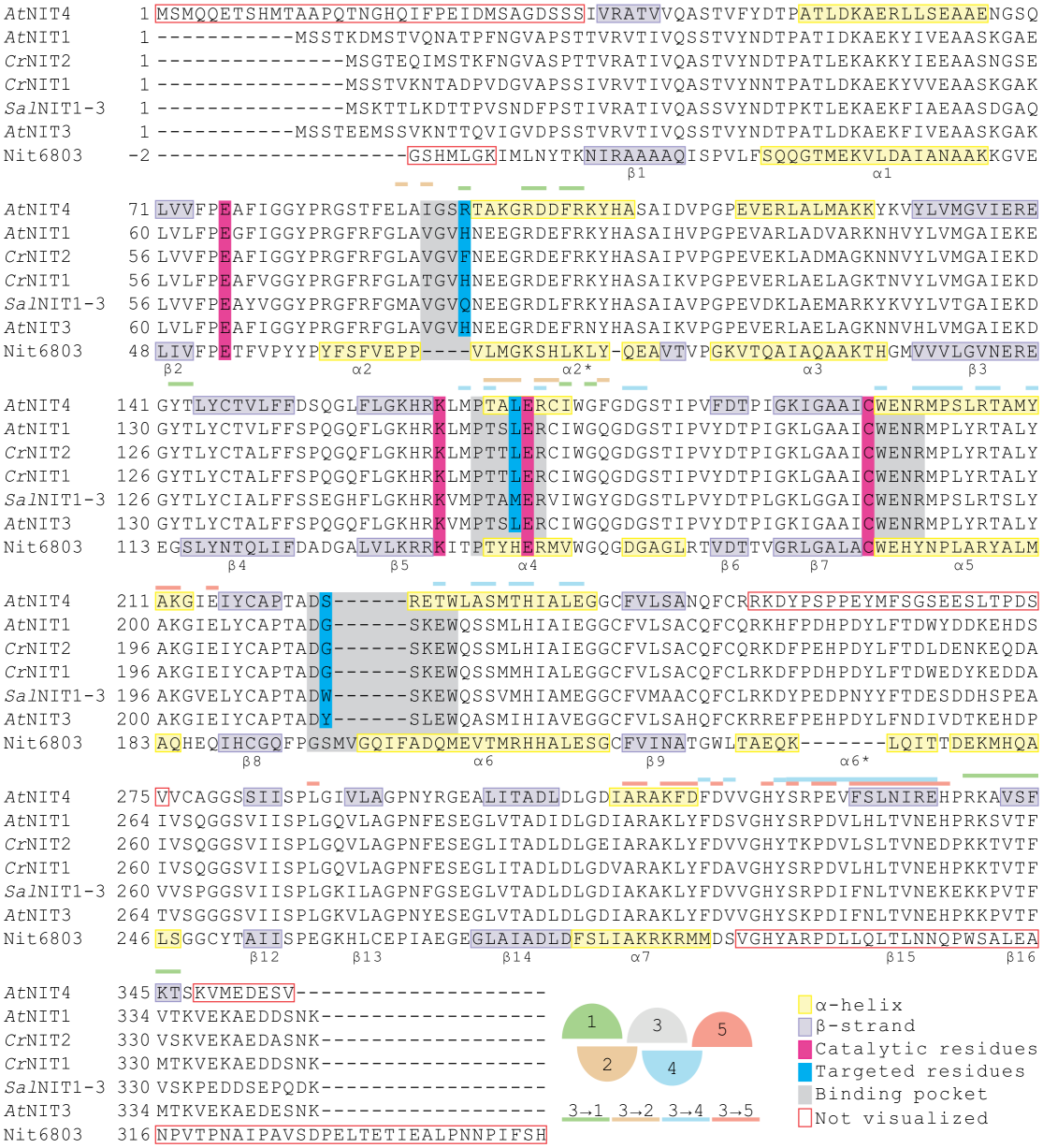
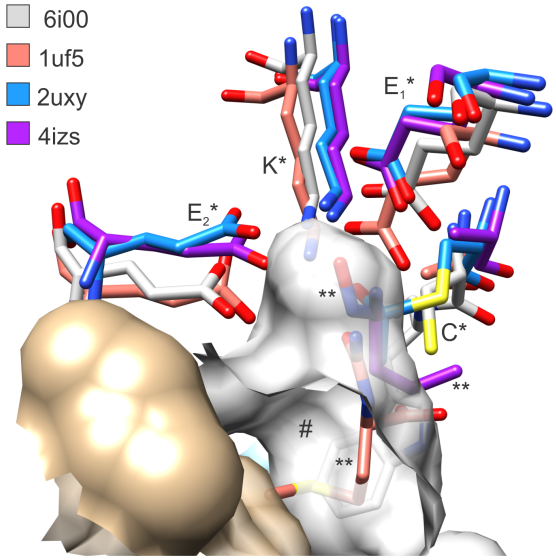
Supplementary Figures



