Trends in Plant Science

CellPress

Review

Cell-penetrating peptides for sustainable agriculture

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Cell-penetrating peptides (CPPs) are short (typically 5–30 amino acids), cationic, amphipathic, or hydrophobic peptides that facilitate the cellular uptake of diverse cargo molecules by eukaryotic cells via direct translocation or endocytosis across the plasma membrane. CPPs can deliver a variety of bioactive cargos, including proteins, peptides, nucleic acids, and small molecules into the cell. Once inside, the delivered cargo may function in the cytosol, nucleus, or other subcellular compartments. Numerous CPPs have been used for studies and drug delivery in mammalian systems. Although CPPs have many potential uses in plant research and agriculture, the application of CPPs in plants remains limited. Here we review the structures and mechanisms of CPPs and highlight their potential applications for sustainable agriculture.

Highlights

Cell-penetrating peptides (CPPs), typically comprising 5–30 amino acids, can deliver impermeable cargo molecules across membranes into the cytosol of eukaryotic cells.

CPPs can transport a broad spectrum of molecules, including peptides, proteins, nucleic acids, and small molecules.

Delivery can be accomplished by covalently attaching a CPP to the target cargo or by the formation of a noncovalent complex between the CPP and cargo.

CPPs penetrate cells via two distinct mechanisms: energy-dependent endocytic pathways or energy-independent direct translocation across the plasma membrane.

CPPs could potentially reduce dependence on chemical fertilizers and pesticides, boost crop yields and resilience to extreme weather conditions, improve nutrient uptake from the soil, and enhance nutrient contents in plants.

New technologies for sustainable agriculture

The growing global population, projected to reach 8–10 billion people by 2050 [1], poses unprecedented challenges for agriculture. In light of the adverse effects of excessive fertilizer and pesticide use on the environment, as well as on the well-being of animals and humans, the concept of sustainable agriculture has gained traction. This approach aims to achieve long-lasting food security while minimizing the reliance on agricultural chemicals, thereby protecting the environment from pollution [2]. To accomplish these goals, growers employ diverse methods to enhance soil health, reduce chemical use, and boost the application of biopesticides and biostimulants. Additionally, it has been proposed that genetically modified organisms (GMOs) can contribute to sustainable agriculture [3]. Indeed, GMO crops have enhanced yields, quality, and tolerance to biotic and abiotic stresses [4]. Unfortunately, strict governmental policies and public apprehension regarding GMOs have hindered their broad adoption in sustainable agriculture [3].

Because natural peptides and proteins offer great promise in addressing important issues in sustainable agriculture, such as biostimulation, defense activation, and biopesticide use, they have increasingly been used [5]. However, the current methods of delivering these peptides and proteins into plant cells are not very efficient, requiring the use of high concentrations and thus making their application expensive [6,7]. Therefore, there is an urgent need for novel approaches to enhance the delivery of these peptides and proteins for sustainable agriculture.

CPPs are short peptides (typically 5–30 amino acids) capable of transporting membrane-impermeable cargo molecules into the cytosol of eukaryotic cells [8]. CPPs have been used to deliver a wide variety of cargo into mammalian cells, including small molecules, peptides, proteins, nucleic acids, and nanoparticles [9]. Several CPPs are currently being evaluated in human clinical trials. Over the past two decades, various applications of CPPs in plants have also been explored, with interesting results. In this review, we provide an overview of the structures and mechanisms of CPPs, discuss their current applications in plants, as well as explore their potential uses in sustainable agriculture.

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Discovery, classification, synthesis, and mode of application of CPPs

The discovery of CPPs traces its roots back to the seminal discovery in 1988 that the transactivator of transcription (TAT) protein of HIV-1 effectively enters mammalian cells and translocates into the nucleus [10,11]. Later studies showed that a short peptide sequence within TAT (^{47}YGRKKRRQRRR^{57}) is necessary and sufficient for TAT cellular uptake [12]. In 1994, Alain Prochiantz's team discovered the first CPP, penetratin, demonstrating that a 16–amino acid peptide derived from the third helix of the homeodomain of Antennapedia facilitates cellular translocation of the entire protein [13]. These groundbreaking studies sparked the field of CPP discovery and application as effective delivery vectors for bioactive compounds. Since then, numerous CPPs have been identified, originating from naturally occurring proteins [(TAT, penetratin, and peptide vascular endothelial-cadherin (pVEC)], artificially designed peptides [polyarginines and multiple antigenic peptide (MAP)], or chimeric peptides [transportan and N-methylpurine DNA glycosylase (MPG)] [8,9]. Since their initial discovery, CPPs have made significant strides in diverse applications, emerging as promising tools for diverse therapeutic and diagnostic purposes for various human diseases. Several recent reviews have summarized the current status of the use of CPPs in the diagnosis and treatment of human diseases [14,15].

CPPs are classified as cationic, amphipathic, and hydrophobic on the basis of their sequence compositions [14,15]. Cationic CPPs (e.g., TAT and penetratin) are characterized by their high contents of positively charged amino acids (arginine and lysine), facilitating interactions with the negatively charged cell membrane. Amphipathic CPPs (e.g., transportan and MAP) contain hydrophilic and hydrophobic residues, allowing them to interact with the polar and nonpolar regions of the cell membrane. Hydrophobic CPPs [e.g., binding immunoglobulin protein (Bip4) and melittin], as the name implies, are rich in hydrophobic amino acids, allowing them to interact with the lipid bilayer of the cell membrane. Notably, many CPPs contain positively charged amino acids, such as arginine and lysine, which is a shared trait among many CPPs. These positively charged amino acids play critical roles during cell entry by interacting with the negatively charged components of the cell membrane, including phospholipids and proteoglycans [12,16,17].

