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Aphid-Proof Plants: Biotechnology-Based Approaches for Aphid Control

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Abstract Aphids are economically significant agricultural pests that are responsible for large yield losses in many different crops. Because the use of insecticides is restricted in the context of integrated pest management and aphids develop resistance against them rapidly, new biotechnology-based approaches are required for aphid control. These approaches focus on the development of genetically modified aphid-resistant plants that express protease inhibitors, dsRNA, antimicrobial peptides, or repellents, thus addressing different levels of aphid-plant interactions. However, a common goal is to disturb host plant acceptance by aphids and to disrupt their ability to take nutrition from plants. The defense agents negatively affect different fitness-associated parameters such as growth, reproduction, and survival, which therefore reduce the impact of infestations. The results from several different studies suggest that biotechnology-based approaches offer a promising strategy for aphid control.

Keywords Agro-biotechnology · Antimicrobial peptide · Aphid · Pest control · Protease inhibitor · Repellent · RNAi

Abbreviations

AMP	Antimicrobial peptide
BPA	Body plan area
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower mosaic virus
CC	Companion cell
dsRNA	double stranded RNA
Eβf	E-beta-farnesene
GM	Genetically modified
GUS	β-glucuronidase
PI	Protease inhibitor
RNAi	RNA interference

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SE	Sieve element
siRNA	small interfering RNA
SHP	Sheath protein

Contents

1 Interactions Between Aphids and Plants

In angiosperms, sieve tubes within the vascular bundles are conduits for nutrition (e.g. photoassimilates) and long-distance signaling. Sieve tubes are composed of longitudinally arranged modules of sieve elements (SEs) and companion cells (CCs). The SEs are connected to one another by sieve pores, which are modified plasmodesmata located in sieve plates at each end of the cell, embedded in the cell wall. Mass flow in the sieve tubes is created by a turgor difference between the source and sink ends [1, 2]. The phloem is subdivided into three functional zones [3]. In the collection phloem, photoassimilates accumulate in the SE/CC complexes within the minor veins of source leaves and are transported to the sieve tube ends in the release phloem of sink tissues such as fruits. The collection phloem and release phloem are connected by the transport phloem, which has a dual function. In the sieve tubes of the transport phloem, photoassimilates are transported from source to terminal sink, but some photoassimilates are released to support growth and maintenance of axial sinks along the pathway [4, 5].

The high nutritional content of sieve tubes makes them an attractive target for bacterial and fungal pathogens as well as insect pests. Therefore, SEs are equipped with defense mechanisms, including chemical components such as protease inhibitors [6] and physical components that lead to SE occlusion and thus the loss of mass flow. These physical mechanisms represent a special challenge for phloem-feeding insects, such as aphids. Ebbing mass flow in the sieve tubes prevents ingestion [7] because this is driven by the high pressure inside the sieve tubes [8]. In this context, there is increasing evidence that callose deposition onto sieve plates and sieve plate occlusion by phloem proteins (P-proteins) are important defense mechanisms against phloem-feeding pests such as aphids (e.g. [9–11].

Aphids are among the most important insect pests in agriculture. There are approximately 4400 aphid species, among which more than 250 are serious pests. In addition to direct damage caused by aphid feeding and the toxic effects of saliva components, the withdrawal of nutrients is detrimental to plant growth and development. Furthermore, aphids can transmit many plant viruses [12], and their excreted honeydew provides nutrition for sooty mold fungi, which can interfere with photosynthesis and reduce the market value of crops.

Aphids as well as other phloem-feeding hemipterans (e.g. whiteflies) have evolved specialized mouthparts (stylets) that penetrate through plant tissues to the sieve tubes, allowing the direct ingestion of sap. The feeding process begins directly after landing, when the aphid presents its labium (the mouthpart containing the stylets) to the surface. The labium is equipped with mechanoreceptors at its apex [13, 14] that can scan the leaf surface, presumably to detect the location of vascular bundles that often have overlying epidermal cells differing in shape from intervening epidermal cells.

The stylet pathway begins with the penetration of the epidermis and continues with stylet movement through the apoplast of the parenchymal tissue [15]. Gel saliva is secreted continuously during this process [16]. As the stylet advances towards the sieve tubes, they briefly perpetrate cortex and mesophyll cells, probably to orient the stylet inside the plant tissue [17]. Aphids take up a small amount of sap from these punctured cells for analysis by the precibarial sensilla located in the food canal between the base of the stylet and the sucking pump [18–20]. After penetrating an SE and identifying it as a source of nutrition, the aphid secretes watery saliva, which is followed by ingestion [21]. Although this feeding behavior has been described in detail by [22], the roles of the two types of saliva are not well understood but may play a key role in aphid–plant interactions [16].

