAIIVSLICYMSPPLMMMINSLPLWNTKGLIALIVLHVTFSEPLYYFLHRSF 150
Glossy1   TRRRRVVRDGVDFDQIDKEWDWDNFLILHALMAAAALCAFP----SLRHLPAWDGRGFAVALVAHAAATEPLSYLAHRAL 146
gl-1      TRRRRVVDDGVDFRQIDTEWDWDNMVIMQTLIAAVLVTSRVFP--ATSDLSAWDLRGWAIAVVLHVAVSEPAFYWAHRAL  83
Cer1      SGKRRIVDKGIDFNQVDRETNWDDQILFNGVLFYIGINLLP----EAKQLPWWRTDGVLMAALIHTGPVEFLYYWLHKAL 149
FvC5SD    HDMMRHPRFLKNQVRLEIMTSLKAFPGMMLLTLPWFQAEVMGYSRLYEGLDTYGYTYLVLSVPLFLLFTDYLVYWVHRIL 160

Wax2      HRNN-YFFTHYHSFHHSSPVPHPMTAGNATLLENIILCVVAGVPLIGCCLFGVGSLSAIYGYAVMFDFMRCLGHCNVEIF 229
Glossy1   HGSSGRLYARYHSLHHSSRVPQPFTAGLATPLEHVALGALMSLPLAAARAAGCASVALAFAYVLAFDSLRAMGHCNVEVV 226
gl-1      HLGP--LFSRYHSLHHSFQATQALTAGFVTPLXXLILTLVAWPHLQG--LHGGTRLRELVYGHISSSTTPVHGVQQRRGH 159
Cer1      HHHF--LYSRYHSHHHSSIVTEPITSVIHPFAEHIAYFILFAIPLLTTLLTKTASIISFAGYIIYIDFMNNMGHCNFELI 227
FvC5SD    HVPV--FYKALHKPHHKWIIPTPFAS----HAFHPVDGYLQSIPYHLFVFIFPLHRILYLILFVAVNFWTILIHDSDMIT 234

Wax2      SHKLFEILPVLRYLIYTPTYHSLHHQEMGTNFCLFMPLFDVLGDTQNPNSWELQKKIRLSAGERKRVPEFVFLAHGVDVM 309
Glossy1   PASLFRAIPALRYVLYTPTYHAIHHTKKEANFCLFMPLFDLLGGTIDRRSWDMQRKMSAGVD---EVPDFVFLAHVVDVM 303
gl-1      LTQDFQDFPPLRYLIYTPSYLSLHHREKDSNFCLFMPLFDALGGTLNPKSWQLQKEVDLGKN--HRVPDFVFLVHVVDVV 237
Cer1      PKRLFHLFPPLKFLCYTPSYHSLHHTQFRTNYSLFMPLYDYIYGTMDESTDTLYEKTLERGD---DIVDVVHLTHLTTPE 304
FvC5SD    G-------HPLETLINGPAHHTLHHIYFTVNYGQYFTWADRAG-------------------------------------- 270

Wax2      SAMHAPFVFRSFASMPYTTRIFLLPMWPFTFCVMLGMWAWS---------------------------------------- 350
Glossy1   QSLHVPFVMRTFASTPFSVQLFLLPMWPFAFLVMLAMWVWSKTFVIS---------------------------------- 350
gl-1      SSMHVPFAFRACSSLPFATHLVLLPLWPIAFGFMLLQWFCSKTFTVSFYKLRGFLHQTWSVPRYGFQYFIPSAKKGINEM 317
Cer1      SIYHLRIGLASFASYPFAYRWFMRLLWPFTSLSMIFTLFYARLFVA---------------------------------- 350
FvC5SD    ----------------NSYRQPEKHLDPLLEVQALGKEEKVE---------------------------------------- 296