Peptides produced using chemical methods such as liquid-phase and solid-phase synthesis can be expensive for large-scale production. Biosynthesis methods, including enzymatic, fermentation, and genetic engineering approaches, are preferred for their cost-effectiveness and the availability of raw materials. Various systems, including bacteria, fungi, and plants, are used for peptide production. Recombinant production is favored for synthesizing long peptides (longer than 25 amino acids) due to its cost-effectiveness, sustainability, and scalability. Efficient recombinant peptide production can lower costs, making peptides more appealing to the pharmaceutical and agricultural industries [18–20].

Diverse cargo molecules, including large proteins, oligonucleotides, and plasmids, have been successfully transported by CPPs into cells while maintaining their bioactive states [21,22]. Delivery may be achieved by covalently conjugating a CPP to the cargo of interest or forming a noncovalent CPP-cargo complex (Figure 1A). Covalent conjugation between a CPP and its cargo can be mediated by noncleavable linkers, such as amides, thioethers, and triazoles, or cleavable linkers, such as disulfides; in the latter case, upon cytosolic entry, intracellular glutathione reduces the disulfide and thus releases the cargo molecule [23,24]. The covalent method proves effective for transporting cargo molecules such as peptides, proteins, and short nucleic acid fragments [25]. For peptide and protein cargos, the CPP and cargo can be genetically fused and recombinantly expressed in *Escherichia coli* or other cells [26] (Figure 1B). However, this strategy is not suitable for delivering plasmid DNAs (pDNAs) due to the lack of convenient

