### **Supplementary Figures**

AtNIT4	1	MSMQQETSHMTAAPQTNGHQIFPEIDMSAGDSSSIVRATVVQASTVFYDT	P <mark>ATLDKAERLLSEAAE</mark> NGSQ
AtNIT1	1	MSSTKDMSTVQNATPFNGVAPSTTVRVTIVQSSTVYNDT	PATIDKAEKYIVEAASKGAE
CrNIT2	1	MSGTEQIMSTKFNGVASPTTVRATIVQASTVYNDT	PATLEKAKKYIEEAASNGSE
CrNIT1	1	WSSTVKNTADPVDGVAPSSIVRVTIVQASTVYNNT	PATLDKAEKYVVEAASKGAK
SalNIT1-3	1	WSKTTLKDTTPVSNDFPSTIVRATIVQASSVYNDT	PKTLEKAEKFIAEAASDGAQ
AtNIT3	1	MSSTEEMSSVKNTTQVIGVDPSSTVRVTIVQSSTVYNDT	PATLDKAEKFIVEAASKGAK
Nit6803	-2	GSHMLGKIMLNYTKNIRAAAAQISPVLF <mark>SO</mark>	<mark>ogtmekvldaianaak</mark> kgve
		β1	α1
AtNIT4	71	LVVFPEAFIGGYPRGSTFELAIGSRTAKGRDDFRKYHASAIDVPGPEVER	LALMAKKYKVYLVMGVIERE
AtNIT1	60	LVLFPEGFIGGYPRGFRFGLAVGVHNEEGRDEFRKYHASAIHVPGPEVAR:	LADVARKNHVYLVMGAIEKE
CrNIT2	56	LVVFPEAFIGGYPRGFRFGLAVGVFNEEGRDEFRKYHASAIPVPGPEVEK.	LADMAGKNNVYLVMGAIEKD
CrNIT1	56	LVLFPEAFVGGYPRGFRFGLATGVHNEEGRDEFRKYHASAIKVPGPEVER	LAELAGKTNVYLVMGAIEKD
SalNIT1-3	56	UVVFPEAYVGGYPRGFRFGMAVGV <mark>O</mark> NEEGRDLFRKYHASAIAVPGPEVDK	LAEMARKYKVYLVTGAIEKD
AtNIT3	60	LVLFPEAFIGGYPRGFRFGLAVGVHNEEGRDEFRNYHASAIKVPGPEVER	LAELAGKNNVHLVMGAIEKD
Nit6803	48	LIVFPETFVPYYPYFSFVEPPVLMGKSHLKLY-OEAVTVPGKVTOA	IAOAAKTHGMVVVLGVNERE
		β2 α2 α2*	α3 β3
A≁NTT4	141	GYTLYCTVLEEDSOGLELGKHRKLMPTALERCTWGEGDGSTIPVEDTPIG	KIGAAI CWENRMPSI. RTAMY
AtNIT1	130	GYTLYCTVLFFSPOGOFLGKHRKLMPTSLERCIWGOGDGSTIPVIDIPIG	KIGAAICWENRMPLYRTALY
CrNTT2	126	GYTLYCTALFFSPOGOFLGKHRKLMPTTLERCIWGOGDGSTIPVIDIPIG	KIGAAICWENRMPLYRTALY
CrNIT1	126	GYTLYCTALFFSPOGOFLGKHRKLMPTTLERCIWGOGDGSTIPVIDIPIG	KI GAAT CWENRMPI YRTAI Y
SalNIT1-3	126	GYTLYCIALFESSEGHFLGKHRKVMPTAMERVIWGYGDGSTLPVYDTPLG	KLGGAICWENRMPSLRTSLY
AtNIT3	130	GYTLYCTAL FFSPOGOFLGKHRKVMPTSLERCIWGOGDGSTIPVIDIPIG	KIGAAICWENRMPLYRTALY
Nit6803	113	EGSI.YNTOI.IFDADGAI.VI.KRRKITPTYHERMVWGOG <mark>DGAGI.</mark> RTVDTTVGI	RIGALACWEHYNPLARYALM
