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Proteinogenic dipeptides, an emerging class of small-molecule regulators

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Abstract

Proteinogenic dipeptides, with few known exceptions, are products of protein degradation. Dipeptide levels respond to the changes in the environment, often in a dipeptide-specific manner. What drives this specificity is currently unknown; what likely contributes is the activity of the different peptidases that cleave off the terminal dipeptide from the longer peptides. Dipeptidases that degrade dipeptides to amino acids, and the turnover rates of the "substrate" proteins/peptides. Plants can both uptake dipeptides from the soil, but dipeptides are also found in root exudates. Dipeptide transporters, members of the proton-coupled peptide transporters NTR1/PTR family, contribute to nitrogen reallocation between the sink and source tissues. Besides their role in nitrogen distribution, it becomes increasingly clear that dipeptides may also serve regulatory, dipeptide-specific functions. Dipeptides are found in protein complexes affecting the activity of their protein partners. Moreover, dipeptide supplementation leads to cellular phenotypes reflected in changes in plant growth and stress tolerance. Herein we will review the current understanding of dipeptides' metabolism, transport, and functions and discuss significant challenges and future directions for the comprehensive characterization of this fascinating but underrated group of small-molecule compounds.

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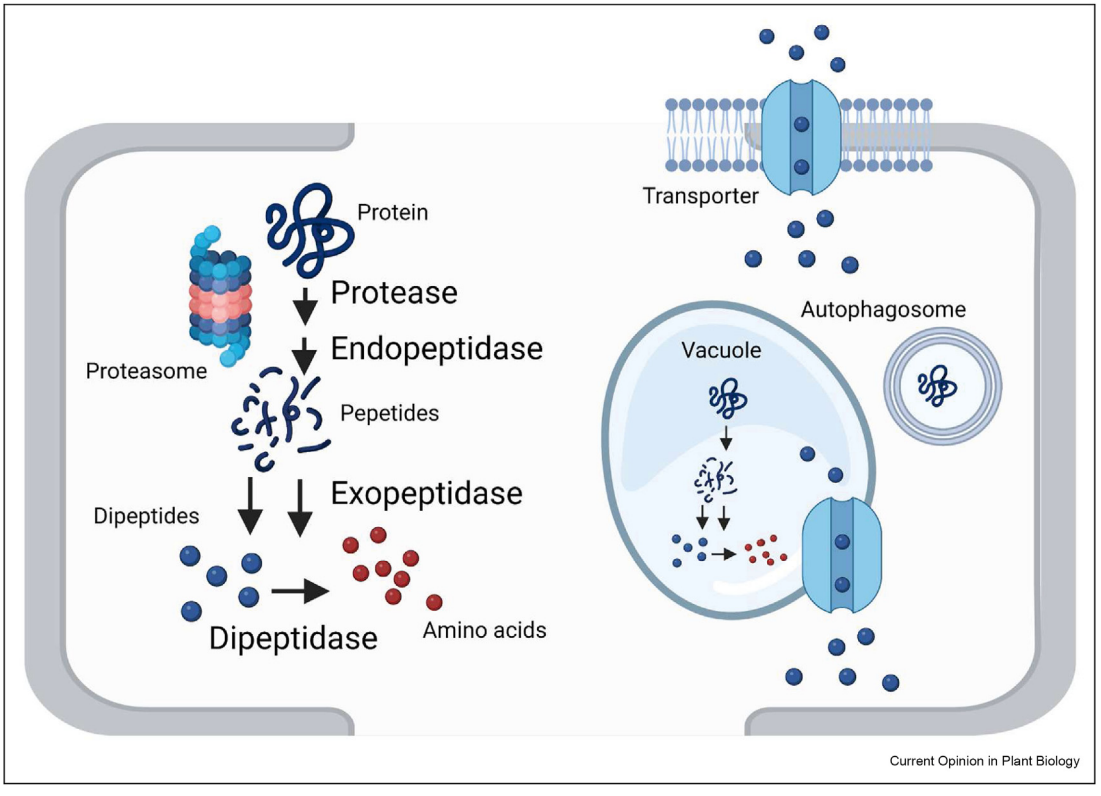
Introduction