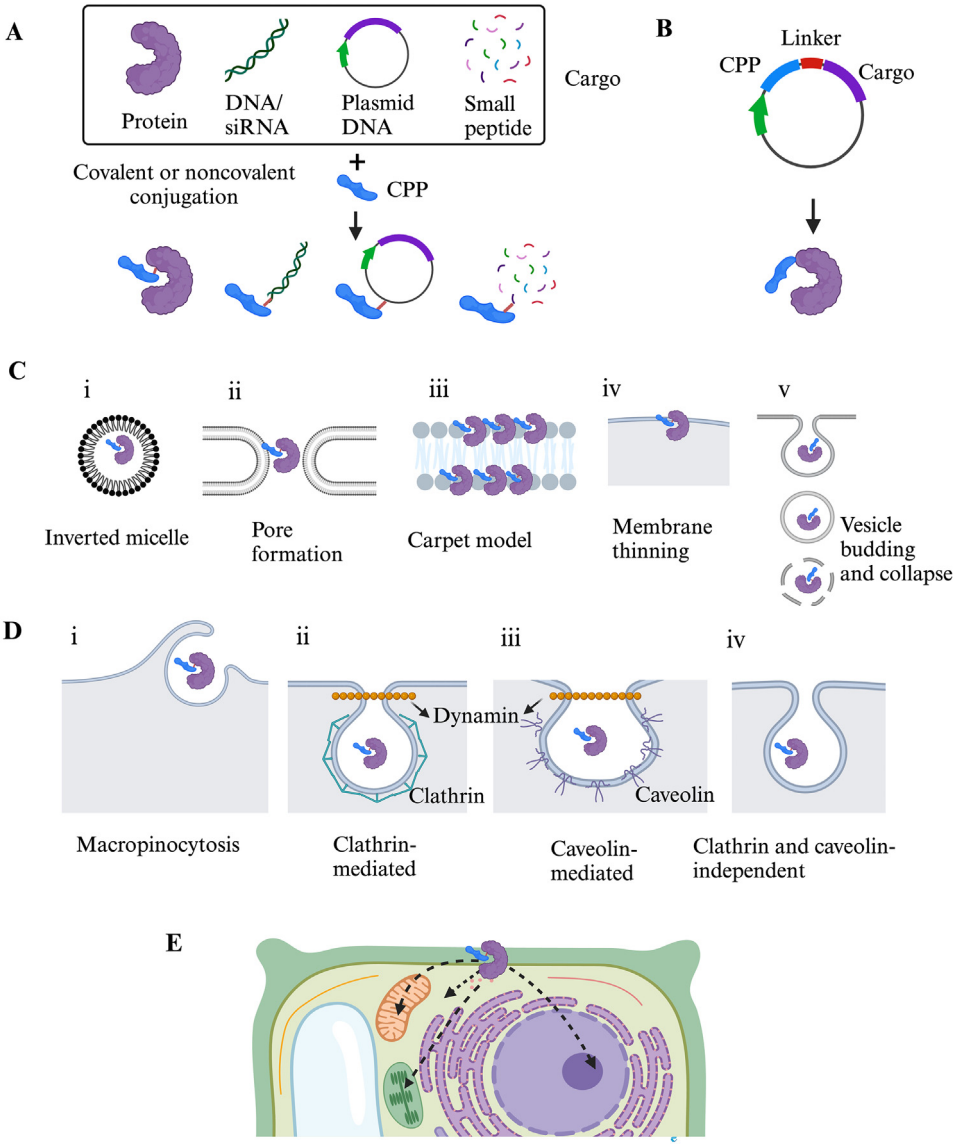

Figure 1. Cell-penetrating peptide (CPP)-cargo complex production and their delivery. There are conventionally two methods to produce CPPs and their associated cargo. (A) Covalently (not suitable for plasmid DNA) as well as noncovalently, the CPP and nucleic acid or other cargo are produced separately. (B) Covalently bonded via engineered plasmid for expressing the CPP and cargo together in any expression systems. There are two possible routes of internalization: (C) direct membrane translocation, which can be through (Ci) inverted micelle formation, (Cii) pore formation, (Ciii) carpet model, (Civ) membrane thinning model, (Cv) vesicle budding and collapse, or (D) the endocytic pathway through (Di) micropinocytosis, (Dii) clathrin-mediated, (Diii) caveolin-mediated, and (Div) clathrin- and caveolin-independent. (E) Once the CPP-cargo complex has crossed the cellular membrane, the complex can be targeted to the nucleus, chloroplast, mitochondria, or cytosol via the signal sequence added. Created with BioRender (www.BioRender.com).

sites for CPP conjugation [27] (Figure 1A). By contrast, negatively charged nucleic acids (e.g., siRNA and pDNAs) readily form noncovalent complexes with positively charged CPPs via electrostatic and hydrophobic interactions. The complexes can enter the cell through endocytosis followed by endosomal escape or direct translocation at the plasma membrane. Many

proteins have been delivered into the cytosol of eukaryotic cells by forming noncovalent complexes with CPPs [28]. The noncovalent method offers flexibility in adjusting the CPP-to-cargo molar mixing ratio, unlike covalent conjugation, where access to specific binding sites is crucial for determining the carrier-to-cargo ratio (Figure 1A).

Mechanism of cell entry by CPPs and CPP-cargo conjugates/complexes

The cell entry mechanism of CPPs has been the subject of intense investigation for three decades and remains contentious to this day. It is generally accepted that CPPs enter the cell via two different mechanisms: energy-independent direct translocation and energy-dependent endocytic pathways at the plasma membrane. In either mechanism, the CPP (and its cargo) must travel across a lipid bilayer (the plasma or endosomal membrane) before reaching the cytosol. Most of the controversy centers on how a CPP and its cargo (often a large biomolecule) crosses a cell membrane without compromising the membrane's barrier function. The concentration and sequence of the peptide, along with the lipid components of each membrane, are crucial in determining the internalization pathways of CPPs into cellular membranes [29]. The uptake route for many cationic CPPs can shift on the basis of peptide concentration; at higher concentrations, rapid cytosolic uptake via direct penetration is observed, whereas lower concentrations predominantly result in endocytosis. The peptide sequence plays a significant role in the uptake mechanism. For arginine-rich CPPs such as TAT and penetratin, the high positive charge due to numerous lysines or arginines enhances local concentrations in the biomembrane through electrostatic interactions. By contrast, amphipathic CPP such as MAP, which rely on helical amphipathicity and a minimum length of four helical turns, have different uptake mechanisms from those of TAT and penetratin [9]. Although the positive charge of CPPs is vital for transporting biomaterials across cellular membranes, it does not completely explain the uptake process. Additionally, the peptide-to-cell ratio can impact the uptake mode, with higher ratios possibly resulting in both direct penetration and endocytosis [30]. The impact of membrane constituents on the uptake mechanism can differ among various CPPs [31]. Several different molecular mechanisms have been proposed over the years and are briefly described later. We posit that CPPs move across both the plasma and endosomal membranes via a recently discovered vesicle budding-and-collapse (VBC) mechanism [32,33].

Direct translocation

Direct translocation often occurs when cells are exposed to high concentrations of cationic CPPs (>10 μM), although this has also been observed for amphipathic and hydrophobic CPPs at very low CPP concentrations (<1 μM) [34]. Several mechanistic models have been proposed to explain how CPPs move across the lipid bilayer, including inverted micelle formation [35], pore formation [36], carpet-like [37] and membrane-thinning models [38], as well as VBC mechanism (Figure 1C). The inverted micelle model involves the invagination of the lipid bilayer, which forms a micelle-like structure that moves across the lipid bilayer. Both positively charged residues (e.g., arginine) and hydrophobic residues (e.g., tryptophan) engage in interactions with the cell membrane, rendering this mechanism less likely for highly cationic CPPs such as TAT and R9 [39]. Pore formation, including the barrel-stave and toroidal pore models, has been proposed for cationic CPPs [15] and amphipathic CPPs with an α-helical structure, such as mastoparan [36]. In the barrel stave model, helical CPPs form a barrel with hydrophobic residues facing the lipid chains, whereas hydrophilic residues create a central aqueous pore. The toroidal model involves lipid bending, keeping the CPPs close to the phosphate headgroups and forming a pore with both CPPs and lipids. In the carpet-like and membrane-thinning models, the interaction between cationic CPPs and negatively charged phospholipids leads to membrane carpeting and thinning, respectively. Local disruption or destabilization of the lipid bilayer allows the CPP and cargo to move across the lipid bilayer. Finally, the VBC mechanism involves the binding of the CPP to the

plasma or endosomal membrane and the clustering of phospholipids into a lipid domain, which buds out as a CPP-loaded vesicle (Figure 1Cv) [32,33]. The subsequent collapse of the budded vesicle releases the CPP (and cargo) into the cytosol. This unique feature renders the VBC mechanism compatible with cargo of any size or with any physicochemical properties. By contrast, all other mechanistic models require the CPP (and cargo) to physically cross a lipid bilayer and therefore partially or totally disrupt the lipid bilayer, making them inherently incompatible with macromolecular cargos.