Gel saliva is secreted onto the leaf surface at the penetration point and continues to be secreted as the stylet advances. It is secreted as a liquid but rapidly forms a solid salivary sheath that envelops the stylet. The most prominent saliva protein, the sheath protein (SHP), may be responsible for sheath hardening due to its high cysteine content. It is assumed that SHPs are solidified (gelled) by oxidation, through the formation of disulfide bonds among cysteine residues [16, 23–25]. Several functions have been proposed for the gel saliva: mechanical support of the stylet, protection of the stylet against molecular plant defenses (e.g. chitinases), lubrication to facilitate stylet movement, and the sealing of stylet penetration sites in the plasma membrane of plant cells [11, 26]. Watery saliva is also secreted during intercellular penetration [27], but in contrast to gel saliva it is secreted when the aphid stylet briefly punctures parenchymal cells and immediately before and during sap ingestion from SEs [28]. Recent proteome studies have identified several proteins (effectors) in watery saliva that potentially interfere with plant cell signaling cascades. Proteases and proteins of unknown function were also detected in the watery saliva, and their roles are the subject of intense research [23, 29].

Several authors have suggested that aphid saliva mediates insect-plant interaction by overcoming plant defenses before and after SE penetration, e.g. [26, 16, 25, 30]. In contrast to the large number of studies involving leaf-chewing insects

[31], direct evidence of the role of individual salivary proteins during aphid-plant interaction are rare [23, 29, 32, 33].

2 Control of Aphids

Chemical insecticides such as imidacloprid and dimethoate are used in conventional agriculture to control aphids, whereas azadirachtin from the Neem tree can be used for organic plant production [34]. An alternative approach is the use of beneficial insects (e.g. hoverfly, ladybeetle and brown lacewing) or entomopathogenic fungi [35]. Insecticides remain the most widely used control mechanism for aphids, even though the number of accredited insecticides has declined due to their negative impact on the environment. An additional problem with insecticides is the emergence of resistant aphid populations (http://www.pesticideresistance.com/). In the context of integrated pest management, biotechnology-based approaches offer an appealing alternative.