Supplementary Figure 1. **Structural sequence alignment[1] between NIT's relevant for this work**. β-strands and α-helices are colored purple and yellow, respectively and numbered according to Thuku et al[2]. 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[3]).



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)[4]; an amidase from *Pseudomonas aeruginosa* with trapped acyl transfer intermediate (pdb id: 2uxy)[5] and a C145A mutant of the amidase from *Nesterenkonia* AN1 complexed with butyramide substrate (pdb id: 4izs)[6].

Supplementary Tables

Supplementary Table 1. Substrate correlation scores.

	Score: ∑(correlated)- ∑(uncorrelated)					
	P_{166}	**T_{167}**	**A_{168}**	**L_{169}**	**E_{170}**	**R_{171}**
D_{223}	2	2	1	5	2	2
S_{224}	7	7	6	**10**	7	7
R_{225}	5	5	4	8	5	5
E_{226}	3	3	2	6	3	3
T_{227}	3	3	2	6	3	3

Supplementary Table 2. Primer sequences

Primer		**T_m**
R95NNS_Forward	GGT TCT **NNS** ACC GCT AAA GCA CGA GAT GAC	68.6° - 78.9°
R95NNS_Reverse	GC GGT **SNN** AGA ACC AAT AGC CAA TTC AAA GG	
L169NNS_Forward	CA GCT **NNS** GAA CGT TGC ATT TGG GGA TTT GG	69.1° - 77.7°
L169NNS_Reverse	CG TTC **SNN** AGC TGT AGG CAT GAG TTT GCG	
S224NNS_Forward	CT GCT GAT **NNS** AGA GAA ACT TGG CTA GCA TCA ATG ACT CAT ATT GCA CTT GAG	80.1° - 82.4°
S224NNS_Reverse	GC TAG CCA AGT TTC TCT **SNN** ATC AGC AGT AGG TGC ACA ATA AAT CTC AAT GCC	
R95T_Forward	GGT TCT **ACT** ACC GCT AAA GCA CGA GAT GAC	71.8°
R95T_Reverse	GC GGT **AGT** 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[7]	**Full coverage[8]**	**Top mutant[9]**	**In top ten[9]**
1 mutation	1610 ± 790	172	80	7
2 mutations	3400 ± 1140	8130	2130	138
3 mutations	5200 ± 1410	3.42×10^5	55500	2970
DH5α	4.08×10^6	$(6.02\pm1.77)\times10^4$	-	-
BL21	7.71×10^5	$(6.02\pm1.77)\times10^4$	-	-
# Plated out	4×10^5	-	-	-

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 ± standard deviation (n = 3) n.d.: not detectable

	Specific enzyme activity [nkat.(mg protein)$^{-1}$]	
Substrate	***At*NIT4**	***At*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.

Supplementary references

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