Endocytosis and endosomal escape

CPPs can be taken up by eukaryotic cells through one or several endocytic mechanisms, including macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis [40] (Figure 1D). In macropinocytosis, extracellular materials (e.g., nutrients) are nonspecifically taken up via invagination of the plasma membrane. Clathrin-mediated and caveolae-mediated pathways involve specific binding to cell surface receptors, triggering the rearrangement of microtubules and actin. Following their endocytic uptake, CPPs and their cargos initially localize inside the early endosome, which matures into a late endosome and eventually fuses with the lysosome, where the intraluminal contents are degraded by hydrolytic enzymes.

To reach the cytosol, which is usually the desired destination for drug delivery, CPPs must escape the endolysosomal compartments, preferably from early endosomes. However, the efficiency of endosomal escape is typically extremely low (approximately 1% or less) for most first-generation CPPs (which are usually linear peptides), resulting in the endosomal entrapment and/or lysosomal degradation of most endocytosed cargos. The mechanistic models described earlier have also been proposed to explain the process of endosomal escape, including the proton sponge model, which describes how histidine-containing CPPs can escape endosomes [41]. Fortunately, second-generation CPPs with vastly improved endosomal escape efficiencies have recently been discovered, including cyclic CPPs [33] and miniature proteins [42]. The availability of highly efficient CPPs allowed the Pei laboratory (one of the coauthors here) to elucidate the mechanism of endosomal escape (i.e., by the VBC mechanism) [43]. Furthermore, we demonstrated that large proteins, such as bacterial toxins, also escape the endosome by the VBC mechanism [44].

Cargo capacity and cytotoxicity of CPPs

CPPs are capable of transporting cargo ranging from small molecules to large proteins up to 120 kDa, both *in vitro* and *in vivo* [8]. Hymel and colleagues observed that the cellular uptake of CPP-cargo complexes is significantly impacted by the cargo's identity [45]. Complexes with positively charged cargoes show increased uptake, regardless of cargo length or the position of arginine residues. Both structured and unstructured CPPs also confirmed the impact of positively charged cargoes, showing a consistent increase in uptake with the addition of positively charged residues. These findings highlight the significance of cargo net charge in the future design of peptide-based reporters or therapeutics [45].

The potential cytotoxicity of CPPs is a significant concern for their bioactivity. Although many studies suggest low cytotoxicity [9,46–50], it is crucial to acknowledge that any substance can become toxic at certain thresholds [9,51]. Liu *et al.* discussed the varying toxicity levels associated with different CPPs [6]. In plant diploid cell transfection, CPPs showed minor effects on the viability of onion epidermal cells and wheat mesophyll cells, as observed through trypan blue and FDA staining [52,53]. Similarly, in wheat haploid transfection, CPPs had weak impacts on microspore cell viability [54]. The toxicity of fusion proteins depends on CPP length and

concentration [55]. Overall, these studies indicate that low CPP concentrations have minimal inhibition on recipient cell viability.

Current applications of CPPs in plants

Compared with the vast volume of research in mammalian cells, the application of CPPs in plants has lagged, likely due to the notion that the plant cell wall would preclude the internalization of large cargo molecules [56]. Plant cells differ markedly from animal cells in their hierarchical structure. The plant cell membrane is surrounded by a rigid cell wall, which comprises a complex network of negatively charged carbohydrates. CPPs are known to become entrapped within the cell wall due to ionic interactions with these negatively charged constituents, and this can destabilize cell walls and plasma membranes [57,58]. The translocation of CPPs through plant cell walls is influenced by their physicochemical properties, including molecular size, shape, and the distribution of positive charges. Consequently, the unique characteristics of plant cells impose specific structural requirements for the internalization of CPPs, which differ from those needed for mammalian cells [59]. Various modifications have recently been implemented in CPPs, including the use of D-form amino acids, branching on backbone sequences, alterations to cyclic structures, and substitutions with nonstandard amino acids, aimed at enhancing their internalization efficiency and stability [6]. Combining CPPs with cationic domains, such as lysine-rich, arginine-rich, and poly(lysine/histidine) domains, has been shown to enhance biomolecule transport efficiency in various plant species [58,60]. Arginine-rich CPPs may exhibit greater persistence in the cell wall of plant cells than lysine-rich CPPs, possibly due to the strong interaction between the arginine side chain and the carboxylate moieties of pectin, a major component of the plant cell wall [61].