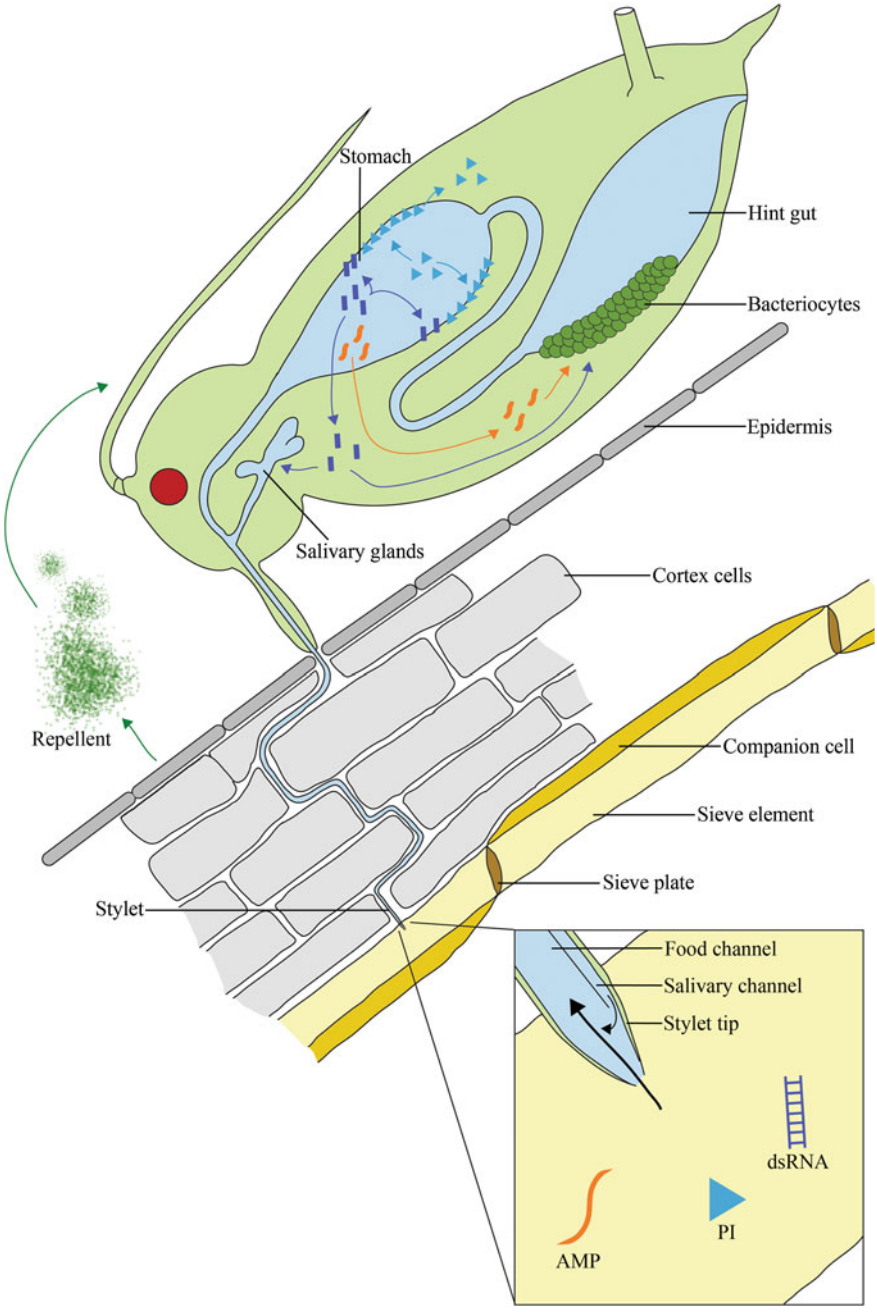
2.1 Biotechnology-Based Approaches

The use of genetically modified (GM) plants to fight insect pests [36–38] as well as fungal plant pathogens [39] has been established for more than 20 years, with most commercial insect-resistant GM crops expressing *Bacillus thuringiensis* (*B.t.*) toxins. Although these toxins are powerful and specific agents against Coleoptera and Lepidoptera [38], they do not affect phloem-feeding insects such aphids [40]. Therefore, alternative strategies are required for phloem-feeders, including the expression of protease inhibitors, RNA interference (RNAi), antimicrobial peptides, and repellents (Fig. 1). This requires a broad understanding of aphid biology as well as aphid-plant interactions to adapt such approaches to the specific properties of this pest.

2.1.1 Controlling the Expression of Defensive Agents in GM Plants

Effective pest control strategies using molecules expressed in plants must take account of the insect feeding strategy. Insects with chewing mouthparts, such as beetles, take up unspecific plant tissues material, whereas aphids have piercing-sucking mouthparts that are adapted for the withdrawal of sap from the xylem and phloem.

The *Cauliflower mosaic virus* (CaMV) 35S promoter is used to control transgene expression in many transgenic plants because it is regarded as a constitutive promoter, but the expression of a β-glucuronidase (GUS) reporter gene using this promoter indicates different levels of activity in different cell types [41]. High levels of


Stomach
Hint gut
Bacteriocytes
Epidermis
Salivary glands
Cortex cells
Repellent
Companion cell
Sieve element
Sieve plate
Stylet
Food channel
Salivary channel
Stylet tip
dsRNA
PI
AMP

◀ **Fig. 1** Overview of target localization for different defense-related agents—protease inhibitors (PIs), double-stranded RNA (dsRNA), antimicrobial peptides (AMPs), and repellents—used for aphid control in GM plants. Aphids penetrate plant tissue with their stylet (epidermis and cortex) and, after reaching the sieve elements, begin to ingest sieve tube sap. Aphids secrete pulses of saliva, produced in salivary glands and released from the salivary channel into the food channel, where it mixes with ingested sap. (E)-β-farnesene functions as an alarm pheromone and is released by specific glandular trichomes on the plant epidermis. It is perceived by chemoreceptors in the aphid antennae and acts as repellent. Repellents are already released prior to feeding following contact between the aphid and the trichomes. PIs, dsRNA, and AMPs are ingested as components of phloem sap. In the gut, PIs target proteases from the saliva and gut, thus interrupting digestion. Additional PI targets are located in the body cavity. Targets for siRNA, a product of dsRNA cleavage, were identified in the gut and salivary glands. There, siRNA induces the silencing of selected proteins required for aphid–plant interactions. Like PIs and dsRNA, AMPs can cross the gut epithelium and target bacterial endosymbionts that are located in the hemolymph or within bacteriocytes (e.g. *Buchnera aphidicola*), thus reducing aphid fitness

GUS activity were observed in the root pericycle cells and in the parenchymal cells of the xylem and phloem tissues in the stem and leaf. However, there was little or no GUS activity in the procambium, phloem, and cortex cells of the root; in the vascular cambium cells of stems; and in the majority of the cortex cells in the leaf midrib. Intermediate levels of GUS activity were observed in leaf mesophyll cells, certain ground tissue cells in the stem and leaf midrib, and in trichome and epidermal guard cells [41]. The activity of the CaMV 35S promoter is downregulated in older root areas and in syncytial feeding cells of nematodes [42]. Despite this inconsistent activity, the CaMV 35S promoter appears to be suitable for the expression of dsRNA to protect plants against coleopteran [43] and aphid pests [44].

