1 Retracing the evolution from antimicrobial to targeting 2 peptides

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21 Abstract

22 We experimentally challenged the endosymbiotic hypothesis that organelle-
 23 targeting peptides derive from antimicrobial amphipathic peptides delivered by
 24 the host cell, to which organelle progenitors became resistant. To explore the
 25 molecular changes required to convert such antimicrobial peptides into *bona fide*
 26 organelle-targeting peptides, we expressed a set of 13 antimicrobial peptides of
 27 various origins in the green alga *Chlamydomonas reinhardtii* that serves as a
 28 model for both mitochondrial and chloroplast import. The peptides were
 29 modified to match distinctive features of mitochondrial and chloroplast targeting
 30 peptides, and we assessed their targeting potential by following the intracellular
 31 localization and maturation of a Venus fluorescent reporter used as cargo
 32 protein. We present a temporal evolutionary scenario that emphasizes the early
 33 contribution of exchanging Lysines with Arginines in the sequence of the
 34 antimicrobial peptide, the evolution of a processing site followed by the addition
 35 of unstructured sequence and protein interaction sites that allow the selective
 36 targeting to the chloroplast.

37 Introduction

38 Antimicrobial peptides (AMPs) have recently been proposed to be at the
 39 evolutionary origin of N-terminal targeting peptides (TPs) that direct proteins
 40 into mitochondria and chloroplasts^{1,2}. Part of the innate immune system, AMPs
 41 are produced by virtually all types of cells in a bid to kill or control microbial
 42 adversaries^{3,4}. The proposed evolutionary scenario posits that early during
 43 endosymbiosis, the host used AMPs to attack the bacterial proto-endosymbiont.

44 A bacterial resistance mechanism, whereby the AMP is imported into the
45 bacterial cytoplasm, would have generated a pathway for host proteins to reach
46 the bacterial cytosol – a plausible first step in the evolution of a protein import
47 machinery. Cationic, Helical-Amphipathic Ribosomally-produced AMPs (HA-
48 RAMPs) and TPs share key physico-chemical properties and they were shown, in
49 some instances, to retain cross-functionality⁵: several TPs showed antimicrobial
50 activity and five HA-RAMPs, fused to a cleavage-site containing TP element, were
51 shown to promote the import of a Venus fluorescent protein into either the
52 mitochondria or the chloroplast of the model green alga *Chlamydomonas*
53 *reinhardtii*.

54 The main part of mTPs and the central element of cTPs appear derived from HA-
55 RAMPs on account of a shared cationic, amphipathic helical structure that often
56 encompasses the entire length of the HA-RAMPs² and mTPs^{6,7}. Although plant
57 cTPs have been described as unstructured⁸, algal cTPs more closely resemble
58 mTPs in being helical⁹. Helices were observed by NMR in membrane-mimetic
59 environments in both algal and plant cTPs^{10–14}, and signatures of amphipathic
60 helices can be detected in a majority of cTPs⁵.

61 In addition to the helices, both mTPs and cTPs contain recognition sites at the C-
62 terminus where processing peptidases in the mitochondrial matrix (MPP) and
63 the chloroplast stroma (SPP) cleave off the cargo protein¹⁵. These recognition
64 sites encompass some 10 residues upstream of the cleavage site and are
65 structurally distinct from the rest of the TPs, even showing a weak sequence
66 conservation^{6,16,17}. While targeting information is usually contained within mTP
67 sequences upstream of the cleavage site, targeting by cTPs shorter than ca. 60
68 amino acids often requires downstream unstructured sequence stretches in the

69 N-terminal domain of the mature protein^{18,19}. Besides the amphipathic helical
70 module and the C-terminal cleavage module shared between mTPs et cTPs, it has
71 been argued that distinct features at their N-termini conferred organelle
72 specificity to each set of targeting peptides^{6,16,20-26}.
73 In this study, we systematically introduced modifications into candidate HA-
74 RAMPs to retrace likely steps along the evolution of TPs that improved
75 targeting^{1,2}. We thus retrace the evolutionary switches from HA-RAMPs to TPs
76 by adding N- and C-terminal TP elements to 13 HA-RAMP sequences, chosen for
77 their similarity to TPs, in a bid to generate targeting to the mitochondria or
78 chloroplast in the model green alga *Chlamydomonas reinhardtii*. Besides
79 providing a guide to the most likely evolutionary sequence of events, this dataset
80 also provides new insights into how different TP elements contribute to
81 differential targeting and to the efficiency of protein import. We show that some
82 HA-RAMPs natively contain such sequence elements, with some HA-RAMPs
83 being prone to chloroplast targeting while others show a preference for the
84 mitochondria. Furthermore, we provide evidence for a critical evolutionary
85 contribution of a substitution of cationic residues from Lysine (K) in HA-RAMPs
86 to Arginine (R) in TPs.

87 Results

88 Figure 1 shows the major sequence features of the 13 HA-RAMPs used in the
89 present study (Fig. 1A) together with those of a typical cTP and a typical mTP
90 (Fig. 1B). On the right-hand side, these peptides are represented according to
91 their proportion of α -helical amphipathic structure. Three HA-RAMPs (Magainin
92 II, Brevinin 1B and Dermaseptin S4) were chosen based on a manual comparison

with TP sequences, and a further ten were chosen based on scoring as highly similar to TPs in a recent analysis⁵, such that different AMP families would be represented.

To explore targeting, the 13 HA-RAMPs were systematically modified (Fig. 2), notably by adding non-helical TP elements from the N- and/or C-terminal domains of *Chlamydomonas* Rubisco activase (RBCA) cTP, or a C-terminal domain of similar length from mitochondrial γ -carbonic anhydrase 2 (CAG2) mTP (Fig. 1B, Fig. 2). Peptides carrying the 15 amino-acid long cTP N-terminus (cTP-N) will be denoted ^cP. Similarly, peptides with C-terminal elements from RBCA-cTP (cTP-C) or CAG2-mTP (mTP-C) will be denoted P^c or P^m respectively. Micrographs for three biological replicates per construct are displayed on Supp. Figs. 1-18.

In the absence of any modifications, only Brevinin-2I (B2I) and Magainin 2 (MII) out of the set 13 HA-RAMPs were capable of organelle-targeting visible in fluorescence microscopy (Fig. 2A). When equipped with B2I, the fluorescent reporter Venus shows mitochondrial localization: the Venus signal is observed as a characteristic network pattern that co-localizes with MitoTracker fluorescence (Fig. 3). By contrast, in the case of MII only a small fraction of fluorescence signal in the Venus channel shows co-localisation with the mitotracker dye, signifying that targeting is only partial (Fig. 3).

K/R content contributes to functional divergence between HA-RAMP and TP

We looked for differences in primary sequence that might impede the ability of the remaining 11 HA-RAMPs to target. As illustrated on Supp. Fig. 19, extant HA-RAMPs and organellar TPs display a few differences in their amino-acid content.