Studies on CPP-mediated protein delivery in plants began about 20 years ago. The first such study demonstrated the internalization of well-known CPPs such as transportan, TP10, penetratin, and pVEC into tobacco (*Nicotiana tabacum*) protoplasts [62]. Another study established the efficient delivery of fluorescent proteins into various tissues of tomato (*Solanum lycopersicum*) and onion (*Allium cepa*) using an arginine-rich intracellular delivery (AID) peptide [52]. Similarly, catalytically active indole-3-acetyl-L-aspartic acid (IAA-Asp) hydrolase was successfully delivered into germinating seeds of mung bean (*Vigna radiata*) using the AID peptide [63]. Strikingly, AID peptides were found to rapidly and efficiently deliver proteins into plant cells through covalent and noncovalent mechanisms [64]. Subsequently, the translocation of various CPPs into immature wheat (*Triticum aestivum*) embryos in the presence of a cell membrane-permeabilizing agent was also reported [53]. The successful delivery of protein cargos into live microspore cells was also accomplished by conjugating R9 to mCherry protein via a cleavable disulfide bond [46]. Additionally, the cell-penetrating efficiency of 55 CPPs, most of which had previously been tested in animal cells, was assessed in dicot and monocot plants [65], and some 'winners' were identified. An investigation into the delivery efficiency of two CPPs into rice (*Oryza sativa*) callus revealed that a 5-day-old callus takes up CPPs more efficiently than a 21-day-old callus [66]. Interestingly, a synthetic CPP containing periodic α-aminoisobutyric acids demonstrated prolonged internalization efficiency in *Arabidopsis thaliana* [67]. A recent study demonstrated that a dual peptide-based system improved the transfection efficiency in callus cells (*A. thaliana* and *O. sativa*) as compared with a single carrier peptide [68]. Interestingly, multiple biomolecules were simultaneously delivered into plant cells with an engineered CPP [22].

The intracellular delivery of nucleic acids via CPPs has emerged as a powerful approach to modulating cellular processes in plants, ranging from transient and stable transformation to gene regulation [6]. pDNA, other double-stranded (ds)DNA, and dsRNA have been successfully delivered into diverse plant cell types using TAT [53,54,57,60,69,70], Pep-1 [69], and other arginine-rich

CPPs [71–73]. Additionally, DNA was successfully delivered into plants using BPCH7 by covalently conjugating BPCH7 to a cyclic DNA-binding domain via a disulfide bond [74]. A CPP/EDP-modified micelle system has been developed to overcome endosomal entrapment and deliver DNA into *A. thaliana* [61]. Still other studies have reported CPP-mediated delivery of dsRNA, single-stranded (ss)RNA, and pDNA [22,75]. For example, a recent study demonstrated that delivery of noncovalent CPP/nucleic acid complexes into plant nuclei and chloroplasts could modulate gene expression [7]. Table 1 provides a comprehensive list of CPPs used for diverse applications in plants.

Role and impacts of cell membrane-permeabilizing agents in the foliar application of CPPs

Cell membrane permeabilizing agents play a significant role in plant biology and biotechnology, primarily by facilitating the introduction of exogenous molecules into plant cells. The key roles of chemical permeabilizing agents on plant cell membranes including enhanced uptake of molecules, transformation and transfection, gene expression and function studies, stress response, and defense mechanisms. The impact of chemical permeabilizing agents remains poorly understood, although according to Koley and Bard [76], Triton X-100 can act as a permeabilizing agent at concentrations below the critical micelle concentration (0.17 mM and lower), making it suitable for cell transfection. However, prolonged exposure to even these low concentrations can result in cell death. Enhanced lipid-detergent micelle interactions disrupt the lipid bilayer, increasing membrane permeability and enabling transfection. If excessive quantities of membrane permeabilizing agents are introduced or cells are exposed to chemicals for extended periods, cell death occurs.

Future directions for sustainable agriculture

As more CPP-related research is conducted in plants, new CPPs with high permeability and low toxicity will become available for sustainable agriculture. These CPPs can be used for the delivery of potent biologicals such as proteins, peptides, dsDNAs, dsRNAs, and small molecules into plant cells for disease and insect management, enhanced growth and yield, abiotic stress tolerance, and nutrient content, without altering the plant's genome. Here we describe some potential applications of CPPs for reducing the use of chemical fertilizers and pesticides, increasing crop yields and resilience to extreme weather conditions, and enhancing plant uptake of nutrients from the soil and nutrient contents (Figure 2).

Delivery of bioinsecticides for insect control

Approximately 14% of crop losses worldwide are attributed to insect pests, which are frequently outcompeted by their natural enemies [77]. Although the potential of biologics as biopesticides has been recognized for decades, until recently, their widespread commercialization has been hindered by challenges in their manufacture and delivery. For example, over a century after its discovery, *Bacillus thuringiensis* (Bt) toxin has become a crucial tool for the management of insect pests in agriculture [78,79]. However, several challenges have impeded the application of Bt crystal proteins, such as high production costs, sensitivity to sunlight, and poor penetration into plant tissues. Additionally, the development of Bt resistance in insects has greatly reduced its efficacy for insect management.

To address these challenges, the use of insect-specific peptide neurotoxins from spiders, scorpions, and centipedes as safe and effective biopesticides and the use of dsRNAs targeting critical genes in insects have recently been explored [80–82]. For example, the topical administration of the spider toxin thioredoxin-ω-HXTX-Hv1a fusion protein had insecticidal effects on *Helicoverpa armigera* and *Spodoptera littoralis* caterpillars [83]. Interestingly, a recent study reported the entry