Wax2      ---------------------------------- 350
Glossy1   ---------------------------------- 350
gl-1      IELAILRADKMGVKVLSLAALNKNEALNGGGTL 350
Cer1      ---------------------------------- 350
FvC5SD    ---------------------------------- 296
```

Figure S6

Supplementary Table1
Strains and plasmids used in this study

Strains and plasmids	Genotype/description	Source/reference
***E. coli* strains**		
DH5α	F'/end A1 hsd R17($r_K^-m_k^+$) glnV44 thi-4 recA1 gyrA (Nal^n) elA1 Δ(lac1ZYA-argF) U169 deoR(ϕ80dlacΔ(lacZ)M15)	
BL21 (DE3)	*E. coli B F⁻ dcm ompT hsdS(rB⁻ mB⁻) gal λ(DE3)*	
F.velutipes strain		
ATCC13547		ATCC
S. pombe strain		
BJ7468	*ura4-D18, leu1-32, and ade6-M216*	Laboratory strain
S. cerevisiae strain		
YlR056W	*erg3::kanMX MATa his3-1 leu2-0 met15-0 ura3-0*	
SC4741 (Wild type)		
Agrobacterium tumifaciens		
EHA105		Laboratory strain
Plasmids		
Fv*C5SD*-pGAL10	Fv*C5SD* cDNA cloned in pGal10HO vector	This study
Fv*C5SD*-pSLF173	Fv*C5SD* cDNA cloned in pSLF173	This study
Fv*C5SD*-pBI121	Fv*C5SD* cDNA cloned in pBI121	This study
Fv*C5SD*-pGEX4T-2	Fv*C5SD* cDNA cloned in pGEX4T-2	This study

Supplementary methods

Separation of protein fractions for Western blot analysis

Total protein extract was prepared from *F. velutipes* in 0.1 M K citrate buffer (pH 3.0) by procedure described earlier [3]. For isolation of microsomal fraction, the total protein extract was subjected to ultracentrifugation at 10,0000xg at 4°C for 90 min. Supernatant was saved as soluble fraction where as pellet was dissolved in microsome solubilisation buffer (50mM Tris HCl–pH 7.4, 150 mM NaCl, 5mM EDTA, 2M Urea, 1% Triton X-100, 0.1M Na_2CO_3-pH11and 20% v/v Glycerol). After shaking at 4°C for 30 min, extract was centrifuged at 10,000 xg at 4°C for 10 min. The supernatant consisted of microsomal fraction.

Expression and purification of GST tagged protein

BL21 (DE) strain of *E. coli* was transformed with Fv*C5SD*-pGEX4T-2 construct. Ampicillin resistant cells were grown at 37°C to an $O.D_{600nm}$ of 0.5 followed by induction with 0.3mM IPTG for 4hrs. Cells were harvested by centrifugation at 5500 rpm at 4°C for 15 min. Cells were washed twice with 1 X PBS. Pellet was resuspended in protein extraction buffer (1mM DTT, 1mM PMSF, 1mM EDTA (pH 8.0) in 1XPBS pH 7.4). Lysozyme was added to the concentration of 1mg/ml and incubated on ice for 30 min. with occasional mixing in between. Triton X 100 (0. 2%) and DNase (5µg/ml) were added to the above mix followed by vigorous shaking. Incubation was done for 1 hr at 4°C with gentle shaking to solubilize the fusion protein. It was centrifuged at 5000 rpm for 30 min. The above supernatant was clarified by filtration through 0.4µ filters. Large scale batch purification of GST tagged protein was done using 50% slurry of glutathione-sepharose resin (GE biosciences).

References

1. Tamura, K.. *et al* . MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0**.** *Mol. Biol. & Evol.* **24**, 1596-1599 (2007).
2. Nugent, T. & Jones, D.T. Transmembrane protein topology prediction using support vector machines. *BMC Bioinformatics* **10**, 159 (2009).
3. Mehta, A. & Datta, A. Oxalate decarboxylase from *Collybia velutipes*: Purification, characterization and cDNA cloning. *J. Biol. Chem.* **266**, 23548-23553 (1991).