The specific feeding strategy of aphids suggests that phloem-specific promoters would be more useful because they achieve targeted and potentially high-level expression in the phloem. This could increase the level of resistance towards phloem-feeding insects in GM plants by increasing the content of defense compounds in phloem sap while reducing the exposure of nontarget insects to the same compounds. Furthermore, this approach would also reduce the GM-associated resource investment by the plant by avoiding the expression of defense compounds in cells/tissues where they would never encounter the pest. The *SUC2* promoter that regulates the CC-specific *AtSUC2* sucrose-H^+ symporter gene is a good candidate because its activity is restricted to the phloem, with no differences between the source and sink tissues [45]. Imlau et al. [46] showed that green fluorescent protein expressed under the control of the *SUC2* promoter is transferred from the CCs via plasmodesmata to the SEs and is then transported along the sieve tubes. This provides proof of concept for the control of agents targeting phloem-feeding insect pests in GM plants. In contrast, the *SUT1* promoter, which regulates *StSUT1* (a sucrose H^+-cotransporter located in the phloem of potato plants), is active solely in the unloading phloem in sink tissues [47]. Therefore, it is unsuitable for the control of defense compounds because pests also infest source tissues such as mature leaves, and the transport of GM-based defense compounds from sink to source has yet to be demonstrated.

Promoters that are used for expression control of defense compounds and that are continuously active throughout the plant lifecycle can be regarded as inefficient because they produce these in the absence of infestation, and they can encourage the emergence of resistant populations. Therefore, promoters should ideally be inactive prior to infestation and/or wounding. Several promoters are inactive when tissues are intact but are activated by wounding, including the mannopine synthase (*mas*) promoter [48], the potato proteinase inhibitor II (*pin*II) promoter [49], and the *PR1-a* promoter [50]. The inducible *PR1-a* promoter is activated by salicylic acid, a chemical involved in wound-induced signaling in plants [50], and its production is triggered by aphid feeding [51]. The ideal promoter for the control of aphid resistance genes would therefore be chimeric, combining the functional elements of wound-inducible promoters (e.g. *PR1-a*) and phloem-specific promoters (e.g. *SUC2*). This would allow the development of GM plants with defense mechanisms triggered only by phloem-feeding insects such as aphids.

2.1.2 Protease Inhibitors

Protease inhibitors (PIs) are small molecules, peptides, or proteins that reduce or inhibit the activity of proteases by directly or indirectly blocking their active site or an adjacent exocite. PIs regulate the activity of endogenous proteases but can also act defensively against proteases secreted by pests and pathogens. They have been grouped into 48 families based on the sequence of the inhibitory domain [52]. As defense molecules, PIs ingested with phloem sap disrupt the digestion of proteins by insect proteases inside the gut, thus attenuating amino acid assimilation, slowing the growth of insects and reducing damage to the plant. Other targets in insects affected by PIs include water balance, molting, and enzyme regulation [53]. In non-GM plants, PIs are detected in storage organs and can be induced by insect feeding and pathogen infection [54]. The expression of trypsin inhibitors and other PI-like chymotrypsin inhibitors has already been achieved in the phloem of transgenic plants [55, 56].

Rhabé and Febvay [57] tested the toxicity of different proteins against the aphid species *Acyrthosiphon pisum* by artificial feeding *in vitro*. They found that the plant lectin concanavalin A was toxic and inhibited growth, whereas PIs were only effective at relatively high concentrations. A broader study of lectin and PI toxicity against five aphid species (*Aphis gossypii*, *Aulacortum solani*, *Macrosiphum euphorbiae*, *Macrosiphum albifrons* and *Myzus persicae*) revealed a dependence on the lectin/PI combination and aphid species [58]. Corcuera [59] suggested that naturally occurring PIs may defend barley against aphids, indicated by infestation induced accumulation of PIs against chymotrypsin and trypsin [60]. The authors used two aphid species (*Schizaphis graminum* and *Rhopalosiphum padi*) and observed that the amount of PI produced depended on the species and the number of aphids. PI activity was significantly greater in barley infested with *S. graminum,* probably reflecting the impact of each species; for example, *S. graminum* causes chlorosis around the feeding site, whereas *R. padi* does not [61]. Furthermore, PIs

significantly affected the survival of *R. padi* but had only a minor impact on *S. graminum*. PIs may also defend white cabbage cultivars and *Arabidopsis thaliana* against the aphid *Brevicoryne brassicae* [62].

The first GM plant expressing a PI for the control of plant-sucking insects was a tobacco (*Nicotiana tabacum*) plant expressing snowdrop lectin from *Galanthus nivalis* [63]. Tobacco plants are infested with the aphid *M. persicae*, but those feeding on the transgenic plants and on artificial diets containing the lectin showed reduced growth, survival, and reproduction. The insecticidal activity of snowdrop lectin was previously demonstrated against chewing insects in GM plants [64] and for planthoppers *in vitro* [65, 66]. Other PIs expressed in GM plants as defense compounds against aphids include oryzacystatin I in rapeseed [67] and eggplants [68], and a cysteine-PI from barley in *A. thaliana* [69]. These generally demonstrated similar effects to those described previously (i.e. reduced survival, growth, and reproduction), as well as a developmental delay. The use of PIs for aphid control therefore appears to be an effective strategy for pest management [63, 67–69].