Table 1. Application of different CPPs in plants

Peptide	Sequence	Plant	Refs
Transportan	GWTLNSAGYLLGK(Fl)INLKALAALAKKIL	Tobacco protoplasts	[62]
TP10	AGYLLGKINLKALAALAKKIL-K(Fl)	Tobacco protoplasts	[62]
Penetratin	Fl-RQIKIWFQNRRMKWKK	Tobacco protoplasts	[62]
pVEC	Fl-LLIILRRRIRKQAHAHSK	Tobacco protoplasts	[62]
TAT	GRKKRRQRRRPPQ	Onion, tomato	[52]
R9	RRRRRRRRR	Onion, tomato	[52,64]
TAT	YGRKKRRQRRR	*Vigna radiata*	[63]
BP100	KKLFKKILKYL	Rice, BY-2 cells, leaves of *Arabidopsis thaliana*, tobacco, tomato, poplar, and rice callus	[7,65,108,109]
TAT	Fl-RKKRRQRRR	Wheat immature embryo	[53]
TAT_2	Fl-RKKRRQRRRRKKRRQRRR	Wheat immature embryo; *Triticale* microspore	[53,54,69,70]
M-TAT	Fl-AKKRRQRRR	Wheat immature embryo	[53]
Transportan	Fl-GWTLNSAGYLLGKINLKALAALAKKIL	Wheat immature embryo	[53]
pVEC	Fl-LLIILRRRIRKQAHAHSK	Wheat immature embryo	[53]
N21	NQGISEKQLDQLLCQLISALL	Tobacco, peach, tomato	[88,110]
BP209	G-KKLFKKILKYL-AGPA-GIGKFLHSAK-OH	*Nicotiana benthamiana*	[111]
BP210	S-KKLFKKILKYL-AGPA-GIGKFLHSAK-OH	*N. benthamiana*	[111]
BP211	G-KKLFKKILKYL-AGPA-KFLHSAK-OH	*N. benthamiana*	[111]
Pep-1	KETWWETWWTEWSQPKKKRKV	*Triticale* microspore	[69]
(KH)9-Bp100	KHKHKHKHKHKHKHKHKHKKLFKKILKYL	*A. thaliana*	[75]
Penetratin-Cys-mCherry	RQIKIWFQNRRMKWKK	Wheat microspore	[46]
R9-Cys-mCherry	RRRRRRRRR	Wheat microspore	[46]
Transportan-Cys-mCherry	GWTLNSAGYLLGKINLKALAALAKKIL	Wheat microspore	[46]
MAP-Cys-mCherry	KLALKLALKALKAALKLA	Wheat microspore	[46]
(BP100)2K8	KKLFKKILKYLKKLFKKILKYLKKKKKKKK	*A. thaliana*	[58]
flg15	RINSAKDDAAGLQIA-OH	Pear	[112]
BP178	KKLFKKILKYLAGPAGIGKFLHSAKKDEL	Rice, tomato	[113–115]
Anti-acpP-CPP1	(KFF)3K- eg1-ctcatactat -30	Apple	[116]
BP100(KH)9	KKLFKKILKYLKHKHKHKHKHKHKHKHKH	*A. thaliana*, *N. benthamiana*, soybean, tomato	[7,58]
BPCH7	KKLFKKILKYLHHCRGHTVHSHHHCIR	*A. thaliana*	[74]
BPKH	KKLFKKILKYLKHKHKHKHKHKHKHKHKH	*A. thaliana*	[74]
D-R9	rrrrrrrrr (D form)	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]
R12	RRRRRRRRRRRR	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]
KLA10	KALKKLLAKWLAAAKALL	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]
M511	FLGKKFKKYFLQLLK	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]
E162	KTVLLRKLLKLLVRKI 16	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]
E165	LLKKRKVVRLIKFLLK	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]

Table 1. (continued)

Peptide	Sequence	Plant	Refs
MG2d	GIGKFLHSAKKWGKAFVGQIMNC	BY-2 cells, leaves of *A. thaliana*, tobacco, tomato, poplar, and rice callus	[65]
KH-AtOEP34	KHKHKHKHKHKHKHKHKHMFAFQYLLVM	*A. thaliana*	[117]
Cytcox-KH	MLSLRQSIRFFKKHKHKHKHKHKHKHKHKH	*Nicotiana tabacum*	[117]
BP100(KH)9	KKLFKKILKYLKHKHKHKHKHKHKHKHKH	Rice callus, *A. thaliana*, *N. benthamiana*, *Oryza sativa*	[66,68,118]
BP100CH7	KKLFKKILKYLHHCRGHTVHSHHHCIR	Rice callus	[66]
dTat-Sar-EED4	rrrqrrkkr-$(Sar)_6$-GWWG	*A. thaliana*, *O. sativa*	[68]
BP358 (flg15-BP16)	RINSAKDDAAGLQIA-KKLFKKILKKL-NH2	Pear	[119]
BP359 (BP16-flg15)	KKLFKKILKKL-RINSAKDDAAGLQIA-OH	Pear	[119]
BP16	KKLFKKILKKL-NH2	Pear	[115]
KH9-BP100	KHKHKHKHKHKHKHKHKHKKLFKKILKYL	*N. benthamiana*, *A. thaliana*, soybean, tomato	[7,22]
BP100-R9	KKLFKKILKYLRRRRRRRRR	*N. benthamiana*, *A. thaliana*, soybean, tomato	[7]
R9-BP100	RRRRRRRRRKKLFKKILKYL	*N. benthamiana*, *A. thaliana*, soybean, tomato	[7]

of four CPPs into insect cells and midgut tissues [84]. Fusing insect toxin peptides with CPPs should enhance the insecticidal effects and reduce the cost of field application. CPPs have been employed to deliver dsRNA/siRNA into crops to confer resistance against insect pests [85–87].