117 HA-RAMPs are richer in helix-breaking Glycines (G) but poorer in Prolines (P),
 118 whereas TPs are enriched in Serines (S) compared to HA-RAMPs. Most of the
 119 other amino acid frequencies are broadly similar between the two sets of
 120 peptides and within range of the uniprot average. As expected, both HA-RAMPs
 121 and TPs are poor in acidic residues (D, E) but enriched in basic residues (K or R).
 122 However, their complement in basic residues is markedly different (Fig. 4A):
 123 HA-RAMPs are rich in Lysines (K) whereas TPs are rich in Arginines (R).
 124 In order to see whether these contrasting differences in K/R ratio contributed to
 125 the functional divergence between HA-RAMPs and TPs, we substituted all
 126 instances of R by K in TPs and of K by R in HA-RAMPs. In the rest of the text, the
 127 basic amino acid mostly present in a given peptide P will be indicated by a
 128 subscript as P_R or P_K . HA-RAMPs with $K \rightarrow R$ transition showed reduced
 129 antimicrobial activity, as illustrated on Fig. 4B by the increased minimum
 130 inhibitory concentrations for MII, Dermaseptin S4 (DS4) or Brevinin 1E (B1E).
 131 Conversely, when the natively R-rich Chlamydomonas Rubisco activase (RBCA)
 132 cTP (${}^cCH_R^c$) and mitochondrial γ -carbonic anhydrase 2 (CAG2) mTP (MH_R^m)
 133 underwent systematic $R \rightarrow K$ substitutions (${}^cCH_K^c$, MH_K^m), their ability to target
 134 the chloroplast and the mitochondria were reduced or abolished (Fig. 4C). Note
 135 that TP helical fragments, devoid of N- and C-terminal domains, are denoted as
 136 MH for mTP and CH for cTP (Fig. 1B). These experiments demonstrate the
 137 respective functional contributions of K and R residues to antimicrobial and
 138 organelle targeting activity. We then systematically re-examined the organelle
 139 targeting ability of the set of 13 HA-RAMP that had undergone $K \rightarrow R$
 140 substitutions (Fig. 2B). $MIIR$ now shows full mitochondrial targeting, and $R2G_R$

(Ranatuerin 2G) as well as CP3_R (Cecropin P3) show partial targeting, as evidenced by significant overlap of Venus and mitotracker fluorescence (Fig. 4D).

Targeting peptide elements contribute to targeting

C-termini matter for targeting

Addition of mTP-C, the cleavage-site containing C-terminal element of CAG2-mTP (Fig. 1B) enabled mitochondrial targeting for ES1_R (Esculentin 1S) and R2G_K and improved the targeting already observed for R2G_R and CP3_R, in addition to continued targeting by B2I and MII (Fig. 2CD). The impact of adding cTP-C, the C-terminal element derived from RBCA-cTP (Fig. 1B) is even more important: cTP-C significantly enabled chloroplast targeting (Fig. 2E,F, Fig. 5B), which could be seen in 9 HA-RAMP constructs involving EHF (Enterocin HF), B15 (Bacillocin 1580), LCA (Leucocin A), S1D (Sarcotoxin 1D) and CP3_R. The addition of cTP-C to the helical part of the CAG2-mTP (MH_R^c) even partially retargets the construct to the chloroplast (Fig. 5C), and deletion of cTP-C abolishes chloroplast targeting by RBCA-cTP (cCH_R). By comparison, deletion of mTP-C reduces but does not totally abolish mitochondrial targeting of CAG2-mTP (MH_R), and addition of mTP-C fails to rescue cCH_R (cCH_R^m). Note that the addition of cTP-C was also compatible with mitochondrial localization by B2I and MII and the addition of mTP-C also enabled chloroplast localization by EHF_R (Fig. 2D-F, Fig. 5A,B).

N-termini matter for chloroplast targeting

The sole addition of cTP-N generated at least partial chloroplast targeting (Fig. 2G,H, Fig. 5D) in 6 HA-RAMP constructs, notably by SI Moricin (SIM) as well as EHF_R, B15_R, E1S_R and CP3_R. However, cTP-N also enabled mitochondrial

targeting (Fig. 2G,H, Fig. 5E) in three HA-RAMPs that had not previously shown mitochondrial localization, B1E, S1D and DDM_R (Dermadistinctin M).

The importance of cTP-N as a chloroplast determinant becomes most evident when combined with a C-terminal element: addition of cTP-N to constructs equipped with mTP-C (Fig. 2I,J) resulted in 10 HA-RAMP constructs targeting the chloroplast (EHF, B15, SIM, E1S, LCA_K, CP3_K) as well as 6 constructs showing dual targeting to the chloroplast and the mitochondria (S1D, B1E, MII_R, B2I_R). Combining cTP-N with cTP-C (Fig. 2K,L) generates at least partial chloroplast targeting in 22 out of 26 HA-RAMP constructs (including 1 instance of dual targeting by R2G_R); only Dermaseptin S4 (DS4) fails to show any chloroplast targeting. Consider B2I with cTP-N (cB2I, Fig. 2G-La) as an example of the effect of combining cTP-N with C-termini, shown in Fig. 5F. Equipped with mTP-C, cB2I_{K^m} targets the mitochondria whereas addition of cTP-C (cB2I_{K^c}) generates partial targeting to the chloroplast. Upon K→R, the presence of mTP-C (cB2I_{R^m}) now supports dual targeting and full chloroplast targeting is observed in the presence of cTP-C (cB2I_{R^c}).

Finally, Fig. 5G shows that while addition of cTP-N to the CAG2-mTP abolished mitochondrial targeting (cMH_{R^m}), the cTP-N/cTP-C combination also retargets MH to the chloroplast, and enables partial chloroplast targeting of a control in which the central element is composed of a poly-Alanine tract (cAA^c) devoid of amphipathic properties and of a no peptide control (c-c). The two latter experiments demonstrate that there are enough determinants for recognition of the chloroplast translocon dispersed between the Nter and Cter of a cTP, to target Venus to the chloroplast, albeit with a lower efficiency than when an amphipathic helix is present in between.

To further explore the role of the N-terminal cTP domain, we performed some *in silico* comparisons of the studied peptides. In Principal Component Analyses (on ACC Z-scales⁵) comparing TP N- and C-termini with our 13 HA-RAMPs (Sup. Fig. 20), cTPs and mTPs could be differentiated much better by N- compared to C-termini, supporting N-termini as important specificity determinants. Furthermore, even though algal cTP N-termini are more charged, less hydrophobic and more disordered than higher plant cTPs (Sup. Fig. 21) (in line with algal cTPs being more mTP-like⁹), *Chlamydomonas* cTP N-termini are recognized as chloroplast-targeting in a model trained on *Arabidopsis* TP and vice versa (Sup. Fig. 21), confirming that essential physico-chemical features of chloroplast determination in cTP N-termini are maintained in both lineages.

HA-RAMPs dominate targeting specificity

Considering Fig. 2 by columns reveals that organelle specificity is, to a large extent, determined by HA-RAMPs. Only 3 HA-RAMPs (B2I, R2G, MII; referred to hereafter as the mt-set) account for >70% of all constructs where mitochondrial targeting is seen. Similarly, 5 HA-RAMPs (E1S, LCA, SIM, B15, EHF; referred to as the cp-set) account for the majority (~57%) of all chloroplast targeting, and for two thirds when excluding the cTP-N/cTP-C combination that generates chloroplast targeting across most HA-RAMPs. In some instances, a set of peptide modifications may switch targeting from the mitochondria to the chloroplast, yet the major effect of modifications, i.e. exchanging K→R (Fig. 4, Sup. Fig. 22) or adding TP elements (Fig. 5, 6), is to enhance the targeting ability to an organelle determined by the HA-RAMP primary sequence properties (Fig. 2).