Until recently, the target for PIs in aphids was uncertain because of conflicting data concerning the protease activity in the aphid gut [70]. Initially, aphids were considered to be unable to digest proteins in the sieve tube sap, thus relying on free amino acids as a nitrogen source [56]. However, an aminopeptidase and a cathepsin-L-like cysteine protease are thought to be immobilized in the gut of *A. pisum* [58, 71, 72]. Aminopeptidase, which represents 15.6 % of the total gut protein, may be a binding site for lectins [72]. An additional study identified cathepsin-B-like proteases in the *A. pisum* gut [70]. More recent findings indicate that several types of proteases, including metalloproteases, are present in the watery saliva of *A. pisum* [23, 29]. Because watery saliva is secreted into pierced SEs and mixes during nutrition uptake with the phloem sap in the stylet [16], plant-derived PIs target aphid proteases in two different environments, the sieve tubes and the alimentary tract. Additional targets for PIs may be present elsewhere in the aphid body because some PIs, such as oryzacystatin I, can cross the gut epithelium [67].

Despite the positive results achieved using different PIs against aphids, key considerations include the potential for aphids to adapt to PIs and the potential impact of ingested PIs on aphid predators and parasitoids. The overexpression of endogenous proteases could outcompete PIs and the expression of insensitive proteases could circumvent them, as previously seen in caterpillars and beetles [53]. A comparable observation was recently described for *M. persicae*, which upregulates expression of cathepsin B following PI ingestion [73]. Furthermore, oryzacystatin I is not only toxic towards the aphid *M. euphorbiae* but also to its parasitic wasp *Aphidius ervi* [74].

2.1.3 RNA Interference

RNA interference (RNAi) is a posttranslational RNA-mediated gene silencing process controlled by the RNA-induced silencing complex (RISC). RNAi is the major antiviral defense mechanism in both plants and insects [75, 76]. In insects,

the short interfering RNA (siRNA) pathway is the principal antiviral pathway and is considered to be part of the insect innate immune system [76].

Double-stranded RNA (dsRNA) derived from an exogenous source (e.g. a virus) or an endogenous source (e.g. pre-miRNA) is cleaved inside the cell by a ribonuclease III known as DICER to generate siRNAs or miRNAs 20–23 nucleotides in length with short tails [77, 78]. These are separated into single strands and the guide strand is integrated into the RISC complex [79], whereas the passenger strand is degraded. The siRNA or miRNA-RISC complex binds to its target mRNA resulting in cleavage (siRNA) or translational repression (miRNA) [78]. This process, when mediated by siRNAs, specifically reduces the abundance of target mRNAs [80].

Artificial exogenous sources of dsRNA can be provided by feeding or by the expression of hairpin RNA constructs in transgenic plants. The latter mechanism is termed host-induced gene silencing because the plant host delivers siRNA to the pest or pathogen [81, 82]. It is still unclear how exogenously administered dsRNA and siRNA enters insect cells [83].

Two early studies demonstrated that plants can be engineered to produce dsRNA, offering protection against specific insect pests. Baum et al. [43] transformed corn to produce dsRNA targeting the V-type ATPase A subunit mRNA, significantly reducing feeding damage by Western corn rootworm larvae (*Diabrotica virgifera*). Mao et al. [84] targeted the gut-specific cytochrome P450 gene of the cotton bollworm (*Helicoverpa zea*), which confers resistance to gossypol, a polyphenol defense compound produced by cotton plants. Bollworm larvae were initially fed on transgenic tobacco and *A. thaliana* plants expressing target-specific dsRNA, which made the insects sensitive towards gossypol present in artificial diets. The target specificity of dsRNA coupled with its ability to suppress genes that are critical for insect–host interaction or insect survival, for example, suggests that dsRNAs can be developed as highly specific pesticides, allowing the control of one or more specific insect pests without off-target effects [85].

In aphids, RNAi-mediated gene silencing has been achieved by injecting dsRNA or siRNAs into the hemolymph [86, 87] or by artificial feeding with dsRNA [85, 88]. In these studies, RNAi was used to investigate the function of proteins, such as the uncharacterized salivary gland protein C002 [33, 87] and a gut-specific aquaporin [88]. Jaubert-Possamai et al. [86] demonstrated that a single dose of dsRNA induces temporal silencing in aphids, with peak inhibition of 30–40 % target mRNA levels 5 days after injection, returning to normal 7 days after treatment. In this context, Mutti et al. [87] reported a 50 % reduction in the expression of a salivary gland protein. In *Tribolium castaneum*, a parental effect was observed in which the inhibition of target genes is transmitted to offspring [89], but no comparable studies have yet been carried out in aphids.