Delivery of defense activators and antimicrobial proteins/peptides for disease control

Plant diseases caused by fungi, bacteria, oomycetes, viruses, and nematodes pose a significant threat to crop yield and food security. Although synthetic chemicals have proved to be effective, their environmental and human health impacts have inspired scientists to search for biological alternatives for disease control. To date, numerous defense activators, antimicrobial proteins/peptides, and dsRNAs have shown inhibitory effects on pathogens under laboratory conditions. However, only a few have been used as commercial products for disease control in the field.

For example, efforts to use the defense activator harpin began in the early 2000s [88]. Despite its effectiveness for certain applications, this product has not gained widespread use for crop production. A key limitation lies in the low bioavailability of harpin protein in plants for foliar application, a challenge that could potentially be addressed by fusing it with a CPP. CPP-based carrier systems successfully enable quick and efficient RNAi-mediated gene silencing in plants such as *A. thaliana*, *Nicotiana benthamiana*, tomato, poplar, *N. tabacum* suspension-cell cultures, and rice callus tissue [73,75,89]. Therefore, the foliar application of conjugates/complexes of CPPs and defense-related proteins, peptides, or dsRNA presents a promising avenue for achieving effective disease control in sustainable agriculture.

Promoting plant growth and yield

Plant growth and development are influenced by phytohormones such as auxin, cytokinin, and gibberellin, which govern intercellular communication via intricate mechanisms. Plant growth–regulating peptides (PGRPs) represent a novel class of phytohormones with signaling properties, with significant biological activities at very low concentrations [90,91]. Seven classes of peptide phytohormones [CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE);

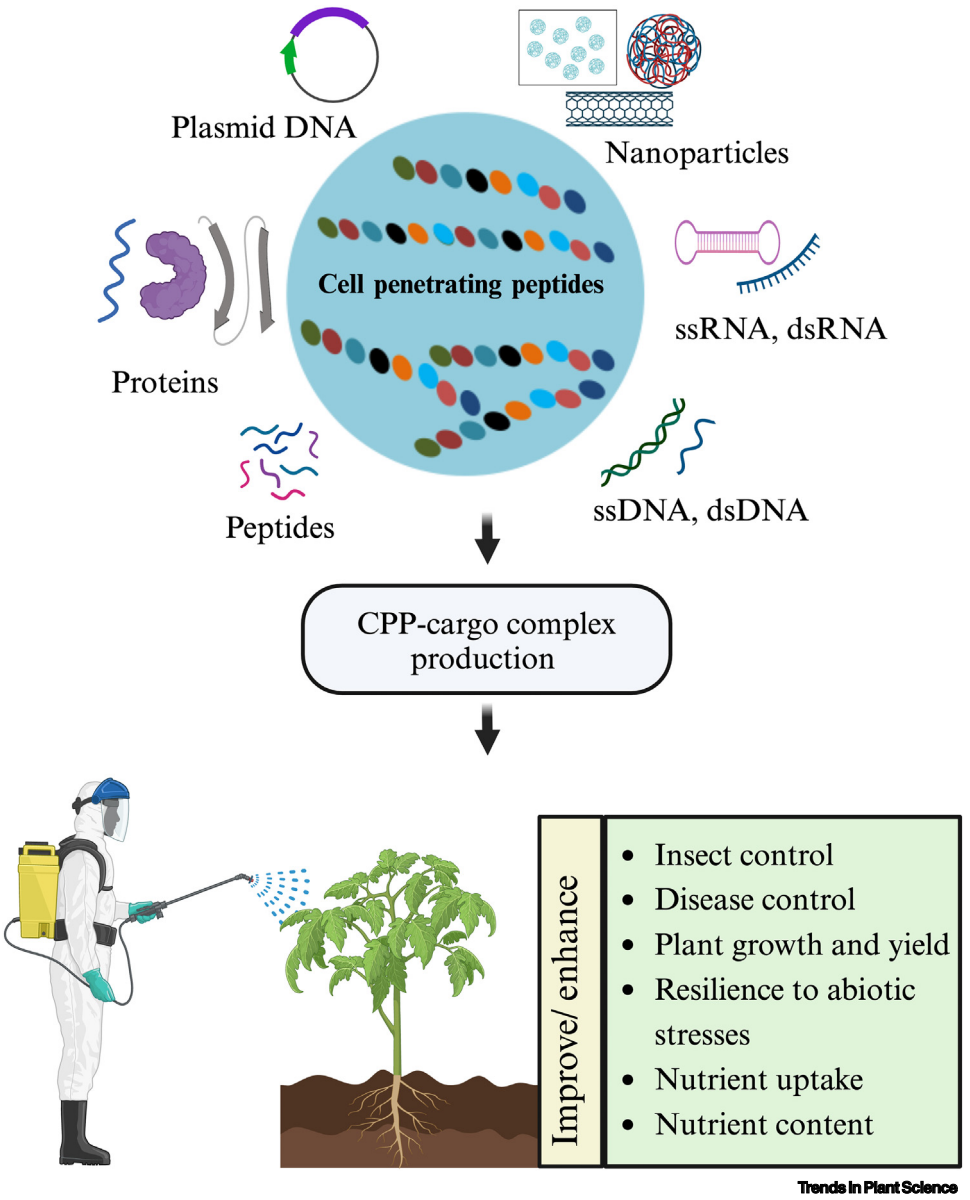

Figure 2. Different applications of cell-penetrating peptides (CPPs) in sustainable agriculture. Created with BioRender (www.BioRender.com). Abbreviations: ds, double-stranded; ss, single-stranded.