Chloroplast targeting involves longer, less helical peptides

To understand what differentiates mt-set from cp-set HA-RAMPs, we examined the sequence characteristics of our 13 HA-RAMPs with those of well-characterized *Chlamydomonas* targeting peptides (Fig. 6). In *Chlamydomonas*, mTPs are *ca.* 35 residues in length (Fig. 6A). By contrast, most cTPs are significantly longer ($p=0.0017$). The difference is even greater than shown here given that many cTPs require a contribution from post-cleavage site residues for successful targeting^{5,18,19}. Consistently, cp-set HA-RAMPs (in green, Fig. 6A) are longer than mt-set HA-RAMPs (in orange; $p=0.0184$) and require further elongation by addition of TP elements before targeting can be observed (Fig. 2). Most mTPs fold into an amphipathic helix for most of the sequence ($\sim 80\%$ on average, Fig. 6B) starting directly from their N-terminus (Fig. 6C). The fraction dedicated to amphipathic helix formation in cTPs is significantly lower (Fig. 6B, $p<0.0001$), with a longer stretch of non-helical sequence at the N-terminus (Fig. 6C, $p<0.0001$). A PCA analysis based on these properties, plus the fraction of R and K, recapitulates the combined properties within each peptide (Sup. Fig. 23). The PCA shows that cp-set HA-RAMPs are localized with cTPs in the upper left area of the graph, consistent with a lower helix fraction (Fig. 1, Fig. 6B) due to longer sequence stretches upstream of the main helix than in the mt-set HA-RAMPs (Fig. 1, Fig. 6C, $p=0.0199$). Consistent with the importance of these helical features, LCA, the most helical among cp-set HA-RAMPs also shows the highest fraction of only partial chloroplast targeting (3 out of 5 targeting constructs, Fig. 2j rows E,F,K). Furthermore, SIM and E1S, the two cp-set HA-RAMPs with the shortest pre-helix segments (Fig. 7C), both require addition of cTP-N for chloroplast targeting (Fig. 2i,k).

cTPs are predicted to be more prone to protein interaction than mTPs

cTPs appear more likely to interact with proteins than mTPs (Sup. Fig. 24), just like cp-set HA-RAMPs. ‘FGLK’ motifs that are reportedly TOC interaction sites in plant cTPs²⁰ appear shortened to ‘GLK’ motifs in *Chlamydomonas* but they are associated with an increased protein interaction potential in cTPs compared to mTPs ($p < 0.0001$), including within cTP-N and cTP-C elements. However, Hsp70-interaction sites²³, which are commonly found at cTP N-termini²⁰, also occur at a high frequency in mTP sequences and HA-RAMPs (Sup. Fig. 24, 25).

Probing cleavage of HA-RAMP-driven reporter constructs by immunoblotting

To characterize the maturation of HA-RAMP-targeted proteins upon organellar import, we performed immunoblotting experiments (Fig. 7). Indicative examples were selected for clarity; a more comprehensive overview is provided in Sup. Fig. 26.

In the absence of a dedicated cleavage site (Fig. 7A), we found some cleavage did occur but preproteins are also maintained: two bands are observed in constructs that were localized in an organelle by fluorescence imaging (lanes d,f-j). The upper band corresponds to unprocessed Venus, which is a fusion of the reporter with the HA-RAMP construct and migrates at varying positions depending on the length of the presequence. The lower band corresponds to the processed form migrating closer to the “free” Venus position (lane c) depending on the exact site of cleavage. The cTP and mTP controls (lanes a,b) contain 23 residues between cleavage site and Venus (Venus+23), and maintain very little preprotein migrating above. In HA-RAMP constructs by contrast, the majority of Venus remained in the unprocessed top band for constructs targeting the mitochondria (lanes f-j). Nonetheless, the presence of a faint processed form in the lower band

263 just above free Venus shows that some cleavage occurs. The proportion of
264 processed versus unprocessed Venus preprotein is much higher in partially
265 chloroplast-targeted ϵ SIM_K (lane d), hinting at more efficient degradation of the
266 unprocessed form in the chloroplast (see also Sup. Fig. 26).

267 Addition of cleavage sites improves cleavage across organelles: In the presence
268 of either cTP-C (Fig. 7B, Blot 1) or mTP-C (Fig. 7B, Blot 2), which contain the
269 RBCA-cTP and CAG2-mTP cleavage sites respectively, targeting constructs
270 appear to be cleaved at a site corresponding to TP controls (Venus+23).
271 Constructs equipped with either of the two cleavage sites deliver a processed
272 Venus of similar size, independently of whether the mitochondria or the
273 chloroplast are targeted (Fig. 7B). These results indicate that mTP and cTP
274 cleavage sites are recognized in both organelles. We note that in the case of HA-
275 RAMP constructs equipped with mTP-C, cleavage appears to occur not at the
276 cleavage site previously identified for CAG2 (Fig. 7B Blot 2 lane a, top band,
277 corresponding to Venus+23), but instead at or near a second site closer to “free”
278 Venus (compare Fig. 7B Blot 2 lane a, bottom band to lanes b-g), implying
279 processing within ten residues upstream of Venus. Unlike mTP-C (Fig. 7B Blot 2
280 lane h), cTP-C appears to be processed in the absence of targeting (Fig. 7B Blot 1
281 lanes a,b) at a site different to the one used in organelles (Fig. 7B Blot 1 lanes c-
282 h).

283 A switch from K to R (Fig. 7C), which improved targeting, also increased the
284 amount of processed form relative to that of the unprocessed form in all cases,
285 whether due to a gain of targeting (lanes f-i) or an increase in efficiency (lanes b-
286 e), suggesting that cleavage can serve as a proxy for targeting. Indeed, there is a
287 remarkable correlation between which constructs show cleavage, as observed in

immunoblots, and which constructs show targeting, as judged from microscopy (Sup. Fig. 26). We note that faint processed bands can be seen for several unmodified HA-RAMPs (Sup. Fig. 26), suggesting further peptides in addition to B2I_K and MII_K (Fig. 2) may be able to support some amount of targeting that is, however, too partial to be seen in fluorescence microscopy.

Discussion

How TPs may have evolved from HA-RAMPs

In an assessment of the hypothesis that extant TPs are derived from a subclass of antimicrobial peptides that harbor an amphipathic helix, the HA-RAMPs^{1,2}, we studied a systematic series of HA-RAMP constructs. This dataset allows us to propose an evolutionary scenario (Fig. 8) for the conversion of such peptides from antimicrobial to *bona fide* targeting peptides whose properties allow differential targeting between mitochondria and chloroplasts. We showed that a K to R switch reduces cytotoxicity of HA-RAMPs, while increasing targeting. This dual effect must produce a strong selection pressure favoring this exchange at an early step of the endosymbiosis process (Fig. 8A). The underlying mechanism warrants further research, but the finding is in line with the fact that Arginines provide a better uptake efficiency than other cationic residues because of the guanidinium group forming bidentate hydrogen bonds with membrane phosphate groups^{27,28}. Differences in bulkiness or in pK_a^{29,30}, or trans-acting factors such as regulation of targeting through ubiquitination of K³¹, may also play a role. Mitochondria appear able to tolerate the presence of HA-RAMP-containing preproteins, suggesting mTPs could have evolved relatively easily

311 from HA-RAMPs. Even unaltered HA-RAMPs can in some cases deliver cargo,
 312 providing a plausible starting point for protein import. Thus addition of cleavage
 313 sites would likely have come as a second step (Fig. 8B), allowing presequences to
 314 be degraded separately from the cargo protein³². Interestingly, Arginines feature
 315 prominently in *Chlamydomonas* cleavage site consensus sequences¹⁷, thus
 316 cleavage sites may be generated by the K→R switch, potentially accounting for
 317 increased chloroplast-localization among cTP-N bearing constructs (Fig. 2GH).
 318 Since cTPs evolved in a cell that already had mTPs, additional discriminating
 319 elements were needed to avoid large scale mistargeting. We found that N-
 320 terminal elements interact with suitable C-termini to determine chloroplast
 321 targeting. The ~15 N-terminal residues have been suggested as specificity
 322 determinants in the literature before^{20–22}, however, the contribution of this
 323 element to chloroplast targeting has remained under debate. Our data suggests
 324 that rather than providing an absence of Arginines^{21,33} (Sup. Fig. 21) or a
 325 difference in Hsp70 binding sites^{20,25,26} (Sup. Fig. 24), cTP N-termini appear to
 326 contribute unstructured sequence upstream of the amphipathic helix. Thus HA-
 327 RAMPs that intrinsically contained relatively unstructured N-termini proved
 328 most able to support chloroplast targeting even in the absence of cTP-N. The
 329 need to contain unstructured sequence⁸ as well as the increased length of cTPs,
 330 even extending beyond the cleavage site in many cases^{5,18,19}, appear
 331 mechanistically related to the import system. Whereas mitochondrial import
 332 makes use of the proton gradient across the inner membrane to power uptake of
 333 positively charged presequences^{34,35}, energized chloroplast import requires the
 334 cTP to be elongated enough to reach across both TOC and TIC and contact the
 335 translocation motor^{20,36}. Highly structured sequence elements such as a helix at