The proof of concept for transgenic plants delivering dsRNA to aphids resulted in the specific inhibition of Rack1 (located in the gut) and C002 (located in the salivary gland) in the green peach aphid *M. persicae* [44]. Both tobacco and *A. thaliana* were transformed with the silencing constructs, inducing up to 60 % silencing in the feeding aphids and reducing their fecundity. Surprisingly, silencing

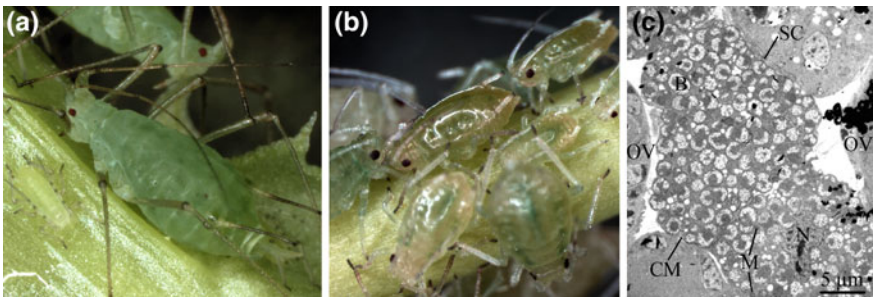


Fig. 2 **a** *Acyrthosiphon pisum* and **b** *Myzus persicae* are model aphids that have been used in most published studies, reflecting the availability of genomic resources for both species. *Buchnera aphidicola*, the primary bacterial endosymbiont of aphids, is located within bacteriocytes and is transmitted vertically to embryos. **c** *Aphis fabae* embryo bacteriocytes and surrounding tissues. Note the close packing of symbionts. *B Buchnera aphidicola* cell; *CM* cell membrane; *M* mitochondrion; *N* lobed nucleus; *OV* part of ovariole; *SC* sheath cell. (*Buchnera aphidicola* image kindly provided by Tom L. Wilkinson, University College Dublin, Ireland)

C002 did not reduce survival, as previously observed with *A. pisum* after siRNA injection [87]. This may reflect species-dependent differences or the impact of different application methods.

Experiments on aphids as a model for piercing–sucking pests suggests that the most promising RNAi targets are salivary proteins [33, 87] and gut proteins [88]. However, additional promising targets include transporters in the bacteriocyte plasma membrane, which are required for the transport of nutrients between the aphid and its obligate bacterial endosymbionts, such as *Buchnera aphidicola*. Most RNAi studies in aphids have focused on *A. pisum* and *M. persicae* (Fig. 2a, b) because the corresponding genome sequences are available, allowing the identification of RNAi target genes (IAGC [90]. The sequencing of additional species such as *A. gossypii*, *Diuraphis noxia*, *M. euphorbiae*, *M. persicae,* and *S. graminum* is in progress (IAGC [91, 92]; http://arthropodgenomes.org/wiki/i5K) and would broaden the scope of RNAi-based aphid control.

2.1.4 Antimicrobial Peptides

Peptide antibiotics are synthesized ribosomally in all organisms and in addition are produced enzymatically in fungi and bacteria. In eukaryotes, peptide antibiotics are termed antimicrobial peptides (AMPs) and generally comprise 12–50 amino acids. AMPs are active against Gram-positive and Gram-negative bacteria in different ways, according to their structure. Three main structural classes have been described: (1) linear α-helical peptides lacking cysteine residues; (2) peptides adopting a β-sheet globular structure stabilized by intramolecular disulfide bridges; and (3) peptides with an unusual bias for certain amino acids, such as histidine, glycine, proline, or tryptophan [93]. The production of peptides with

direct microbicidal activity is considered to be the most ancient mechanism of immunity. The formation of peptide-induced transmembrane pores in bacteria or other peptide-mediated mechanisms of membrane disruption abolishes the maintenance of membrane potential and causes bacterial cell death. Other AMPs have intracellular modes of action, such as the inactivation of bacterial DnaK [94].

AMPs represent the innate immune system, which is the only form of immunity in arthropods [95, 96]. Many insect species produce diverse AMPs [97, 98], but these are not present in aphids, nor do aphids produce components enabling the recognition and signaling of bacterial infection [99, 100]. The lack of an antibacterial defense response may reflect the close relationship between aphids and their endosymbiotic bacteria; for example, *B. aphidicola* is localized in specialized aphid cells known as bacteriocytes [101]; Fig. 2c). Additional facultative bacterial endosymbionts include different strains of *Hamiltonella*, *Serratia*, *Rickettsia*, and *Regiella* spp. [102]. Facultative endosymbionts may be intracellular and/or free within the hemolymph [103–105]. Aphids benefit from symbiotic bacteria because they convert nonessential amino acids in the phloem sap into essential amino acids [90], which are normally present at minimal levels [106]. Facultative symbiotic bacteria also confer resistance to parasitoid wasps [107], pathogenic fungi [108, 109], and heat [110, 111], as well as better performance on different host plants [112, 113]. The reliance of aphids on bacterial endosymbionts makes the latter a useful target for AMPs expressed in plants [114] based on the observation that eliminating different aphid bacterial endosymbionts using antibiotics reduces fecundity and delays aphid development, e.g. [115].