		β4 β5 α4 β6	β7 α5
AtNIT4	211	AKGIEIYCAPTAD <mark>S</mark> <mark>RETWLASMTHIALEG</mark> GCFVLSANQFCRRKD	YPSPPEYMFSGSEESLTPDS
AtNIT1	200	AKGIELYCAPTAD <mark>G</mark> SKEWQSSMLHIAIEGGCFVLSACQFCQRKH	FPDHPDYLFTDWYDDKEHDS
CrNIT2	196	AKGIEIYCAPTAD <mark>G</mark> SKEWQSSMLHIAIEGGCFVLSACQFCQRKD	FPEHPDYLFTDLDENKEQDA
CrNIT1	196	;AKGIEIYCAPTA <mark>DG</mark> SKEWQSSMMHIALEGGCFVLSACQFCLRKD	FPDHPDYLFTDWEDYKEDDA
SalNIT1-3	196	AKGVELYCAPTAD <mark>W</mark> SKEWQSSVMHIAMEGGCFVMAACQFCLRKD	YPEDPNYYFTDESDDHSPEA
AtNIT3	200	AKGIEIYCAPTAD <mark>Y</mark> SLEWQASMIHIAVEGGCFVLSAHQFCKRRE	FPEHPDYLFNDIVDTKEHDP
Nit6803	183	AQHEQIHCGQFPGSMV <mark>GQIFADQMEVTMRHHALESG</mark> CFVINATGWL <mark>TAEQ</mark> I	K <mark>LQIT</mark> T <mark>DEKMHQA</mark>
		β8 α6 β9	α6*
AtNIT4	275	VVCAGGSSIISPLGIVLAGPNYRGEALITADLDLGD <mark>IARAKFD</mark> FDVVGHY	SRPEVFSLNIREHPRKAVSF
AtNIT1	264	IVSQGGSVIISPLGQVLAGPNFESEGLVTADIDLGDIARAKLYFDSVGHY:	SRPDVLHLTVNEHPRKSVTF
CrNIT2	260	IVSQGGSVIISPLGQVLAGPNFESEGLITADLDLGEIARAKLYFDVVGHY	TKPDVLSLTVNEDPKKTVTF
CrNIT1	260	IVSQGGSVIISPLGQVLAGPNFESEGLITADLDLGDVARAKLYFDAVGHY:	SRPDVLHLTVNEHPKKTVTF
SalNIT1-3	260	VVSPGGSVIISPLGKILAGPNFGSEGLVTADLDLGDIAKAKLYFDVVGHY:	SRPDIFNLTVNEKEKKPVTF
AtNIT3	264	TVSGGGSVIISPLGKVLAGPNYESEGLVTADLDLGDIARAKLYFDVVGHY	SKPDIFNLTVNEHPKKPVTF
Nit6803	246	LSGGCYTAIISPEGKHLCEPIAEGEGLAIADLD <mark>FSLIAKRKRMM</mark> DS <mark>VGHY</mark>	ARPDLLQLTLNNQPWSALEA
		β12 β13 β14 α7	β15 β16
AtNIT4	345	KTSKVMEDESV	-
AtNIT1	334	VTKVEKAEDDSNK	a-helix
CrNIT2	330	VSKVEKAEDASNK	p-strand
CrNIT1	330	MTKVEKAEDDSNK 2	Targeted residues
SalNIT1-3	330	VSKPEDDSEPQDK	Binding pockot
AtNIT3	334	MTKVEKAEDESNK <u>3→1 3→2 3→4 3→5</u>	Not wisualized
Nit6803	316	NPVTPNAIPAVSDPELTETIEALPNNPIFSH	Luoc visuatized

