An NPF Transporter Exports a Central Monoterpene Indole Alkaloid Intermediate from the Vacuole

Richard M. E. Payne,[a] Deyang Xu,[b,c] Emilien Foureau,[d] Marta Ines Soares Teto Carqueijeiro,[d] Audrey Oudin,[d] Thomas Dugé de Bernonville,[d] Vlastimil Novak,[b,c] Meike Burow,[b,c] Carl-Erik Olsen,[c] D. Marc Jones,[e] Evangelos C. Tatsis,[a] Ali Pendle,[f] Barbara Ann Halkier,[c] Fernando Geu-Flores,[c,g] Vincent Courdavault,[d] Hussam Hassan Nour-Eldin,[b,c] Sarah E. O'Connor[a*]

[a] The John Innes Centre, Department of Biological Chemistry, Norwich Research Park, Norwich NR4 7UK, UK

[b] DynaMo Center, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Denmark.

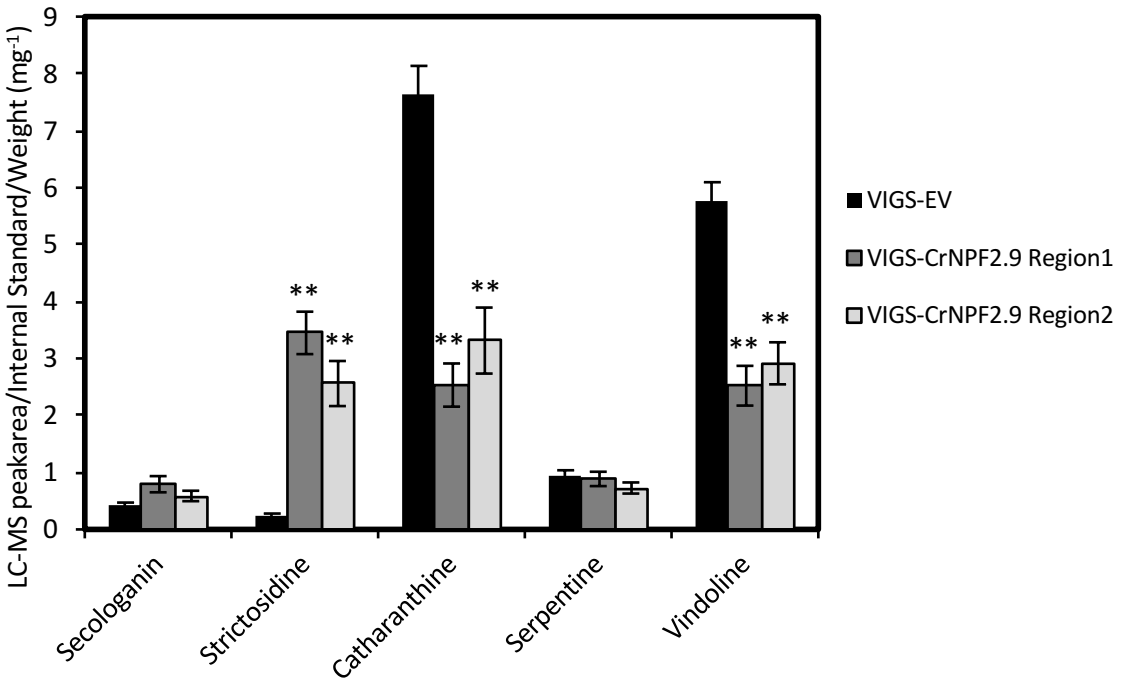
[c] Copenhagen Plant Science Center, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, 1871 Frederiksberg C, Denmark

[d] Université François-Rabelais de Tours, EA2106 Biomolécules et Biotechnologies Végétales, Département de Biologie et Physiologie Végétales, UFR Sciences et Techniques, Parc de Grandmont 37200 Tours, France