the Venus N-terminus³⁷ can thus impede chloroplast targeting in the absence of a sufficiently long presequence. HA-RAMPs with suitably unstructured C-termini, such as SIM in this study, were thus most likely to generate chloroplast targeting even in the absence of an added TP C-terminus. Similarly, higher chloroplast targeting among cTP-C constructs may be due to mTP-C being more structured, containing a long predicted amphipathic helix. Unstructured elements at N- and C- termini may also contribute to chloroplast targeting by enabling interactions with protein receptors associated with TOC. In basal algal lineages, a conserved F at cTP N-termini appears to play a role in contacting TOC through an interaction with cytoplasmic POTRA domains of TOC75^{38–40}, mimicking Omp85-interactions in the bacterial cytoplasm mediated by C-terminal sequence motifs⁴¹, which may make a TOC-interacting N-terminus an ancient feature of cTPs. In the green lineage, TOC-interaction has instead been attributed to semi-conserved ‘FGLK’ motifs^{25,42–44}, although the requirement for F appears to be relaxed in *Chlamydomonas* (Fig. 5G)⁴⁵. The presence of GLK sites with high predicted interactivity in cTP-N and cTP-C may explain the high chloroplast targeting potential of constructs equipped with both elements. Thus the evolution of the chloroplast import machinery and chloroplast-specific TPs likely went hand in hand. To generate unstructured, protein interactive N- and C-termini, cTPs may have evolved from HA-RAMPs that already contained unstructured sequence elements (Fig. 8C), as seen for cp-set HA-RAMPs. Alternatively, cTPs may have co-opted existing mTPs that already contained R and a cleavage site, yet were still recognized by cyanobacterial HA-RAMP importers which were part of their resistance mechanism to antimicrobial attack. In this case, the key innovation that generated cTPs may simply have been the addition of an unstructured,

possibly TOC-interacting N-terminus to mTPs (Fig. 8D), which we showed here to be sufficient to retarget mt-set HA-RAMPs equipped with TP C-termini at least partially to the chloroplast.

The legacy of HA-RAMPs: Amphipathic helices and membrane interaction

A direct interaction with the membrane bilayer, rather than protein receptors, would provide a basic mechanism for a first step in organelle targeting and could explain how TPs can be functionally specific whilst diverse in sequence. Core components of the outer membrane translocons, such as the translocation pores TOM40 and TOC75 are widely conserved, and both yeast TOM40 and plant TOC75 retain the ability to interact with TPs in the absence of dedicated receptor proteins^{46–48}. In contrast to the core components, dedicated TP receptor proteins are not conserved between the key eukaryotic lineages and should be regarded as later addition to translocation machineries^{49,50}. Where receptor interactions are required for targeting, binding sites need to be restored when using heterologously expressed proteins, e.g. a leading F needs to be added to plant cTPs to generate cyanelle-targeting in glaucophytes⁵¹ (see previous paragraph). Yet for the most part, heterologous expression of TPs across even large phylogenetic distances generates reliable targeting in both plastids and mitochondria^{52–54}, such as ophistokont mTPs in trypanosomes and cyanelle cTPs in plant chloroplasts. Most strikingly, chromatophore targeting peptides from *Paulinella chromatophora*, an amoeba that has undergone a primary endosymbiosis with an α -cyanobacterium ca. 1 Mya, target tobacco chloroplasts⁵⁵. TP-enabled targeting must therefore use a universally shared mechanism. In HA-RAMPs, amphipathic helices serve to insert into target

membranes. Individual mTPs and cTPs have also been shown to interact with membrane bilayers^{6,14}. Our finding that targeting specificity is largely determined by the sequence of the HA-RAMPs points to amphipathic helical elements being directly involved in determining target specificity. While N- and C-terminal elements aid specificity determination, particularly for chloroplast import, their influence fails to explain why some HA-RAMPs target preferentially the mitochondria or exclusively the chloroplast. In analogy to Rubisco small subunit cTP interacting with chloroplast-mimetic membranes only in presence of the chloroplast-specific galactolipid MGDG⁵⁶, we suggest amphipathic helices may interact specifically with the membranes of the targeted organelle³. Further understanding of lipid interactions, the relationship between extant translocons and bacterial resistance importers, as well as of the peptidases involved in the degradation of TP, should shed new light on the evolutionary process that allowed the stable integration of proto-organelles through endosymbiosis.

Materials and Methods

Construct generation

Venus expression constructs were designed in SnapGene (v4.3.11) and generated by integrating PCR-amplified (Q5 hot start high-fidelity, #M0515, New England Biolabs) DNA fragments into plasmid pODC53⁵⁷ upstream of Venus using Gibson assembly (NEBuilder HiFi DNA assembly, #E5520S, New England Biolabs). Chlamydomonas TP sequences were amplified from genomic DNA extracted from strain T222+ (CC-5101). Templates for codon optimized HA-RAMP, RP and R→K modified TP sequences were obtained by gene synthesis

(Eurofins Genomics). Correct assembly was verified by sequencing (Eurofins Genomics). Linear transformation cassettes were generated through restriction digest with *EcoRV* (New England Biolabs).

Transformation and fluorescence screen conditions

Constructs were transformed into wild-type strain T222+ (CC-5101) using the protocol described previously⁵⁸, except using 4µl of DNA at 2µg/µl. Transformants selected for Paromomycin resistance were grown in 200µl TAP in 96-well plates under 50µmol photons m⁻² s⁻² for 3-5 days and then screened for Venus expression in a fluorescence plate reader (CLARIOstar, BMG Labtech) as described previously⁵⁷.

Microscopy

Cells were grown in 96-well plates and epifluorescence microscopy was carried out as described before⁵. Strains with suspected mitochondrial targeting were subjected to 0.1 µM MitoTracker Red CMXRos (ThermoFisher) in growth medium for 30 min in the dark and washed with TAP prior to taking epifluorescence images with the same settings as before⁵, using a white light LED and a beam splitter at 535nm (423052-0104-000, Zeiss) in combination with a custom-made filter set (559/34 BrightLine HC, Beamsplitter T 585 LP, 607/36 BrightLine HC; AHF Analysentechnik AG) for detecting the MitoTracker signal. Images were adjusted in Fiji⁵⁹ (ImageJ version 2.0.0) as described before⁵, and final figures assembled in PowerPoint (Microsoft PowerPoint for Mac 2011, Version 14.6.3).