Functional diversity reflects the immense chemical diversity of living organisms. Plants produce hundreds of thousands of small molecule compounds, most of which remain to be chemically and functionally characterized [1,2], including compounds previously deemed as solely metabolic intermediates. Prime examples are RNA and protein degradation products such as 2′, 3′-cyclic nucleotides and proteinogenic dipeptides, intermediates of the nucleotide, and amino acids recycling. In recent years both groups of compounds have emerged as signals and regulators. In plants, 2′, 3' - cAMP involvement in stress responses was shown independently by [3–5]. Analogously, also dipeptides have been linked to the regulation of organismal stress responses. Here, we will focus on what is known about dipeptides in plants, but there are numerous published examples of dipeptides having dipeptide-specific bioactivities in animal and fungal cells. To name just a few, dipeptide Tyr-Leu, but not retro-dipeptide Leu-Tyr, was shown to have potent antidepressant-like activity in mice [6,7]. A different tyrosine containing dipeptide Tyr-Ala extends the lifespan of *C.elegans* [8], and prevents acute liver failure in mice [9] and kyotorphin (Tyr-Arg), is a neuroactive dipeptide that has a role in pain regulation in the brain [10]. Accumulation in the X-Leu dipeptides in the rare chronic myelogenous leukemia (CML) stem cell populations activates amino-acid signaling and blocking such accumulation inhibits CML stem cell activity [11]••. Similarly to plants (see below), coral holobionts accumulate N-terminal lysine and arginine dipeptides in response to heat stress [12], whereas in budding yeast, dipeptide levels increase sharply before the diauxic shift when the glucose levels drop in the growth media [13]. Sequential binding of dipeptides with, respectively, basic and bulky-hydrophobic N ′terminal residue to a yeast E3 ubiquitin ligase, UBR1, induces recruitment and subsequent degradation of the dipeptide transporter CUP9 [14]. The above examples support the regulatory functions of dipeptides beyond their role as intermediates of amino acid recycling. But analogous to plants, the exact mechanism underlying dipeptide-associated bioactivities remain poorly characterized.

Dipeptide metabolism

Dipeptides are products of protein degradation (Figure 1). The two major proteolytic pathways responsible for removing misfolded proteins and protein aggregates in eukaryotic cells are the Ubiquitin Proteasome System (UPS) and autophagy [15,16]. Whereas UPS mainly degrades single, misfolded proteins, autophagy can deal with larger macromolecular complexes, such as protein aggregates and whole organelles. In chloroplast and mitochondria, proteases, including the CLP core complex, degrade unwanted proteins and, together with PREP1 and PREP2, also transit peptides from processed preproteins [17,18]. The initial products of protein degradation, irrespective of the proteolytic pathway, are peptides; for instance, proteasome degradation typically yields peptides circa 5−24 residues long [19]. These are subsequently cleaved by a suit of diverse endo- and exo-peptidases. Exopeptidases cleave the terminal peptide bonds and, in the process, can release amino acids, but also dipeptides and tripeptides from either the N' (aminopeptidase) or C′ termini (carboxypeptidase). Known plant dipeptidyl peptidases include organellar oligopeptidase (OOP) [20], cathepsin B [21], and Nudix hydrolase 3 [22]. Exopeptidases can have broad but also very narrow cleavage specificity; for instance, prolyl oligo-peptidases would cleave peptide bonds at the C′ terminal side of prolines generating X-Pro dipeptides. Dipeptides are further degraded to amino acids by the activity of dipeptidases that as exopeptidases can vary significantly in their cleavage specificity. Examples of plant peptidases with dipeptidase activity include leucine aminopeptidases LAP3 and LAP-2 [23], prolyl aminopeptidases PAP1 [24] and PAP2 [25], and glutamate carboxypeptidase AMP1 [26]. Because protein degradation and the corresponding suite of exopeptidases occur in multiple subcellular compartments, we anticipate that dipeptides will be also produced in the different compartments. Mutants defective in the activity of the dipeptidyl peptidases and dipeptidases have pronounced phenotypes, not surprisingly given the importance of protein degradation for amino acid turnover, but also signaling and regulation. For instance, *amp1* mutants are characterized by the alteration to the development of a shoot apical meristem, which the authors hypothesized may be related to the processing of yet unknown