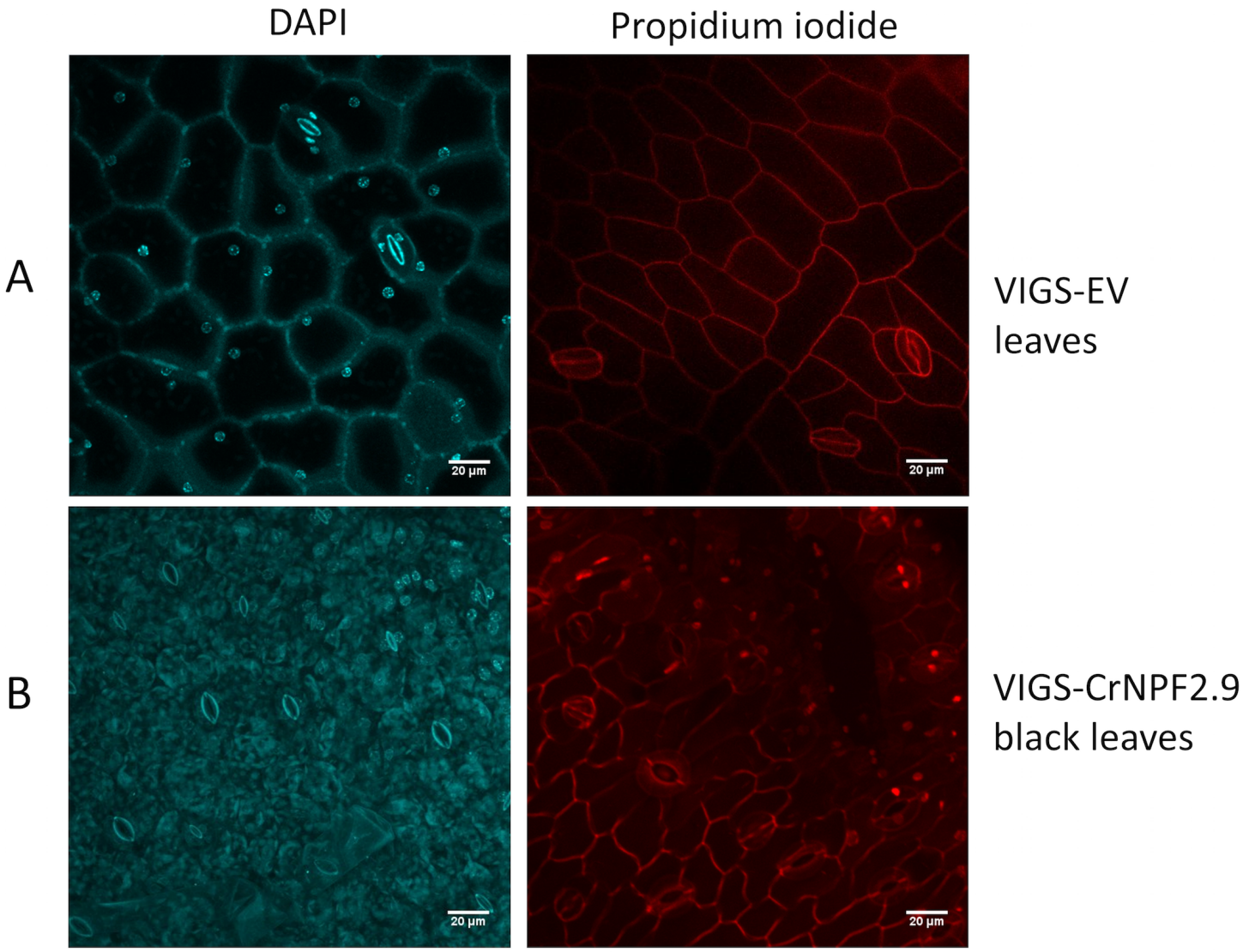
[e] The John Innes Centre, Department of Computational and Systems Biology, Norwich Research Park, Norwich NR4 7UK, UK

[f] The John Innes Centre, Department of Cell and Developmental Biology, Norwich Research Park, Norwich NR4 7UK, UK