Western Blots

Cells were grown up in 10ml TAP Erlenmeyer Flasks under 30 μ mol photons m⁻² s⁻¹, shaken at 160RPM until late mid-log phase. 2ml Aliquots were harvested by centrifugation at room temperature in a tabletop centrifuge, resuspended in 30 μ l storage buffer (1 $\times$ Roche cOmplete™ Mini proteinase inhibitor cocktail, 10 mM NaF, 0.2 M DTT, 0.2 M NaCO₃) and kept at -20°C. 20 μ l boiling buffer (1 $\times$ Roche cOmplete™ Mini proteinase inhibitor cocktail, 10mM NaF, 50 g/L SDS, 200 g/L sucrose) was added to thawed aliquots, which were then boiled for 50 seconds. Cell debris was removed by centrifugation (tabletop centrifuge, maximum speed, 15 minutes, 4°C). Chlorophyll content of 5 μ l supernatant in 1 ml water was estimated as 1 μ g chlorophyll μ l⁻¹ = 0.11 $\times$ (OD₆₈₀ – OD₇₇₀), with Absorbances at 680 nm (OD₆₈₀) and 770nm (OD₇₇₀) recorded in a spectrophotometer. Samples were diluted to load identical volumes, and a constant 10 μ g chlorophyll were loaded onto large gels (35 $\times$ 27cm; resolving gel: 12% Acrylamide, 0.32% Bisacrylamide, 0.25 μ l/ml Tetramethylethylenediamine, 250 mg/L Ammonium Persulfate, 375 mM Tris/HCl pH 8.8; stacking gel: 5% Acrylamide, 0.133% Bisacrylamide, 0.666 μ l/ml Tetramethylethylenediamine, 666 mg/L Ammonium Persulfate, 125 mM Tris/HCl pH 6.8) alongside a molecular size marker (Precision Plus Dual Extra Prestained Protein Standards, BioRad), and gels run overnight at room temperature at 18 A. Proteins in the size range <25 kDa to >75 kDa were transferred onto 0.1 μ m nitrocellulose membranes for 1h at 0.8 A cm⁻², using 5 layers of 3 mm filter paper (Whatman) soaked in transfer buffer 1 (40 mM Aminocaproic acid, 20% Isopropanol, 25 mM Tris/HCl pH 9.4) between at the cathode, followed by gel, membrane and 2 filter papers soaked in transfer buffer 2 (20% Isopropanol, 25 mM Tris/HCl pH 10.4), and finally 3 filter papers

soaked in transfer buffer 3 (20% Isopropanol, 300 mM Tris/HCl pH 10.4) at the anode. Proteins were fixed and visualized by Ponceau Red staining, incubated for 1h at room temperature in block (30 g/L skimmed milk powder, 0.1% tween-20, 1x PBS: 140 mM NaCl, 30 mM KCl, 100 mM Na₂HPO₄, 15 mM KH₂PO₄) and immunolabelled overnight at 4°C using an α-FLAG 1° antibody (Sigma Aldrich F1804, diluted 1:10 000 in block). Membranes were thoroughly washed (0.1% tween-20, 1x PBS), treated for 1h at room temperature with horseradish peroxidase-conjugated α-mouse 2° antibody (diluted 1:10 000 in block), washed again and revealed using ECL recorded on a ChemiDoc (Biorad). Blots were analyzed and converted into TIFF format using the Image Lab software (Version 6.0.0 build 26, BioRad), final figures were assembled in PowerPoint (Microsoft).

Antimicrobial activity assay

Minimum inhibitory concentration assays were performed as described before⁵.

Sequence Data Set

TPs with experimentally-confirmed cleavage sites were recovered from proteomic studies: *Chlamydomonas reinhardtii* cTP (Terashima 2011), mTP (Tardif 2012); *Arabidopsis thaliana* cTP (Ge 2008, Rowland 2015), mTP (Huang 2009). For each peptide, we recovered the full-length protein sequence from NCBI and UNIPROT. Cytoplasmic control sequences were generated by taking N-terminal sequence stretches of random length, matching the distribution of peptide lengths observed in our *Chlamydomonas* TP dataset, from a random subset of *Chlamydomonas* proteins with a validated cytoplasmic location in UNIPROT. For calculating amino acid frequencies, the same HA-RAMP sequences were used as before⁵.

Amphipathic helix prediction

Amphipathic α -helices are predicted as described previously⁵, following the principle of the Heliquest algorithm⁶⁰. Briefly, the approach aims to establish the longest sequence stretch that contains identifiable hydrophilic and hydrophobic faces. The algorithm is iterated such that multiple non-overlapping helices can be found within a given peptide (Fig. 1). Consequently, helix fractions are calculated as number of residues within all predicted helices divided by the total number of residues in the peptide; for evaluating the number of upstream residues, only the longest helix was considered (Fig. 7, Sup. Fig. 23).

ACC terms

In order to evaluate the physicochemical properties of our peptides we used the approach we previously described⁵. Briefly, each amino acid is described in terms of 3 'Z-scale' values⁶¹ that can be interpreted as representing a residue's hydrophobic, steric and electronic properties. Auto-cross-covariances (ACC) between nearby residues are calculated up to a distance of four amino acids, generating a quantitative representation of a given peptide in terms of 36 ACC terms.

Visualization

We performed a principal component analysis (PCA) to visualize the relationships between peptides as described by their 36 ACC terms (Sup. Fig. 20) or by their 5 salient TP properties (Sup. Fig. 23) using python package sklearn v0.22.1⁶².

Analysis of TP N-termini

TP N-termini, defined as the N-terminal 15 amino acids, were analysed (Sup. Fig. 21) as follows: Charge profiles were generated as described in the literature²⁵. Hydrophobicity of TP N-termini was estimated using the Heliquet standalone application⁶⁰. To evaluate disorder, we used IUpred2A, a software that calculates the probability for each residue of being in a disordered region⁶³. We used the default 'Long' setting, which has been reported to be more accurate than the alternative 'Short' setting⁶⁴. The disorder of a given sequence was taken as the mean of the probability values for each residue (average residue disorder probability).

Statistical prediction

To evaluate the predictive power of ACC terms⁵ obtained for 15 residue-long TP N-termini with regards to localization (Sup. Fig. 21) we used a binomial logistic regression classifier. We performed one hundred 5-fold cross validation runs. In each set of one hundred runs, we randomly selected sequences such that the same number of mTPs and cTPs were used. For *C. reinhardtii* we used 33 mTP and 33 cTP and for *A. thaliana* we use 29 mTP and 29 cTP sequences. We used an elastic net penalty in a saga solver with an l1-ratio (a-parameter) of 0, which is equivalent to using only ridge penalty, where all features take part in the model, and a C parameter ($1/\lambda$) of 0.1. The a and $1/\lambda$ parameters were optimised with 10-fold cross validation. Firstly, when $1/\lambda = 1$, the best accuracy (0.82) was obtained with a between 0 and 0.09. Secondly, with a = 0, the best accuracy (0.82) was obtained with $1/\lambda = 0.1$. Custom scripts were written in python (v3.7.6).

Interaction site prediction

Values for the Boman index, a quantitative proxy for whether a peptide is more likely to interact with proteins (high values) or lipids (low values), were calculated as described in the literature⁶⁵. Anchor2 interactivity values, a second proxy for protein interaction potential developed for disordered sequences⁶⁶, were calculated using the IUPred2A standalone application and webserver^{63,67}. Interaction sites for Hsp70 were predicted as described by Ivey and coworkers²³ based on experimental affinity values for individual amino acids. Putatively TOC-interacting 'FGLK' sites were established by searching for presence of F and [P or G] and [K or R] and [A or L or V], and absence of [D and E], within each 8-residue window of a sequence, corresponding to rule 22 by Chotewutmontri and coworkers²⁵ that was recommended by the authors in personal communication. 'FGLK-1' sites were established the same way, but requiring the presence of only three of the four positive determinants. Custom scripts were implemented in R⁶⁸ (v3.6.1) using RStudio⁶⁹ (v1.2.1335).

Statistical analysis

Chlamydomonas TP distributions (Fig. 6, Sup.Fig. 24) were compared using two-sided t-tests ($n=34$ for mTPs and $n=85$ for cTPs), and associated mt-set and cp-set HA-RAMPs were compared using one-sided t-tests based on the trends set by TP (s) ($n=3$ for mt-set and $n=5$ for cp-set HA-RAMPs) in R⁶⁸ using RStudio⁶⁹. Multiple Kruskal statistical tests are performed (same Chlamydomonas mTPs and cTPs as above, plus $n=382$ HA-RAMPs) to evaluate the distribution of amino acids (Fig. 4A, Sup. Fig. 19) in the different groups, followed by Dunn post-hoc tests (scipy v1.4.1).

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Competing interest statement

The authors declare no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Code availability statement

Custom code generated in the course of this project will be made available without restrictions upon request to the authors.