Supplementary Figure 1. Structural sequence alignment<sup>1</sup> between NIT's relevant for this work.  $\beta$ -strands and  $\alpha$ -helices are colored purple and yellow, respectively and numbered according to Thuku et al<sup>2</sup>. Residues making up the catalytic tetrad are shown in magenta and the residues targeted here for site-saturation mutagenesis are shown in cyan. Regions forming the borders of the substrate-binding pocket are shown in gray. Helical interfaces are shown in light green, tan, light blue and orange and explained in the inset, residues not visualized in the model are outlined in red. *At*NIT1, *At*NIT3, *At*NIT4: nitrilase 1, 3 and 4 from *Arabidopsis thaliana*; *Cr*NIT1, *Cr*NIT2: nitrilase 1 and 2 from *Capsella rubella*; *Sal*NIT1-3: nitrilase 1-3 from *Sinapis alba*; Nit6803: nitrilase from *Synechocystis sp.* PCC6803 (3wuy<sup>3</sup>).



**Supplementary Figure 2. NIT4 and nitrilase superfamily members with bound substrates/ intermediates.** The positions of the CEEK catalytic tetrad residues (\*) are conserved between *At*NIT4 and other nitrilase superfamily members after structural alignment. Substrates/ substrate intermediates (\*\*) localize to the edge of the *At*NIT4 binding pocket (gray surface, #) and extend towards the lid loop (tan). The structures used were: a C171A/V236A mutant of N-carbamyl-D-amino acid amidohydrolase from *Agrobacterium sp.* KNK712 complexed with N-carbamyl-D-methionine (pdb id: 1uf5)<sup>4</sup>; an amidase from *Pseudomonas aeruginosa* with trapped acyl transfer intermediate (pdb id: 2uxy)<sup>5</sup> and a C145A mutant of the amidase from *Nesterenkonia* AN1 complexed with butyramide substrate (pdb id: 4izs)<sup>6</sup>.

# **Supplementary Tables**

	Score: ∑(correlated)- ∑(uncorrelated)					
	P <sub>166</sub>	<b>T</b> <sub>167</sub>	<b>A</b> <sub>168</sub>	L <sub>169</sub>	E <sub>170</sub>	<b>R</b> <sub>171</sub>
D <sub>223</sub>	2	2	1	5	2	2
<b>S</b> <sub>224</sub>	7	7	6	10	7	7
R <sub>225</sub>	5	5	4	8	5	5
E <sub>226</sub>	3	3	2	6	3	3
<b>T</b> <sub>227</sub>	3	3	2	6	3	3

## Supplementary Table 1. Substrate correlation scores.

#### Supplementary Table 2. Primer sequences

Primer		T <sub>m</sub>
R95NNS_Forward	GGT TCT <b>NNS</b> ACC GCT AAA GCA CGA GAT GAC	68.6° -
R95NNS_Reverse	GC GGT <b>SNN</b> AGA ACC AAT AGC CAA TTC AAA GG	78.9°
L169NNS_Forward	CA GCT <b>NNS</b> GAA CGT TGC ATT TGG GGA TTT GG	69.1° -
L169NNS_Reverse	CG TTC <b>SNN</b> AGC TGT AGG CAT GAG TTT GCG	77.7°
S224NNS_Forward	CT GCT GAT <b>NNS</b> AGA GAA ACT TGG CTA GCA	
	TCA ATG ACT CAT ATT GCA CTT GAG	80.1° -
S224NNS_Reverse	GC TAG CCA AGT TTC TCT <b>SNN</b> ATC AGC AGT	82.4°
	AGG TGC ACA ATA AAT CTC AAT GCC	
R95T_Forward	GGT TCT <b>ACT</b> ACC GCT AAA GCA CGA GAT GAC	71.8°
R95T_Reverse	GC GGT <b>AGT</b> AGA ACC AAT AGC CAA TTC AAA GG	72.8°

**Supplementary Table 3**. Library assembly bottlenecks. The number of CFU's estimated from a 1% sample and the number required at the 95% confidence level.