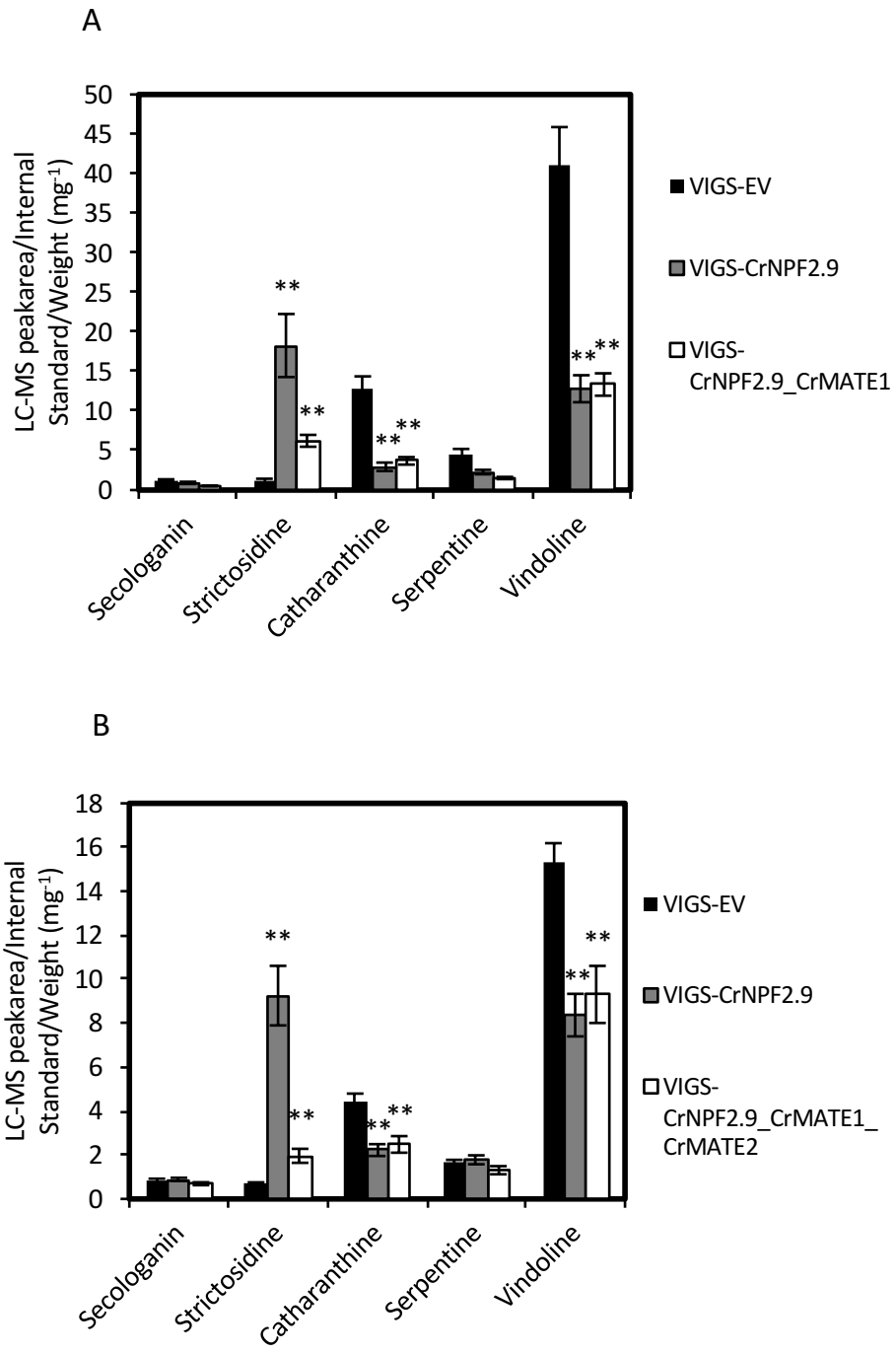
[g] Section for Plant Biochemistry, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, 1871 Frederiksberg C, Denmark



Supplementary Figure 1. *In planta* silencing of CrNPF2.9 using additional VIGS vectors harbouring alternative gene fragment. To ensure that the response to silencing was specific, an additional gene region was selected and tested for CrNPF2.9. Alkaloid profile for leaf tissue transformed with vector VIGS-CrNPF2.9 Region1, VIGS-CrNPF2.9 Region 2 relative to empty vector control tissue in Little Bright Eyes (VIGS-CrNPF2.9 Region1 (n= 10), VIGS-CrNPF2.9 Region2 (n= 11), VIGS-EV (n=9)) ** $p<0.01$. All data show are mean $\pm$ SEM.

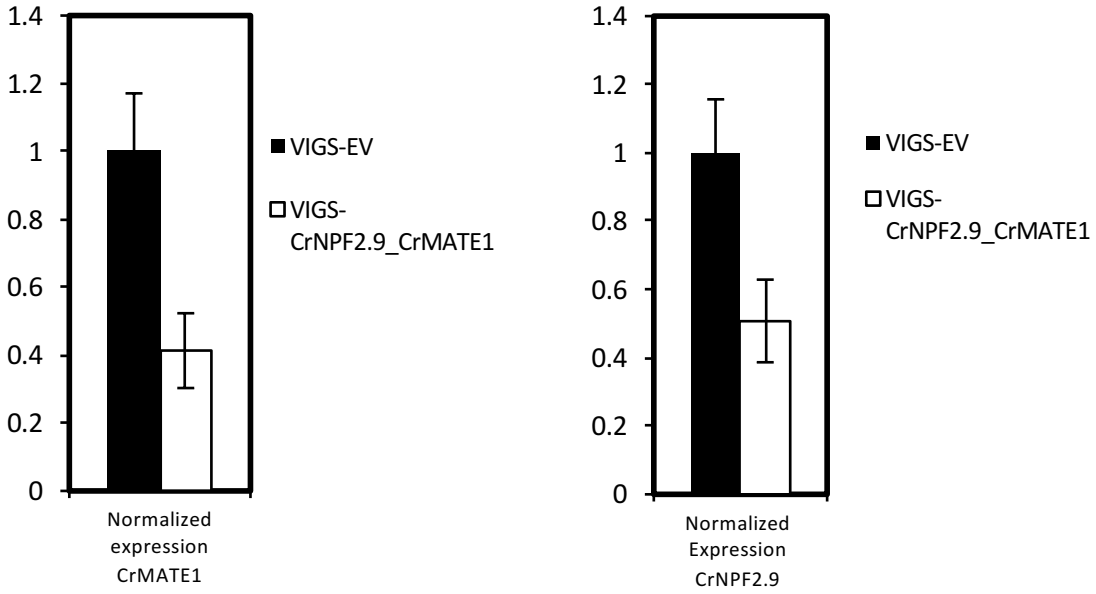


Supplementary Figure 2. Confocal microscopy of VIGS-EV leaf tissue and VIGS-CrNPF2.9 leaf tissue displaying the black phenotype. A. DAPI and propidium iodide staining of VIGS-EV leaf tissue **B.** DAPI and propidium iodide staining of VIGS-CrNPF2.9 leaf tissue.