Materials availability statement

All constructs and an informative subset of expressing strains will be made available through the Chlamydomonas resource centre.

Author contributions

FAW, IL and YC conceptualized the project and acquired funds. ODC devised, conducted and visualized the wet-lab experimental part of the investigation, and was chiefly responsible for assigning subcellular localization interpretations based on microscopic evidence. CG carried out the dry-lab/bioinformatic part of the investigation and associated visualization under the supervision of IL, writing custom software to do so (ODC contributed to the analysis of FGLK and Hsp70-binding sites). ODC wrote the original draft of the manuscript. FAW, IL, ODC and YC reviewed and edited the manuscript. All authors approved the final manuscript.

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769

770 **Figure captions**

A) Studied HA-RAMPs

B) TP controls

C) Negative control peptides

Legend:

771

772 **Figure 1. Peptide sequences under study.** Amino acid sequences are shown
773 using the one-letter code. Positively charged residues are highlighted in red. The
774 fraction of the sequence predicted to fold into amphipathic helices (see Methods)
775 is provided by a pie-chart to the right of the sequence; for TPs in (B) this was

776 calculated up to the cleavage site indicated by a downward arrow. Predicted
777 amphipathic helices are highlighted using a cylinder cartoon, with residues
778 contributing to the hydrophilic/hydrophobic face indicated on the top/bottom.
779 No helix could be predicted within RBCA-cTP, and thus the indicated helix is
780 taken from a published NMR-structure obtained in membrane-mimetic
781 conditions¹¹. Note that the two helices of E1S are at an angle to each other and
782 therefore cannot form a single continuous amphipathic helix. Abbreviations: **A)**
783 B2I: Brevinin 2ISb, R2G: Ranatuerin 2G, MII: Magainin 2, B1E: Brevinin 1E, DS4:
784 Dermaseptin S4, DDM: Dermadistinctin M, CP3: Cecropin P3, S1D: Sarcotoxin 1D,
785 E1S: Esculentin 1S, LCA: Leucocin A, SIM: SI Moricin, B15: Bacillocin 1580, EHF:
786 Enterocin HF; **B)** TP – targeting peptide, cTP: chloroplast TP, mTP: mitochondrial
787 TP, RBCA: Rubisco activase, CAG2: γ -carbonic anhydrase, cTP-N: cTP N-terminal
788 element, CH: cTP helix, MH: mTP helix, cTP-C: cTP C-terminal element, mTP-C:
789 mTP C-terminal element; **C)** RP: random peptide.

Figure 2. TP modifications enable HA-RAMP targeting. Chimeric constructs were generated by combining TP elements with HA-RAMPs (see Fig. 1 for sequences; in the ^cAA^c construct in column s, all residues of the helical element “CH” within RBCA-cTP are replaced by Alanines). The overview graph shows in each column (a-s) one of the peptides with a cartoon indicating the position of the predicted amphipathic helices within the sequence; and in each row (A-L) a combination of peptide, K/R modification and TP-element indicated by a cartoon and the following shorthand: P = peptide, _K = contains mostly Lysines, _R = contains mostly Arginines, ^m = mTP element, ^c = cTP element (^cP = cTP-N, ^{P^c} = cTP-C). In each case, an overview of observed targeting is provided by a colour

code (see figure legend). Images for all constructs are shown in Supplementary Fig. 1-18. Note that the present results for B2I^c, MII^c, S1D^c, B15^c and EHF^c (row E, columns a,b,h,l,m) confirmed our previous report on these strains⁵.

Figure 3. Unmodified HA-RAMPs can target the mitochondria. Indicated peptides were inserted upstream of a Venus fluorescent protein reporter in a bicistronic expression system for *Chlamydomonas*⁵⁷. AR^P: hybrid *HSP70a-RBCS2* promoter, i1: *RBCS2* intron 1, i2: *RBCS2* intron 2, FLAG: FLAG-tag, |: stop codon, *: bicistronic bridge sequence tagcat, AphVIII^R: Paromomycin resistance gene, R2^T: *RBCS2* terminator. Epifluorescence microscopy images of selected examples are shown. False-coloured yellow fluorescence from the Venus channel reports on the subcellular localization of the fluorescent reporter. MitoTracker fluorescence, false-coloured in cyan, indicates the position of mitochondria (although parts of the cell exterior are sometimes also stained), with salient features highlighted with orange arrows to indicate co-localisation with the Venus channel. Chlorophyll autofluorescence, shown in magenta, indicates the location of the chloroplast. Scale bars are 5μm. Refer to Fig. 1 for sequences and

Sup. Fig. 1 and 2 for biological replicates. Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting.

Figure 4. K is for killing, R is for targeting. A) Lysine (K) and Arginine (R) frequencies for Chlamydomonas TPs and HA-RAMPs are shown as boxplots (center line: median; box limits: upper and lower quartiles; whiskers: min/max values within 1.5x interquartile range). To give a baseline for comparison, their average across the Uniprot database is given as red horizontal line. Statistically

828 significant differences are indicated with stars: (Multiple Kruskal-Wallis tests
829 followed by Dunn post-hoc tests, *** $p < 0.0001$). See Sup. Fig. 19 for all amino
830 acids. **B)** Grayscale photos of antimicrobial activity growth assays in the
831 presence of dilutions of three selected antimicrobial peptides against *E. coli*
832 strain BW25113 show reduced activity when natively K-rich sequences (K, left
833 hand columns) are altered by replacing all Ks by Rs (R, right hand columns). **C**
834 **and D)** Epifluorescence images of selected chimeric TP control (C) or HA-RAMP
835 (D) constructs are shown as in Fig. 3. MH: mTP helical fragment, CH: cTP helical
836 fragment. Orange arrows highlight co-localisation of Venus signal with the
837 MitoTracker dye, demonstrating mitochondrial localisation; green arrows
838 highlight the presence of Venus signal in the pyrenoid, demonstrating
839 chloroplast localization. Where a construct was interpreted as generating
840 reporter localization in mitochondria or chloroplast, this is indicated by an
841 orange 'm' or a green 'c' respectively.

Figure 5. TP N- and C-termini enable chloroplast targeting by HA-RAMPs.

Exemplary epifluorescence images are shown as in Fig. 3; same convention as in Fig. 4 for Venus localisation. Note that cTP-C contains a pyrenoid localization motif⁷⁰, explaining the particularly pronounced Venus accumulation in the pyrenoid in constructs carrying this element. The result for B2I_K^c in (B) confirms our previous report on this construct⁵.

Figure 7. Chloroplast and mitochondrial targeting HA-RAMPs match cTPs and mTPs respectively. For salient properties, *Chlamydomonas* mTPs and cTPs are compared to our 13 HA-RAMPs. TP distributions are shown as boxplots (center line: median; box limits: upper and lower quartiles; whiskers: min/max values within 1.5x interquartile range), coloured points represent individual peptides. The position of the CAG2-mTP and RBCA-cTP is circled in each graph. The non-zero value for RBCA-cTP in (B) and the single circle in (C) report on the amphipathic helix established by an NMR-study¹¹, since no helix could be predicted by our approach. HA-RAMPs are colour-coded by preferred targeting: orange = mt-set; green = cp-set (cf. Fig. 2). **A)** cTPs are significantly longer than mTPs ($p=0.0017$), and cp-set are longer than mt-set HA-RAMPs ($p=0.0184$). **B)** Helices make up a significantly smaller fraction of cTPs than mTPs ($p<0.0001$), and HA-RAMPs show a similar trend (0.1122). **C)** cTPs contain significantly longer sequence stretches upstream of the longest predicted helix than mTPs ($p<0.0001$), as do cp-set compared to mt-set HA-RAMPs ($p=0.0205$). Reported p -values were obtained through two-way t-tests for TPs and one-way t-tests for HA-RAMPs.