Figure 1



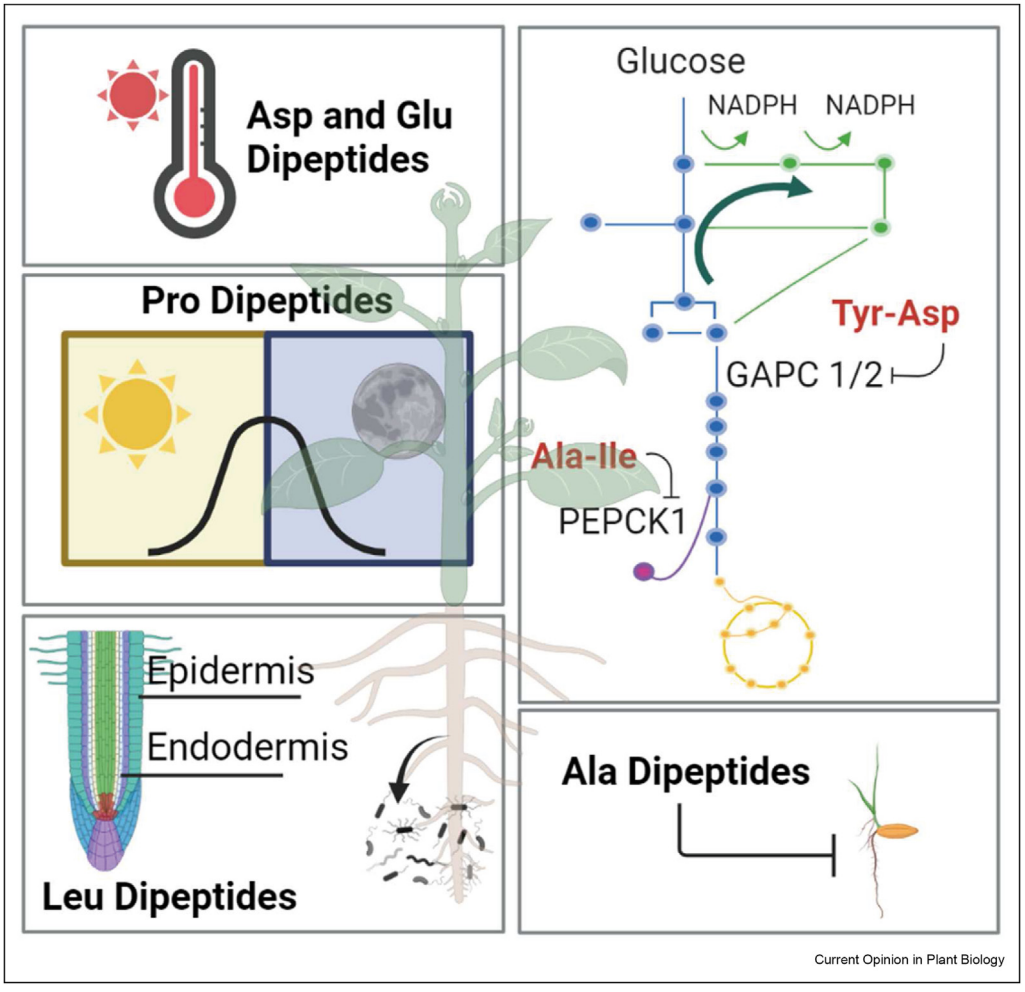
Dipeptide metabolism and transport. Proteinogenic dipeptides are products of protein degradation. The diverse and often highly specific exopeptidases would cleave dipeptides of either N′ or C′ termini of the peptide substrates. Dipeptidase would be further degraded to amino acids by the activity of dipeptidases. The figure depicts two major proteolytic routes, UPS and autophagy, but dipeptides also arise from other known proteolytic pathways. Dipeptides transporters are found in plasma- and vacuole membranes. Figure was created using Biorender.

signaling peptides [26]. In comparison, the *pap1* and *pap2* mutants are compromised in their stress tolerance, which was attributed to proline deficiency [24]. Given the emerging regulatory role of dipeptides (see below), it would be interesting to investigate whether the observed phenotypes can also be related to the altered levels of specific dipeptides. Lastly, it has to be pointed out that although the primary source of dipeptides is protein degradation, there are examples of enzymes, such as kyotorphin synthases in mammals and amino acid ligases in bacteria, that can produce specific dipeptides [27,28].