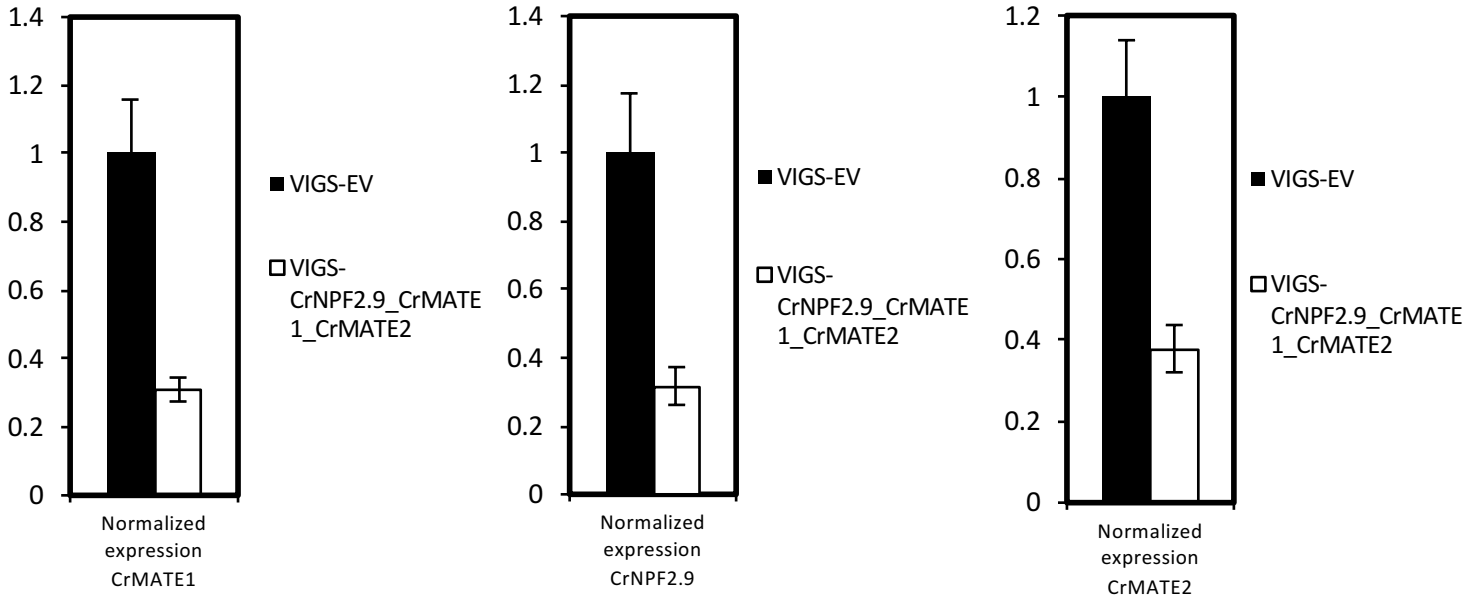


Supplementary Figure 3. Combinatorial (simultaneous) *in planta* silencing of CrNPF2.9, CrMATE1 and CrMATE2.

C



D



Supplementary Figure 3. Combinatorial (simultaneous) *in planta* silencing of CrNPF2.9, CrMATE1 and CrMATE2.

Supplementary Figure 3. Combinatorial (simultaneous) *in planta* silencing of CrNPF2.9, CrMATE1 and CrMATE2. A. Alkaloid profile for tissue that has been transformed with a VIGS vector that targets both-CrNPF2.9 (Region 1) and CrMATE1 (Region 1) relative to empty vector control tissue, and tissue transformed with only VIGS-CrNPF2.9 (Region 1) (VIGS-CrNPF2.9 Region1 (n = 10), VIGS-CrNPF2.9-CrMATE1 double (n = 11), VIGS-EV (n = 9)) ** $p < 0.01$. **B.** Alkaloid profile for tissue that has been transformed with a VIGS vector that targets the three genes CrNPF2.9 (Region 1), CrMATE1 (Region 1) and CrMATE2 relative to empty vector control tissue and tissue transformed with only VIGS-CrNPF2.9 (Region 1) (VIGS-CrNPF2.9 Region1 (n = 12), VIGS-CrNPF2.9-CrMATE1-CrMATE2 triple (n = 15), VIGS-EV (n = 11)) ** $p < 0.01$. **C.** Normalized expression levels of the CrNPF2.9 and CrMATE1 genes in empty vector control tissue and the double silenced CrNPF2.9_CrMATE1 tissue as measured by qPCR (VIGS-CrNPF2.9_CrMATE1 (n = 8) VIGS-EV (n = 8)). All data shown are mean $\pm$ SEM. **D.** Normalized expression of CrNPF2.9, CrMATE1 and CrMATE2 genes in empty vector and the triple silenced CrNPF2.9-CrMATE1-CrMATE2 tissue (VIGS-CrNPF2.9-CrMATE1-CrMATE2 (n = 8), VIGS-EV (n = 8)). All data shown are mean $\pm$ SEM.