There has been one report thus far describing the influence of AMPs on aphids, using indolicidin as a model [116]. Indolicidin is a cationic AMP present in bovine neutrophils [117]; it shows activity against fungi [118] and bacteria such as *Escherichia coli* [117], which is closely related to *B. aphidicola*. Le-Feuvre et al. [116] demonstrated that the ingestion of indolicidin reduces the number of bacteriocytes in *M. persicae*, disrupts their structure, and reduces the number of bacteria, ultimately reducing the performance, survival, and reproduction of the aphids. Although antibiotic and AMP feeding generate distinct results, perhaps reflecting the secondary effects on gut cells or other internal tissues [116], these findings nevertheless indicate that AMPs produced by GM plants offer a promising experimental approach for pest control. Beyond that, proof of concept has been demonstrated for the control of fungal infections by AMPs expressed in plants, offering a new dimension to the defense system of plants that remain infested with unchallenged pests, e.g. [39, 119].

2.1.5 Repellents

Aphids detect odors via receptors in the primary and secondary rhinaria, which are antennal segments present in the Sternorrhyncha [120–124]. It has been suggested that the detection of plant volatiles is restricted to the primary rhinaria [120, 124, 125]. The overall response of these receptors to odors can be studied by

electroantennography, which measures the average output of antennal nerves to the brain for a tested odor [126]. Plant volatiles are used by aphids for long-range orientation [127] and responses have been recorded in species such as *S. avenae*, *Metopolophium dirhodum* [124, 128], *Aphis fabae* [122], *Megoura viciae* [129, 130], *A. pisum* [125], *B. brassicae,* and *M. persicae* [131]. Like other animals, aphids use pheromones for intraspecific communication, and these are also perceived by the antenna.

Pheromones are chemicals secreted into the environment to induce a social reaction from conspecifics. As well as aggregation and mating pheromones, chemicals such as (E)-7,11-dimethyl-3-methylene-1,6,10-dodecatriene (also known as E-β-farnesene or Eβf) function as alarm pheromones in aphids such as *R. padi*, *M. dirhodum*, *S. avenae* and *M. persicae* [132] and also in some beetles and wasps. Receptors for alarm pheromones are located in the two primary rhinaria in aphids [133]. The alarm pheromone is secreted by endangered aphids (e.g. in the presence of a predator) and induces others to stop feeding and escape, thus interrupting the feeding cycle and increasing alertness and the time spent walking or dropping off the plant at the expense of resource accumulation [134, 135].

Several plants, including wild potato species, have been shown to synthesize Eβf as a natural aphid repellent [136, 137]. In this context, the volatile is termed an allomone—that is, a substance that induces a reaction in a different species without any benefit to that species. Gibson and Pickett [137] suggested that the allomone is secreted by specific glandular hairs on the leaf surface and demonstrated that aphids remain a distance of 1–3 mm from the leaf surface during choice experiments. Nevertheless, the authors observed that not all aphids treated with air from Eβf-emitting potato species were disturbed during feeding on susceptible plants. As well as showing alarm responses, groups of aphids react to Eβf by producing a higher ratio of winged offspring (migratory morphs) after application [138], which has also been demonstrated in the field [139]. These observations suggest that plants producing aphid alarm pheromones benefit from a reduced number of feeding aphids and a higher ratio of winged offspring tending to leave the host plant [138]. The aphid resistance of a recently described melon line may reflect the same phenomenon [140], and it has been suggested as a strategy to produce aphid-resistant versions of economically-relevant cultivars [137].

Eβf also shows kairomonal effects by attracting *Adalia bipunctata* [141], *Coccinella septempuctata* [142], *Coleomegilla maculate*, *Hippodamia convergens*, *Harmonia axyridis* [143], the primary aphid parasitoid wasps *Aphidius uzbekistanicus* [144] and *A. ervi* [145], and the hoverfly *Episyrphus balteatus* [146]. This dual effect as a pest repellent and an attractant for beneficial insects increases the benefits of Eβf production by GM plants for aphid control. A recent study indicated that the dispersal of herbivore-induced plant volatiles affects insects to a range of 8 m from the release site [147], corroborating the idea that plant emitted volatiles affect a wide range around the release site.