Dipeptide accumulation patterns

Proteinogenic dipeptides are routinely detected in untargeted mass spectrometry-based metabolomic analysis. However, there are only a few studies that would specifically focus on dipeptides, and related the majority of the published datasets would comprise a handful rather than a comprehensive panel of dipeptide identifications. Absolute quantification of 36 dipeptides across 15 mice tissues/organs revealed organ-specific accumulation patterns, and the concentration of the different dipeptides varied from mid to high fmol/mg [29]•. Similar systematic, absolute quantification of dipeptides is missing in plants. However, we anticipate that analogously to the mice study, also in plants, the concentrations and accumulation pattern of the different dipeptides will vary (Figure 2). For instance, of the 332 dipeptides present in our reference compound library, 63 were measured in a high-density Arabidopsis time-course stress experiment comprising eight environmental conditions varying in temperature and light at 22 time points [30]•. The measured dipeptides not only accumulated in response to the stress conditions, but the different groups of dipeptides responded differently. The most striking was the accumulation of the aspartic acid or glutamic acid-

Figure 2



Schematic overview of dipeptide accumulation and mode of action. Dipeptides accumulate in response to the changing environment. Leu-containing dipeptides are found in the root epidermis, endodermis, and root exudates. Dipeptides were shown to inhibit the seedling establishment and modulate the activity of central carbon metabolism enzymes. The figure was created using Biorender.

containing dipeptides that responded rapidly to multiple stresses, with the levels increasing under heat and dark conditions but decreasing in the cold treatment. In addition to dipeptides also several amino acids, but neither aspartic nor glutamic acid, responded to heat and dark [31]. Guided by the dipeptide-transcript co-expression analysis and by examining autophagy-deficient mutants, we provided evidence implicating autophagy in the observed accumulation of dipeptides in response to the combined heat and dark stress. A different, equally interesting dipeptide accumulation pattern was reported for the diel cycle in Arabidopsis [32]. Among the 179 measured dipeptides, 16 stood out based on their oscillation under short-day conditions and accumulation in *raptor1b* mutant characterized by the substantial reduction of TOR kinase activity. The levels of these 16 dipeptides correlate with the changes in the diurnal carbon status. Seven of the 16 dipeptides contained proline, and four had valine or leucine. Differential dipeptide accumulation was also reported in the developing maize kernels in response to drought [33], in wheat in response to the *Fusarium* virulence factor [34], and in root exudates of Arabidopsis plants [35]•. Activation of the stress-induced mitogen-activated kinases associated with microbial response, MPK3, and MPK6 induced accumulation of leucine, isoleucine, and phenylalanine-containing dipeptides [36], whereas the co-cultivation with a root colonizing fungus *Piriformospora indica* changed the dipeptide composition of the Arabidopsis root exudate [37]. Together these results imply that dipeptides may have a role in shaping plant-microbe interactions. Dipeptide levels respond to changes in the environment. What about during development and in the different cell types? Metabolic profiling of five Arabidopsis root cell types yielded 14 differential dipeptides, the highest levels measured in the endo- and epidermis, attesting to cell-specific accumulation patterns [38]•. In a similar vein, of the 45 dipeptides measured across early leaf growth, four accumulated in the proliferating whereas five in expanding leaf primordia [39]. What determines the specific dipeptide accumulation patterns is an open question, an important one to understand dipeptide functions. We speculate that the key role belongs to the net activity of the different, and often very specific, exo- and dipeptidases. An additional contributing factor may be the identity and turnover rates of the protein and peptide substrates. For instance, the processing of the glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic (GIP) peptides in mice was shown to produce bioactive dipeptides, His-Ala and Tyr-Ala, respectively. Interestingly, and analogously to GIP and GLP1, also His-Ala and Tyr-Ala, regulate glucose homeostasis [40]. Whether cleavage and activation of signaling peptides could also be a source of regulatory dipeptides in plants needs to be investigated.