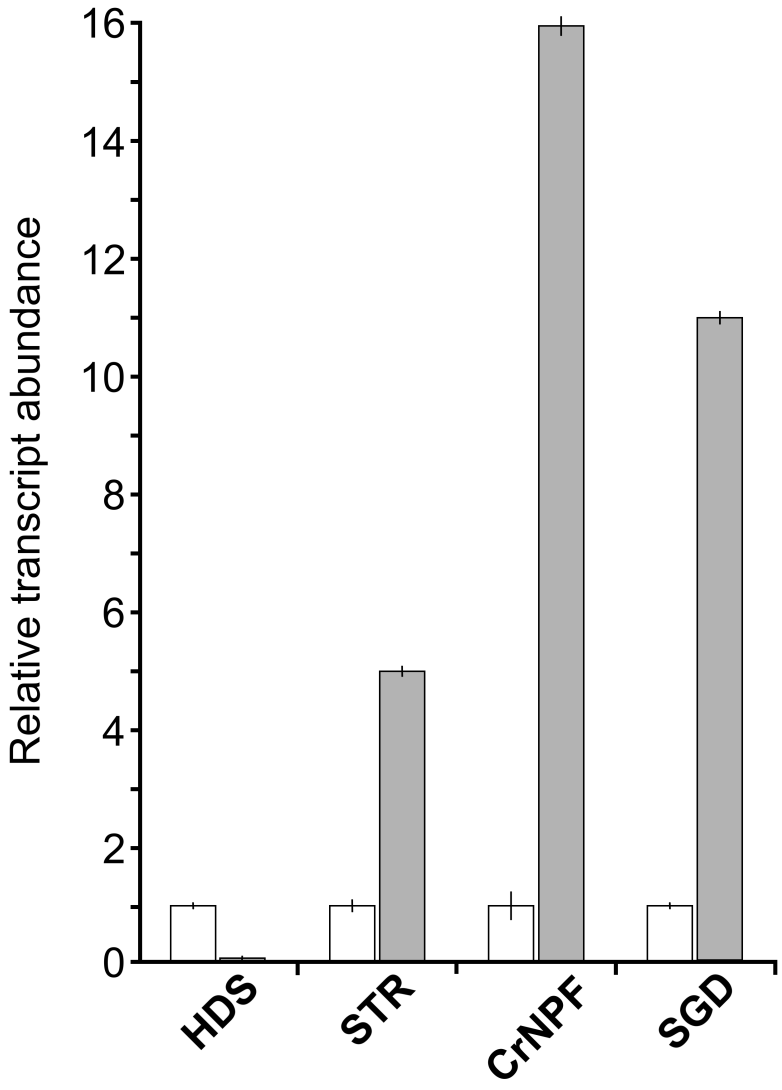
A

```
AtGTR1          MKSRVILNHRDRRDKNHNNNNTNHYTQVDTMERKPLEVEPSTTTTNTDVV
AtNRT1.1        MSLPE-------------------------------------TKSDDILL
CrNPF2.9        MGD---------------------------------------TEAQ-
NAXT            MASSV-------------------------------------TGDAETAI

AtGTR1          DS---FEEEQRKIVYRGWKVMPFIIGNETFEKLGIIGTLSNLLVYLTSVF
AtNRT1.1        DAWDFQGRPADRSKTGGWASAAMILCIEAVERLTTLGIGVNLVTYLTGTM
CrNPF2.9        L-----LQPGH-KQKGGWITFPFILATRTLLTLAVAGFSSNLIVYLINEF
NAXT            S-----ADSSTKRRGGGWITFPFMIATLLGLTIAAWGWLLNLIVYLIEEF

AtGTR1          NLKSYTAATIINAFSGTINFGTFIAAFLCDTYFGRYKTLSVAVIACFLGS
AtNRT1.1        HLGNATAANTVTNFLGTSFMLCLLGGFIADTFLGRYLTIAIFAAIQATGV
CrNPF2.9        NVNRIDSAQIYNVVNGCMALFPLLLAIIADTFLGCFNVIWISTLISLMGM
NAXT            NVKSIAAAQIANIVSGCICMVPAVAAIASDSFFGTIPVISVSAFISLMGV

AtGTR1          FVILLTAAIPSLHPVACG-N-KISCEGPSVGQILFLLMGLGFLVVGAGGI
AtNRT1.1        SILTLSTIIPGLRPPRCNPTTSSHCEQASGIQLTVLYLALYLTALGTGGV
CrNPF2.9        ALLTLTSSITSLRPQPCA-EGSTFCQQPSAYQSSILFLALALPSIGFAGT
NAXT            ALLTLTASLDTLRPRPCE-TASILCQSPSKTQLGVLYTAITLASIGTGGT

AtGTR1          RPCNLAFGADQFNPKSESGKKGINSFFNWYFFTFTFAQIISLTAVVYIQS
AtNRT1.1        KASVSGFGSDQFDETEPKERSKMTYFFNRFFFCINVGSLLAVTVLVYVQD
CrNPF2.9        SFTVGTMGAHQLDDPKHQ-----ENFFNWFLFIWNAASVISGIGIVYVQD
NAXT            RFTLATAGANQYEKTKDQ-----GSFFNWFFFTTYLAGAISATAIVYTED

AtGTR1          NVSWTIGLIIPVALMFLACVIFFAGDRLYVKVKA-SGSPLAGIARVIAAA
AtNRT1.1        DVGRKWGYGICAFAIVLALSVFLAGTNRYRFKKL-IGSPMTQVAAVIVAA
CrNPF2.9        NVSWSWGFGICVASNLLGLIIFLAGKRLYRDVQPQKSSPFKDLACVVVAA
NAXT            NISWTLGFGLSVAANFFSFLVFVSGKRFYKHDKP-LGSPFTSLLCVIFAA

AtGTR1          IKKRGLKPVKQPWVNLYNHIPSN-------YANTTLK---YTDQFRFLDK
AtNRT1.1        WRNRKLELPADP--SYLYDVDDIIAAEGSMKGKQKLP---HTEQFRSLDK
CrNPF2.9        LSKKKLSLSNKE-EDYYSELPDNAEEQQQQEGVTLLPATVPDESFKFLNH
NAXT            LRKRKAVVSTNE-KDYHNES-----------------ITMPTKSFRFFNR

AtGTR1          AAIMTPEEKLNSDGTASDPWKLCTLQQVEEVKCIVRVIPIWFASTIYYLA
AtNRT1.1        AAIRDQEAG--VTSNVFNKWTLSTLTDVEEVKQIVRMLPIWATCILFWTV
CrNPF2.9        AALVTS-ADIQPDGSIKKSWKLCTVKQIEDLKTLIRLFPLWTTGFLLTIP
NAXT            AALKQE-DEVKPDGTIRNPWRLCSVQQVEDFKAVIRIIPLALATIFLSTP

AtGTR1          ITIQMTYPVFQALQSDRRLGSGGFRIPAATYVVFLMTGMTVFIIFYDRVL
AtNRT1.1        HAQLTTLSVAQSETLDRSIG--SFEIPPASMAVFYVGGLLLTTAVYDRVA
CrNPF2.9        MGVLSSLTTLQALTMDCSTF-WGLKYPVGSMSVFTLLAGAISLTFIDRLI
NAXT            IAMQLSLTVLQGLVMDRRLG-PSFKIPAGSLQVITLLSTCLFIIVNDRVL

AtGTR1          VPSLRRVTGLETGISLLQRIGAGFTFAIMSLLVSGFIEERRRNFALTKPT
AtNRT1.1        IRLCKKLFNYPHGLRPLQRIGLGLFFGSMAMAVAALVELKRLRTAHAH--
CrNPF2.9        FPICRKMA---KPIRPLQRIAIGHIINVISVVIAAIVEHKRLQLARAQKF
NAXT            YPFYQKLTG--KHLTPLQRVGIGHAFNILSMAVTAIVEAKRLKIVQKGHF

AtGTR1          LGMAPRTGEISSMSALWLIPQLTLAGIAEAFAAIGQMEFYYKQFPENMKS
AtNRT1.1        -GP--TV-KTLPLGFYLLIPQYLIVGIGEALIYTGQLDFFLRECPKGMKG
CrNPF2.9        QGK--TDSVVVPMSVFWLIPQLALSGTGEAFHFPGQALLYYKEFPASLKS
NAXT            LGS----SSVADMSVLWLFPPLVIVGIGEAFHFPGNVALCYQEFPESMRS

AtGTR1          FAGSIFYVGAGVSSYLASFLISTVHRTTAHSPSGNWLAEDLNKAKLDYFY
AtNRT1.1        MSTGLLLSTLALGFFFSSVLVTIVEKFTGK--AHPWIADDLNKGRLYNFY
CrNPF2.9        TSTAMLAILIAIGYYMGTFVIDVVRKVT------DWLPEDINHGRLDNLY
NAXT            TATSITSVVIGICFYTSTALIDLIQRTT------AWLPDDINHGRVDNVY

AtGTR1          FMLTGLMVVNMAYFLLMARWYRYKGGNDEDITEIETNEEETKQQQLQDKN
AtNRT1.1        WLVAVLVALNFLIFLVFSKWYVYKEKRLAEVG-IELDD------EPSIPM
CrNPF2.9        WLVAVLGVLNFVYYLACAGAYEYSSVMEDE-------DE-----TNDNKI
NAXT            WILVIGGVLNLGYFLVCSWLYRYRNLKDD--------DH-----KQAANV

AtGTR1          SV
AtNRT1.1        GH
CrNPF2.9        YM
NAXT            SH
```