		# CFU's		
	Counted <sup>7</sup>	Full coverage <sup>8</sup>	Top mutant <sup>9</sup>	In top ten <sup>9</sup>
1 mutation	1610 ± 790	172	80	7
2 mutations	3400 ± 1140	8130	2130	138
3 mutations	5200 ± 1410	3.42×10 <sup>5</sup>	55500	2970
DH5α	4.08×10 <sup>6</sup>	(6.02±1.77)×10 <sup>4</sup>	-	-
BL21	7.71×10 <sup>5</sup>	(6.02±1.77)×10 <sup>4</sup>	-	-
# Plated out	4×10 <sup>5</sup>	-	-	-

**Supplementary Table 4. Nitriles included in the selection assay.** All of the nitriles were supplied by Sigma-Aldrich with % purity shown. Those nitrile plates that produced bacterial colonies are shown followed by brackets indicating the number of colonies observed on the plate and the number of days required for visible colony growth.

Nitrile (purity, # colonies, # Days)			
Saturated aliphatic mononitriles	Benzene derivatives		
Potassium Cyanide ≥98% (1, 9)	Benzonitrile 99.9%		
Acetonitrile 97%	Phenylacetonitrile 98%		
Propanenitrile 99% (7, 2)	3-Phenylpropionitrile 99%		
Butanenitrile ≥99% (92, 2)	4-phenylbutyronitrile 99%		
Pentanenitrile 99.5%	Mandelonitrile 97%		
Octanenitrile 97%	3-Hydroxy-3-phenylpropionitrile ?%		
Nonanenitrile 98%	2-Amino-2-phenylacetonitrile 98%		
Dodecanenitrile 99% (150, 13)	Aryl nitriles		
Fluoroacetonitrile 98% (300, 2)	4-hydroxyphenylacetonitrile 98%		
Unsaturated aliphatic mononitriles	3- Cyanophenol 99%		
Acrylonitrile 99.5% (20, 9)	p-Tolunitrile 98%		
3-Butenenitrile 98% (43, 13)	o-Tolunitrile ≥97% (1, 13)		
4-Pentenenitrile 97% (4, 9)	m-Tolunitrile 99%		
6-Heptenenitrile 98% (5, 9)	2-Nitrobenzonitrile ≥99%		
Branched aliphatic mononitriles	3-Nitrobenzonitrile 98%		
Isovaleronitrile 98% (3, 4)	3-Aminobenzonitrile 99%		
ß-cyano-L-alanine 95%	3,5 Dibromo-4-hydroxybenzonitrile (Bromoxynil) 99.6%		
Saturated aliphatic dinitriles	3-Chlorobenzonitrile 99%		
Malononitrile ≥99%	4-Chlorobenzonitrile 99%		
Adiponitrile 99% (22, 2)	Unsaturated aliphatic dinitriles		
Heterocyclic compounds	Crotonitrile 99%		
2 – Furonitrile 99%	Fumaronitrile 98%		
2-Cyanopyridine 99% (1, 9)	Aromatic dinitriles		
3-Cyanopyridine 98% (25, 13)	Benzylidenemalononitrile 98%		
4 Cyanopyridine 98% (300, 2)	1,4-dicyanobenzene 98%		

# **Supplementary Table 5**. Substrate profiles of *At*NIT4 and *At*NIT4 R95T L169A S224Q. The data show means $\pm$ standard deviation (n = 3) n.d.: not detectable

Specific enzyme activity [nkat.(mg protein) <sup>-1</sup> ]			
Substrate	AtNIT4	<i>At</i> NIT4 R95T L169A S224Q	
Fumaronitrile	n.d.	n.d.	
4-cyanopyridine	n.d.	27.24 ± 1.01	
Fluoroacetonitrile	n.d.	34.31 ± 4.88	
2-furonitrile	n.d.	232.89 ± 6.82	
2-cyanopyridine	n.d.	340.58 ± 18.63	
Potassium cyanide	n.d.	378.56 ± 19.37	
ß-cyano-L-alanine	314.16 ± 71.52	n.d.	

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