phytosulfokines (PSK); PLANT PEPTIDES CONTAINING SULFATED TYROSINE (PSY); INFLORESCENCE DEFICIENT IN ABSCISSION (IDA); C TERMINALLY ENCODED PEPTIDES (CEPs); PLANT ELICITOR PEPTIDES (PEP); RAPID ALKALINIZATION FACTOR (RALF)] have been identified from plant-interacting microbes [92]. In plants, these peptides undergo processing into mature forms, which interact with receptors and trigger growth responses. Besides these peptides, numerous microRNAs (miRNAs) play crucial roles in regulating plant development by affecting cell division, cell proliferation, and both vegetative and reproductive growth [93]. Therefore, CPPs could be used to deliver growth-regulating peptides or miRNAs into plants, representing a promising approach to fostering plant growth.

Enhancing plant tolerance to abiotic stresses

Abiotic stresses such as drought, cold temperature, and salinity are some of the most important problems currently faced by agriculture. Several signaling peptides that play important roles in plant responses to abiotic stresses have been identified and used to increase stress tolerance in plants [94]. Additionally, numerous miRNAs have been identified as potential targets for enhancing tolerance to abiotic stresses, particularly cold, heat, salinity, and drought [93,95,96]. For example, miRNAs were reported to be involved in tolerance against drought (miRNA156 and miR393), salt (miR319 and miR393), and temperature stress (miR9748, miR169, and miR1320) [97,98]. Therefore, CPPs offer a potential solution for improving plant tolerance to abiotic stresses through the delivery of peptides and miRNAs.

Enhancing nutrient uptake from soil

Fertilizers have played a critical role in enhancing yields over the past half-century. However, the excessive use of fertilizers is becoming a major environmental concern due to nutrient leaching that causes water eutrophication and promotes toxic algal blooms. This issue underscores the pressing need for new technologies to improve nutrient use efficiency in crop plants. Recent research indicates that many small peptides are involved in nitrogen signaling and acquisition [99]. For instance, CLE peptides and CEPs are important for systemic nitrogen-demand signaling [100,101]. These peptides stand out as promising candidates for CPP-mediated regulation of nutrient absorption of nitrogen in plants through foliar application.

Improving nutrient contents in plants

Biofortification, whether through conventional breeding or genetic engineering, offers a promising solution to enhance micronutrient availability in crops. Although modern biotechnology has successfully generated transgenic plants with enhanced nutrient contents, strict regulations and public concerns have hindered the broad acceptance of these crops. CPPs offer a potential alternative by delivering essential micronutrient biosynthesis-related proteins/enzymes to enhance nutrient bioavailability in plants. For example, overexpressing bacterial ketolase and hydroxylase genes enhanced ketocarotenoids, such as astaxanthin, in transgenic crops such as potato (*Solanum tuberosum*) and tomato [102,103]. The foliar application of CPP-fused ketolase and hydroxylase proteins represents an attractive strategy for enhancing nutrient levels in crops before harvest.

Concluding remarks

CPPs offer a promising approach for enhancing crop production and protecting plants from biotic and abiotic stresses. The foliar application of CPP-fused biostimulants, defense activators, biopesticides, and key metabolic enzymes may provide a quick and efficient way to enhance crop production, stress resilience, or nutritional content without generating transgenic crops. However, two significant challenges remain: the low cell-penetrating efficiency and metabolic stability of linear CPPs and the difficulty in identifying optimal cargos for crop production and stress resistance. To overcome these challenges, it is imperative to substantially improve the cell permeability, intracellular stability, and specificity of CPPs for the intended compartments within plant cells or tissues. Furthermore, the identification of more effective cargo molecules, such as proteins, peptides, and nucleic acids tailored to specific crop traits, remains crucial. The continuous discovery of new CPPs, facilitated by artificial intelligence (AI) technologies, promises to provide a broader array of CPPs for plants. For instance, AI can accelerate CPP discovery via machine learning predictors, deep-learning frameworks, and online databases that aid in prediction and characterization, advancing CPP development for biomedical and biotechnological uses [104–107]. With the endless possibilities for CPPs, we are only beginning to explore their potential impacts on sustainable agriculture (see also Outstanding questions).

Outstanding questions

How can we increase cell selectivity and penetrating efficiency of CPPs in different plants, especially monocots with thick cuticle layers on the leaf and stem surfaces?

How can we enhance the metabolic stability of CPP-cargo complexes inside the plant cell?

How can we rapidly select the best CPP-cargo combinations for sustainable agriculture?

Can AI and other advanced algorithms identify CPPs that are highly efficient in plant cells?

Is the foliar application of CPP-cargo fusion proteins in crops acceptable to government agencies as well as consumers?

Acknowledgments

This work was supported by grants from the U.S. National Science Foundation's EAGER program (2154863), the Ohio State University's Accelerator Awards program, the Nationwide AgTech Innovation Hub program, the IDI Interdisciplinary Seed Grant Program, the CFAES's Internal Grants Program (2023-028), and the National Institutes of Health (GM122459).

Declaration of interests

The authors have no interests to declare.

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