B

```
CrNPF2.9
MGDTEAQLLQPGHKQKGGWITFPFILATRTLLTLAVAGFSSNLIVYLINEFNVNRIDSAQIYNVVNGCMALFPLLLA
IIADTFLGCFNVIWISTLISLMGMALLTLTSSITSLRPQPCAEGSTFCQQPSAYQSSILFLALALPSIGFAGTSFTV
GTMGAHQLDDPKHQENFFNWFLFIWNAASVISGIGIVYVQDNVSWSWGFGICVASNLLGLIIFLAGKRLYRDVQPQK
SSPFKDLACVVVAALSKKKLSLSNKEEDYYSELPDNAEEQQQQEGVTLLPATVPDESFKFLNHAALVTSADIQPDGS
IKKSWKLCTVKQIEDLKTLIRLFPLWTTGFLLTIPMGVLSSLTTLQALTMDCSTFWGLKYPVGSMSVFTLLAGAISL
TFIDRLIFPICRKMAKPIRPLQRIAIGHIINVISVVIAAIVEHKRLQLARAQKFQGKTDSVVVPMSVFWLIPQLALS
GTGEAFHFPGQALLYYKEFPASLKSTSTAMLAILIAIGYYMGTFVIDVVRKVTDWLPEDINHGRLDNLYWLVAVLGV
LNFVYYLACAGAYEYSSVMEDEDETNDNKIYM
```