Figure 8. Import is associated with maturation of the preprotein. Western Blots used an α -FLAG antibody on selected constructs as indicated above blots. Where a construct was interpreted as generating reporter localization by fluorescence microscopy (cf. Fig. 2, Sup. Fig. 1-18) in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. In A) some lanes were spliced for clarity; the uncropped blot is provided in Sup. Fig. 26.

Figure 9. A proposed progression for the evolution of targeting peptides from antimicrobial origins. **A)** HA-RAMPs would have first changed K to R and **B)** acquired a cleavage site to become mTPs. To generate cTPs, either **C)** more unstructured HA-RAMPs were recruited directly to become cTPs by undergoing a K to R shift and acquiring a cleavage site separately, or **D)** mTPs acquired an N-terminal non-helical domain. Early cTP N-termini likely interacted with TOC75 POTRA domains via a motif starting with F^{39,40}. **E)** Further evolution would have reinforced the differences between cTPs and mTPs to limit mis-targeting.

Supplementary Fig. 1. Microscopy for biological replicates of Brevinin 2ISb.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 2. Microscopy for biological replicates of Magainin 2.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5µm.

Supplementary Fig. 3. Microscopy for biological replicates of Ranatuerin

2G. Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5 μm.

Supplementary Fig. 4. Microscopy for biological replicates of Brevinin 1E.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 5. Microscopy for biological replicates of Dermaseptin

S4. Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 6. Microscopy for biological replicates of *Dermadistinctin* M. Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 7. Microscopy for biological replicates of Cecropin P3.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 8. Microscopy for biological replicates of Sarcotoxin 1D.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 9. Microscopy for biological replicates of Esculentin 1S.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 10. Microscopy for biological replicates of Leucocin A.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 11. Microscopy for biological replicates of SI Moricin.

Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5µm.

Supplementary Fig. 12. Microscopy for biological replicates of Bacillocin 1580. Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange ‘m’ or a green ‘c’ respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5μm.

Supplementary Fig. 13. Microscopy for biological replicates of Enterocin

HF. Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting.

Scale bars are 5µm.

Supplementary Fig. 14. Microscopy for biological replicates of negative control Random Peptide 1. Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-F), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5µm.

1001 **Supplementary Fig. 15. Microscopy for biological replicates of negative**

1002 **control Random Peptide 2.** Three independent transformant lines (Strains 1-3)

1003 are shown for each combination of modifications (A-L), represented by a cartoon

1004 and a shorthand description (cf. Fig. 2). Where a construct was interpreted as

1005 generating reporter localization in mitochondria or chloroplast, this is indicated

1006 by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics

1007 for partial targeting. Scale bars are 5μm.

Supplementary Fig. 16. Microscopy for biological replicates of TP control

Rubisco activase cTP helical element (CH). Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5µm.

Supplementary Fig. 17. Microscopy for biological replicates of TP control mitochondrial γ -carbonic anhydrase 2 mTP helical element (MH). Three independent transformant lines (Strains 1-3) are shown for each combination of modifications (A-L), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively in bold for full targeting or in italics for partial targeting. Scale bars are 5 μ m.

Supplementary Fig. 18. Microscopy for biological replicates of no-peptide and Alanine-screen controls. Three independent transformant lines (Strains 1-3) are shown for each combination of elements (A-G), represented by a cartoon and a shorthand description (cf. Fig. 2). Where a construct was interpreted as generating reporter localization in mitochondria or chloroplast, this is indicated by an orange 'm' or a green 'c' respectively, in bold for full targeting or in italics for partial targeting. Scale bars are 5 μm.

Supplementary Fig. 19. Comparison of amino acid frequencies reveals K/R shift. A) Amino acid frequencies are shown as boxplots (center line: median; box limits: upper and lower quartiles; whiskers: min/max values within 1.5x interquartile range) for *Chlamydomonas* cTPs in green, *Chlamydomonas* mTPs in orange and HA-RAMPs in blue. To give a baseline for comparison, the average across uniprot is given for each amino acid as red horizontal line. For each residue, different letters underneath distributions indicate a significant difference: groups that share the same letter are significantly different at $p < 0.05$ (Multiple Kruskal Wallis tests followed by Dunn post-hoc tests) from groups attributed a different letter.

Supplementary Fig. 20. Principal component analyses reveal that N- but not C-termini differ between cTPs and mTPs. Principal component (PC) analyses of auto-cross-correlated Z-scale values⁵ for **A)** N-termini (15 residues) and **B)** C-termini (33 residues, encompassing -10 to +23 relative to the cleavage site for TPs) respectively of *Chlamydomonas* cTPs (in green) and mTPs (in orange) as well as the 13 HA-RAMPs under study (in blue; crosses denote HA-RAMPs after K→R).

Supplementary Fig. 21. Unusual algal cTP N-termini share physicochemical differences against mTP N-termini with plant counterparts. **A)** Plots detailing the percentage of uncharged residues²⁵ (i.e. excluding K/R and D/E) for window sizes ranging from 5 to 17 residues (refer to legend for colour code) show that Chlamydomonas cTPs (on the left) are less uncharged at the N-terminus than Arabidopsis cTPs (on the right). Dashed grey lines indicate the average value for randomized sequences (including mature proteins), to provide an estimate of what would be expected by chance. **B)** Equivalent graphs (as in A) for mTPs show a similar charge profile across Chlamydomonas and Arabidopsis. **C-E)** Distributions of salient values as boxplots (center line: median; box limits: upper and lower quartiles; whiskers: min/max values within 1.5x interquartile

range) for mTPs in orange and cTPs in green with *Chlamydomonas reinhardtii* (Cr) on the left and *Arabidopsis thaliana* (At) on the right show that: C) *Chlamydomonas* cTP N-termini contain R at almost the same frequency as mTP N-termini, whereas *Arabidopsis* cTP N-termini contain fewer R; D) *Chlamydomonas* TP N-termini are less hydrophobic than *Arabidopsis* TP N-termini, with plant cTP N-termini showing the highest hydrophobicity; E) *Chlamydomonas* cTP N-termini are more disordered than mTP or *Arabidopsis* cTP N-termini. Points represent individual peptides (note that for integer values, point positions are randomized within ± 0.5 in y as well as in x to increase point separation), population means are shown as black diamonds. **F)** Binomial logistic regression classifier separating TPs using auto-cross-covariance of Z-scales⁵ of the N-terminal 15 residues for *C. reinhardtii*. The left-hand graph shows the distribution of cTPs in green and mTPs in orange over the model output 'mTP score'. Black bars at the bottom of the graph represent scores for cTPs of *Arabidopsis*, showing that plant cTPs are recognized as cTPs by the model trained on algal cTP N-termini (89% recognized as cTPs, with an mTP score < 0.5). Values for our 13 HA-RAMPs are given below the graph. The right-hand graph shows receiver operating characteristic (ROC) curves, plotting the true positive rate (TPR) against the false positive rate (FPR), where the area under the curve (AUC) serves as estimate of the model quality with values above 0.5 indicating that the model is better than random. **G)** Equivalent model (as in F) trained on *A. thaliana* TPs, with black bars now representing *Chlamydomonas* cTPs showing that the majority of algal cTPs are recognized as cTPs by the plant model (75% recognized as cTPs, with an mTP score < 0.5).

Supplementary Fig. 22. K→R generally improves HA-RAMP targeting. A) An overview graph showing how many, out of 13 HA-RAMPs studied, show targeting to either mitochondria (mt) or chloroplast (cp), or both (dual) before and after K→R represented. B-E) Epifluorescence microscopy images of selected examples are shown as in Fig. 3; same convention for Venus localization as in Fig. 4.