Dipeptide transport

Dipeptides can also be acquired from the soil or growth media. Because of their efficient up-take, dipeptide mobilization and transport throughout the plant contribute to the nitrogen (N) reallocation between N source and sink organs [41]. Plant dipeptides' transporters are known, and analogously to yeast and animals belong to the proton-coupled peptide transporters (NTR1/PTR) that import the di- and tripeptides in co-transport with protons (Figure 1). Dipeptide transporters are characterized by substrate multi-specificity and can presumably recognize and transport all 8400 di/tripeptides [42]. However, the affinity towards the different dipeptides may significantly differ. For instance, the best-characterized yeast transporter PTR2 has the highest affinity toward dipeptides with aromatic residues, especially at the N-terminus [42]. As these are also energetically costly amino acids, these would be preferentially up-taken from the media. In Arabidopsis, the family contains 53 members, of which four AtPTR1 [43]••, AtPTR2 [44], AtPTR3 [45], and AtPTR5 [43] were shown to transport di- and tripeptides in either the yeast complementation assays or electrode voltage clamp studies using *Xenopus laevis* oocytes. Two more proteins AtPTR4 and AtPTR6 are discussed as dipeptide transporters because of their homology with AtPTR2, but they couldn't be functionally validated [46]. The Arabidopsis PTR dipeptide transporters differ in their subcellular localization and expression pattern. The plasma−membrane-localized AtPTR1 and AtPR5 are responsible for the dipeptide uptake by roots and germinating pollen, respectively [43]. AtPTR2, AtPTR4, and AtPTR6 are localized to the tonoplast. At*PTR2* transcript is highest in seeds, and loss-of-function (LOF) plants are characterized by delayed flowering and defects in seed development and germination [47,48]. *AtPTR3* is highly expressed in the seeds, but also in senescing leaves, and its expression is strongly induced by wounding and pathogen infection [45]. *AtPTR3* LOF lines are sensitive to biotic and abiotic stresses. The expression of dipeptide transporter genes and the phenotypes of the LOF plants support the role of dipeptides in N reallocation between source and sink organs. Senescing leaves are a significant source of the organic N from proteolysis for the flowers and seeds during reproductive growth, whereas mobilization of proteins and N from endosperm plays an essential role during germination and early seedling development [41]. The LOF lines of the different dipeptide transporters can also be seen as a tool for studying the role of dipeptides. For instance, given the role of autophagy in dipeptide accumulation in response to heat stress, it would be interesting to look at the consequences of trapping the dipeptides inside the vacuole by interfering with the vacuole transporters. Blocking dipeptide uptake using a chemical inhibitor, was in the past successfully used by [11]•• to demonstrate the role of

dipeptide accumulation for CML stem cell activity *in vivo*.