Supplementary Figure 4. Sequence of CrNPF2.9. **A.** Sequence alignment of AtNRT1.1/AtNPF6.3, AtGTR1/AtNPF2.10, NAXT1/AtNPF2.7 and CrNPF2.9. Highlighted in yellow is the conserved EXXE(R/K) motif implicated in proton coupling that is present in AtNRT1.1/AtNPF6.3 and AtGTR1/AtNPF2.10 and absent in both the NAXT/AtNPF2.7 and CrNPF2.9 transporters. **B.** Protein sequence of CrNPF2.9. Highlighted in yellow is the putative (D/E)$X_{3\text{-}5}$L(L/I) motif that is predicted to act as a tonoplastic targeting sequence.



Supplementary Fig. 5. CrNPF2.9 is mainly expressed in leaf epidermis. Relative expression of HDS, CrNPF2.9, STR and SGD in epidermis enriched fractions of *C. roseus* leaves compared to the whole leaf fraction (HDS, hydroxymethylbutenyl 4-diphosphate synthase; STR, strictosidine synthase; SGD, strictosidine glucosidase). Epidermis enriched transcript fractions were generated by a carborundum abrasion and both fraction types were retro-transcribed before determination of gene expressions by qPCR. Transcript copy numbers were normalized using CrRPS9 and expressed relatively to the amount of transcript measured in the whole leaf fraction. Each assay was performed in triplicate, and expression measurements were performed at least twice with independent experimental replicates.

VIGS vectors	Name	PCR target		Primer Sequence (5'-3')
Single Vectors	VIGS-CrNPF2.9 Region1	CrNPF2.9	Fwrd	GGCGCGAUCGCTTGCGGTAGCTGGATTTA
			Rev	GGTTGCGAUCCCATGACCAACTCACATTATCTTGAAC
	VIGS-CrNPF2.9 Region 2	CrNPF2.9	Fwrd	GGCGCGAU GAA TAG CCA TTG GTC ACA TCA TCA ACG
			Rev	GGTTGCGAU CAA CAT CAA TAA CGA AAG TTC CCA TAT AAT ACC GAA TG
	VIGS-CrMATE1 Region1	CrMATE1	Fwrd	GGCGCGAU CCAAACAAAACTATGAAATAAACCAACCACTGT
			Rev	GGTTGCGAUGTTTTGAGAATATGTAAACAAACATAATAACAATGCCTGTTA
Fusion Vectors	VIGS-CrNPF2.9-CrMATE1 double	CrNPF2.9	Fwrd	GGCGCGAUCGCTTGCGGTAGCTGGATTTA
			Rev	ATTCCAAU GCCACTGATTACTGAAGCAGCATT
		CrMATE1	Fwrd	ATTGGAAUCCAAACAAAACTATGAAATAAACCAACCACTG
			Rev	GGTTGCGAUGTTTTGAGAATATGTAAACAAACATAATAACAATGCCTGTTA
	VIGS-CrNPF2.9-CrMATE1-CrMATE2 triple	CrNPF2.9	Fwrd	GGCGCGAUCGCTTGCGGTAGCTGGATTTA
			Rev	ATTCCAAU GCCACTGATTACTGAAGCAGCATT
		CrMATE1	Fwrd	ATTGGAAUCCAAACAAAACTATGAAATAAACCAACCACTG
			Rev	ACAAACAU AAT AAC AAT GCC TGT TAG GGT TAG AAG AAT TG
		CrMATE2	Fwrd	ATGTTTGU TGG ACA GAC CTT GAA GCT GG
			Rev	GGTTGCGAU ATCCCCTGGCAACACCTG

Supplemental Table 1. Primers for VIGS constructs.