Supplementary Fig. 23. HA-RAMP properties determine their targeting propensities. PCA analysis of cTPs (green dots), mTPs (orange dots), cp-set HA-RAMPs (green crosses), mt-set HA-RAMPs (orange crosses) and other HA-RAMPs (dark crosses) based on their length (Peptide Length: PL), number of residues before the longest predicted amphipathic helix (Long Helix Start: LHS), fraction of residues forming the predicted amphipathic helix (Helix Fraction: HF) and fraction of K (Lysine Fraction: KF) and R residues (Arginine Fraction: AF). Arrows on the graph represent the eigenvalues of the individual variables. The first principal component explains 43% of the variance and distinguishes peptides principally according to KF, PL, LHS (pointing left) and RF residues (pointing right). The second principal component explains 24% of the variance and distinguishes peptides principally according to HF. The cp-set HA-RAMPs

group with the most distinguishable cTPs, being longer, with a higher LHS and a lower HF compared to mt-set HA-RAMPs and mTPs.

Supplementary Fig. 24. Higher protein interactivity predicted for cTPs than mTPs. **A, B and F)** For salient properties, Chlamydomonas mTPs and cTPs are compared to HA-RAMPs (cp-set in green, mt-set in orange). Distributions are shown as boxplots (center line: median; box limits: upper and lower quartiles; whiskers: min/max values within 1.5x interquartile range), coloured points represent individual peptides **A)** cTPs show increased protein interactivity ($p=0.0003$), as do cp-set HA-RAMPs ($p=0.0355$), as estimated through Boman values⁶⁵, a proxy developed for AMPs where a value of ca. >2 indicates increased protein interaction potential, and <2 points to membrane interaction. **B)** cTPs show increased protein interactivity ($p<0.0001$), and cp-set HA-RAMPs show a similar tendency ($p=0.0638$), as estimated with Anchor2⁶⁷, developed to predict

1127 the protein interaction potential of disordered sequences. **C)** Both cTPs and
1128 mTPs show a high fraction of peptides (~50%) that have an Hsp70 interaction
1129 site over a 6-residue window at any given position along the sequence in the top
1130 graph, with a peak towards the N-terminus. HA-RAMP constructs shown in the
1131 bottom graph also display a peak in predicted Hsp70-binding sites at the N-
1132 terminus, independently of construct localisation. The value obtained after
1133 randomizing the position of residues is given as dashed grey line to provide an
1134 estimate of how often sites would be expected to occur given amino acid
1135 frequencies (~50%). Randomization was done over the entire sequence
1136 including the cargo protein. **D)** For Arabidopsis cTPs in the top graph, the
1137 percentage of peptides that have a full 'FGLK' site²⁵ (black curve; presence of F
1138 and G/P and L/V/A and K/R and absence of D/E within an 8-residue window)
1139 far exceeds the value obtained after randomizing the position of residues
1140 (dashed black line for full 'FGLK' sites), a proxy for the frequency of the motif
1141 expected at random, for most positions along the sequence up to ~60 residues.
1142 Randomization was done over the entire sequence including the cargo protein.
1143 Reduced 'FGLK'-1 sites containing three out of four elements appear to occur
1144 mostly in the context of full FGLK sites, except for 'GLK' sites for which
1145 randomization is also shown (dashed red line). Arabidopsis mTPs (bottom
1146 graph) also contain 'FLGK' sites at a frequency higher than expected at random
1147 between residues 20-40, and 'GLK' sites upstream of residue 40, but motifs are
1148 less prevalent than in cTPs. **E)** Equivalent graphs (as D) for Chlamydomonas
1149 show that full 'FGLK' sites (black lines) are rare in cTPs (top graph) and actually
1150 more common in mTPs (bottom graph) upstream of residue 40. Reduced 'GLK'
1151 sites (red lines) by contrast far exceed the prevalence expected at random

(dashed red line) ca. up to residue 40. **F)** Protein interactivity, estimated through Boman values, for only those parts of peptides that correspond to FGLK-1 sites is increased for cTPs ($p < 0.0001$), and cp-set HA-RAMPs show a similar tendency ($p = 0.0627$). Note that cTP-C and cTP-N, but not mTP-C, contain 'FGLK-1' sites with predicted protein interactivity. *DS4 contains no 'FGLK-1' sites. Reported p-values were obtained through two-way t-tests for TP and one-way t-tests for HA-RAMPs.

A) Studied HA-RAMPs

B2I	SFLTTFKDLA <u>TKAAKSAGQSVLSTLSCKLSNTC</u>
MII	<u>GIGKFLHSAKKFGKAFVGEIMNS</u>
R2G	<u>GLLDTLKGAAKDIAIGALEKLCKITGCKP</u>
B1E	<u>FLPLLALGAAFLPKIFCKITRKC</u>
DS4	<u>ALWMTLLKKVLKAAAKAALNAVLVGANA</u>
DDM	<u>ALWKTMLKKLGTMALHAGKAAFCAAADTISQ</u>
CP3	<u>WLSKTAKKLENSAKKRISEGAIAIAIKGGSR</u>
S1D	<u>GWIRDFGKRIERVQHTRDATIQITIAVAQQAANVAATLKG</u>
E1S	<u>GLFSKFNKKIKSGLIKIITKTAGKEAGLEALRTGIDVIGCKIKGEC</u>
LCA	<u>KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGNGFW</u>
SIM	<u>GKIPVKAIAKKAGAAIGKGLRAINTASTAHDVVSFFKPKHKKK</u>
B15	<u>VNYGNGVSCSKTKCSVNWGIITHQAFRVTSQVSG</u>
EHF	<u>KYYGNGVSCNKKGCSVDWGKAIGIIGNNAAANLTTGGKAGWK</u>

Interaction sites:

TOC — 'GLK'
HSP70 — 'FGLK'

B) TP controls

	cTP-N	CH	cTP-C
RBCA-cTP	MQVTMKSSAVSGQVRVGARVATRSVRRALQVAVSSRKQMGWRWSIDAGVDASDDQ		
CAG2-mTP	MLKRVGQSLVPFARAGLTQTAESFRGVSSQFFDAPNGPSVKQVLIIEDEW		
	MH		mTP-C

C) Negative control peptides

RP1	NIVVYWNFTLWHMDINARNAGCDGEGS
RP2	DEVNNDNCRIKFKGDISSESDEKMNINY

Supplementary Fig. 25. Hsp70 and (F)GLK sites are present in HA-RAMPs.

Predicted Hsp70 binding sites are underlined in blue. Putative TOC interaction sites are overlined in green: 'FGLK' sites in dark green and 'GLK' sites in light green. See Fig. 1 for detailed annotations. Notably, both TOC and Hsp70-

interaction sites are contained within native HA-RAMPs (A) and within our TP controls (B).

Supplementary Fig. 26. Western Blots for K-bearing constructs. Samples were immunolabelled using an α -FLAG primary antibody. Constructs representing different combinations of TP-element additions are shown in A-F, as indicated by a shorthand description and a cartoon (cf. Fig. 2). HA-RAMPs are consistently arranged in lanes a-m. Note that the order of HA-RAMPs differs from the one in Fig. 2. Control constructs in lanes o-r carry the R $\rightarrow$ K modification. Additional technical controls are present in lanes labeled with greek letters. Sections containing HA-RAMPs, control constructs and technical controls are separated by dotted black lines. Technical controls (cf. Fig. 1): - is no peptide,

1176 cCH_R^c is Rubisco activase cTP, MH_R^m is mitochondrial γ -carbonic anhydrase 2
 1177 mTP, c- is Rubisco activase cTP N-terminal element (15 residues), -^c is Rubisco
 1178 activase cTP C-terminal element (33 residues, -10 to +23 relative to cleavage
 1179 site), e.v. is empty vector (no Venus expression). Constructs that generated
 1180 chloroplast and/or mitochondrial localization are marked with a green 'c' and/or
 1181 an orange 'm' respectively, in bold for full or in italics for partial targeting. Note
 1182 that E) contains two separate blots for the left-hand section up to lane i and the
 1183 right-hand section following lane j.