Dipeptides, a novel class of small-molecule regulators

The early report of dipeptides having bioactivities distinct from the constituent amino acids in plants comes from herbicide research. Alanine-containing dipeptides, the byproducts of corn gluten hydrolysate, effectively inhibited root growth of the germinating grass seeds (Figure 2) [49]. Growth inhibitory effects of two more dipeptides, Ala-Phe and Lys-Asp, in lines overexpressing the AtPTR5 transporter were presented by [43]. Moreover, supplementation with as low as 100 nM Gly-Ala and Gly-Asp changed root architecture and induced root hair formation in tobacco [50]. So how do the dipeptides exert these bioactivities? Certain dipeptides, such as those containing histidine, were shown to have reactive oxygen-scavenging properties [51]. Moreover, we and others could show that dipeptides bind to proteins [13,52–56]. Hence, we speculate that the dominant mode of dipeptide actions is modulating the function of their protein targets. For instance, of the 237 dipeptides measured in the co-fractionation mass-spectrometry (CF-MS) experiment from Arabidopsis cell cultures, 106 were protein-bound [53]. In contrast, the remaining 131 dipeptides were only present in the protein-free, metabolite-only fractions. In brief, the abovementioned CF-MS experiment entailed the separation of protein-metabolite complexes using size exclusion chromatography followed by the untargeted analysis of the collected fractions. The coelution was used to delineate putative interactors. The protein-bound dipeptides were enriched in valine, leucine, isoleucine, phenylalanine, tyrosine, and glutamine residues, which agrees with reported bioactivities of tyrosine and branched-chain amino acids containing dipeptides, such as Tyr-Leu [7], Tyr-Arg [7,57] and Tyr-Ala. Notably, the elution profiles of the 106 protein-bound dipeptides spanned the whole protein separation range, from large complexes to single proteins, indicating the presence of multiple targets. Yet, the co-eluting dipeptides shared amino-acid residues, such as proline, that point to shared binding specificity. Obtained results are unsurprising given the large chemical space covered by the 106 protein-bound dipeptides. Analogous CF-MS-based experiments in yeast [13] and the thermophilic fungus *Chaetomium thermophilum* [54] similarly retrieved multiple dipeptides separating in the protein complexes. Functional characterization of the selected dipeptide-protein pairings derived from the CF-MS experiments, such as between Tyr-Asp and glyceraldehyde-3-phosphate dehydrogenase (GAPC1/2), revealed a direct inhibitory interaction associated with a significant change in carbon flux and steady-state metabolite levels [58]••. Plant feeding with Tyr-Asp was sufficient to shift glycolytic flux towards the pentose phosphate pathway (PPP) and nicotinamide adenine dinucleotide phosphate (NADPH) production [58], and to mitigate growth penalty associated with oxidative and salt stress (Figure 2). A different dipeptide, Ala-Ile inhibited the activity of a gluconeogenic enzyme PEPCK1. The role of dipeptides in regulating enzymatic activities is highly intriguing as it would constitute a direct regulatory link between protein degradation and central metabolism. Also, in yeast [13] and in *C. thermophilum* [54], dipeptides co-elute with enzymes of central carbon metabolism [55], and dipeptide Ser-Leu was shown to bind and increase the activity of a glycolytic enzyme phosphoglycerate kinase (PGK1) [13]. In addition to enzymes, putative dipeptide interactors derived from the protein-metabolite interaction studies encompass RNA-binding proteins, protein chaperones, and proteasomal subunits. Further functional dissection of the protein-dipeptide interaction network will provide important insight into dipeptides' regulatory and signaling roles.

Outlook

Proteinogenic dipeptides constitute a large group of small molecules covering large chemical diversity; therefore, we also anticipate functional diversity. Dipeptide's presence in protein complexes revealed by CF-MS experiments is unsurprising, given their reported bioactivities in various model organisms. We expect that the functional dissection of protein-dipeptide interactions will provide insight into dipeptide roles and mode-of-action, exemplified by the regulatory interactions between dipeptides and enzymes of the central carbon metabolism. Identifying the exact binding sites will enable genetic strategies to learn about the physiological importance of dipeptide regulation. A different essential question concerns dipeptide biogenesis. However, we and others could demonstrate specificity in the dipeptide accumulation; what drives this specificity is unknown. The contributing factors to consider are (i) the activity of the dipeptidases involved in dipeptide build-up and cleavage, (ii) the turnover rates of the substrate peptides, (iii) dipeptide transport, (iv), and the poorly speculative existence of biosynthetic enzymes, *on par* with kyotorphin synthase. Because of the diversity of possible factors, not an easy question to address but essential to grasp how dipeptide-mediated regulation is exerted and controlled.

Disclosure statement

Given her role as Guest Editor, Aleksandra Skirycz had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Alexandra Dickinson.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Aleksandra skirycz reports financial support was provided by National Science Foundation. Aleksandra Skirycz reports a relationship with National Science Foundation that includes: funding grants.

Data availability

No data was used for the research described in the article.

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- •• of outstanding interest

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