	Primer Name	PCR Target	Sequence (5'-3')
qPCR primers	qCrNPF2.9_ Fwrd	CrNPF2.9	AAGAAGCTCTCCTTGTCAAACAAAGAAGAAG
	qCrNPF2.9_ Rev	CrNPF2.9	CTTGAAACTTTCATCAGGTACAGTAGCAGG
	qCrMATE1_ Fwrd	CrMATE1	CCG GTG TTG CTG TTG GAT GT
	qCrMATE1_ Rev	CrMATE1	CAAGTT TGA AGT AGA ACC CAA GAA GTG CA
	qCrMATE2_ Fwrd	CrMATE2	CTT GAA CTT GCC GCT GCA AAT CTT
	qCrMATE2_ Rev	CrMATE2	CCT TGT CCA CAC AAA CTT TCC AGT GC
	qRps9_ Fwrd	40S ribosomal protein 9	TTG AGC CGT ATC AGA AAT GC
	qRps9_ Rev	40S ribosomal protein 9	CCC TCA TCA AGC AGA CCA TA

Supplemental Table 2. Primers for qPCR.

Oocyte primers	Primer Name	PCR Target	Sequence (5'-3')
pNB1u vector	CrNPF2.9_pNB1u_Fwrd	CrNPF2.9	GGCTTAAU ATG GGA GAC ACC GAA GCA C
	CrNPF2.9_pNB1u_Rev	CrNPF2.9	GGTTTAAU TTA CAT ATA TAT TTT ATT ATC ATT TGT TTC ATC TTC ATC CTC CAT
Primers to generate template for cRNA synthesis	T3T7_pNB1u_Fwrd	CrNPF2.9	AATTAACCCTCACTAAAGGGTTGTAATACGACTCACTATAGGG
	3'UTR_pNB1u_Rev	CrNPF2.9	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTATACTCAAGCTAGCCTCGAG

Supplemental Table 3. Primers for *Xenopus laevis* expression constructs.

Localization primers	Primer Name	PCR Target	Sequence (5'-3')
pSCA_YFP vector	CrNPF2.9_YFP_Fwrd	CrNPF2.9	CTGAGAACTAGTATGGGAGACACCGAAGCACA
	CrNPF2.9_YFP_Rev	CrNPF2.9	CTGAGAACTAGTTTACATATATATTTTATTATCATTTGTTTCATCTTCATCCT
	CrMATE1_YFP_Fwrd	CrMATE1	CTGAGAACTAGTATGGGTTCCAAACAAAACTATGAAATA
	CrMATE1_YFP_Rev	CrMATE1	CTGAGAACTAGTTTCATTGGACAAAGATTTTGGCT
pSCA_CFP vector	TPK_Fwrd	AT5G55630	CTGAGAACTAGTATGTCGAGTGATGCAGCTCGTAC
	TPK_Rev	AT5G55630	CTGAGAACTAGTTCCAGATCCCCTTTGAATCTGA
qPCR primers	qCrMATE1_loc_Fwrd	CrMATE1	CGGTATGATTGGTGGGACATTGAT
	qCrMATE1_loc_Rev	CrMATE1	ATTCGGGGATATCTGTTGAAAAACTT
	qCrMATE2_loc_Fwrd	CrMATE2	GCTTGTGGGCTCTTGTTACTTTCAT
	qCrMATE2_loc_Rev	CrMATE2	TGTCCCCTTAAAAATCCCAACAAATA
	qCrNPF2.9_loc_Fwrd	CrNPF2.9	TGTATTATCTTGCTTGTGCTGGGG
	qCrNPF2.9_loc_Rev	CrNPF2.9	GAAATTGGGATAGTATAACTATATAACC
	qSTR_loc_Fwrd	Strictosidine synthase	CATAGCTCTGTGGGTATATTAGTGT
	qSTR_loc_Rev	Strictosidine synthase	CATAGCTCTGTGGGTATATTAGTGT
	qSGD_loc_Fwrd	Strictosidine glucosidase	CTTCGACAACTTCGAATGGAA
	qSGD_loc_Rev	Strictosidine glucosidase	CTTCTTGACTAACTCAACTAGT
	qHDS_loc_Fwrd	HDS (MEP pathway)	GTCCCTTACTGAACCTCCAGAG
	qHDS_loc_Rev	HDS (MEP pathway)	AATCACCTGTCCTGCGTTGG
	qRps9_loc_Fwrd	40S Ribosomal protein S9	TTACAAGTCCCTTCGGTGGT
	qRps9_loc_Rev	40S Ribosomal protein S9	TGCTTATTCTTCATCCTCTTCATC

Supplemental Table 4. Primers for *Catharanthus roseus* localization constructs and analysis of transcript distribution.