Only one study has thus far shown the direct benefits of Eβf produced by GM plants [148]. *A. thaliana* producing Eβf were created by introducing the *Mentha x piperita* (peppermint) encoding Eβf synthase under the control of the CaMV 35S

promoter. Aphids showed more frequent alarm responses when exposed to a droplet of hexane containing entrained volatiles from a transgenic plant or to air from the headspace above Eβf-producing *A. thaliana* plants. Furthermore, the authors showed that the released Eβf attracted the parasitoid wasp *Diaeretiella rapae*, which spent more time on the transgenic plants than on comparable plants lacking the pheromone. There appeared to be no metabolic costs of Eβf synthesis because the transgenic plants showed no differences in growth or seed production compared to wild-type controls. Current work at Rothamsted Research (UK) focuses on the production of aphid-resistant wheat based upon the results of Beale et al. [148].

In contrast to Beale et al. [148], Kunert et al. [149] found that transgenic *A. thaliana* plants producing Eβf were not resistant to infestation by *M. persicae*, and did not affect reproduction or the ratio of winged and wingless offspring. The amount of Eβf produced by the plants was not influenced by aphid infestation [149]. The absence of a repellent effect may have reflected an adaptation to Eβf due to the continuous release by the transgenic plants. In contrast to the GM plants, wild-type plants release Eβf via glandular hairs in pulses, mimicking the Eβf emission of aphids when they are attacked by predators.

3 Testing Aphid Resistance in Plants

Developed GM plants are initially tested using molecular biology tools (e.g. quantitative PCR) to confirm transgene integration and the expression of the corresponding products and to compare plant lines produced by independent transformation experiments. GM pest/pathogen-resistant plants must then be tested for their efficiency against targeted pests/pathogens.

3.1 Aphid Fitness Parameters for Pest Control

Aphid fitness parameters such as development, body size, reproduction, and survival are relevant for plant infestation and thus are used to determine the efficiency of pest resistance. These parameters depend upon access to nutrition and its quality. The key parameter relevant to plant infestation by aphids is the remarkable rate of reproduction. Most aphid species show cyclical parthenogenesis under natural conditions with a switch from asexual to sexual reproduction. Reproduction begins approximately 1 week after birth; thus the development of aphids is rapid compared to similar-sized insects whose development lasts approximately 3 weeks. This reflects the so—called telescoping of generations in which aphid embryos begin to develop in their grandmothers. All these factors lead to a high rate of reproduction and make aphids ideal r-strategists with a total reproduction potential of several millions of progeny per season distributed over several generations.

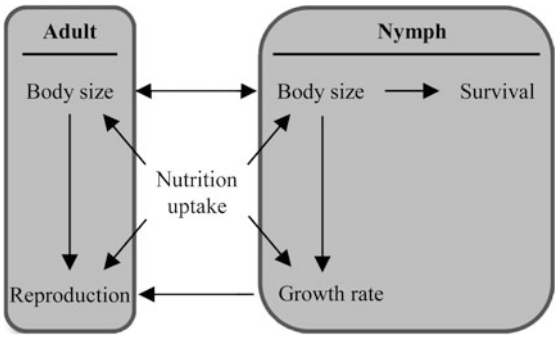


Fig. 3 Influence of nutrition uptake on different fitness-associated parameters and their interplay in aphid adults and nymphs

As discussed above, biotechnology-based approaches have a negative impact on parameters such as development and body size (Box 1) by reducing the intake of nutrition and thus the fitness of adults and offspring (Fig. 3). Nymphs with a low birth weight grow slowly and produce smaller nymphs in the next generation. Furthermore, slower growth increases the time to maturity and reproduction starts later in contrast to larger nymphs, reducing the total reproduction time over the lifespan of each aphid [150]. The final body size is also positively correlated with the reproductive weight [151, 152]. Larger and faster-developing nymphs also show higher survival rates and less parasitization by wasps than smaller nymphs [153]. The negative impact of reduced nutrition on reproduction implies that aphid control strategies do not necessarily have to focus on killing. Approaches that reduce infestation below an economically relevant level are also of interest because they follow the concept of integrated pest management.

Box 1: Measuring the body size and development of aphids

Two technical approaches are used to measure the size of adult aphids. Groups of up to 10 aphids can weighed and the mass of a single individual deduced. This is necessary because the small size of aphids of 1–10 mm (species dependent) means individuals weigh less than 1 mg, which makes accurate determination challenging. Alternatively or in addition, it is possible to measure the so-called body plan area (BPA) by taking images of single aphids using a microscope and a connected digital camera. Image analysis software can be used for size determination, which is calculated on the basis of a scale bar [154]. It is also possible to correlate the BPA with the developmental stage (larval stage 1–4 and adult).

3.2 Observation of Behavior Reveals the Mode of Plant Resistance

Aphid behavior provides additional insights into the interaction between host plants and pests, allowing observers to distinguish between aphids that are repelled by a plant, unable to access the plant, or have disrupted nutrition uptake. Three

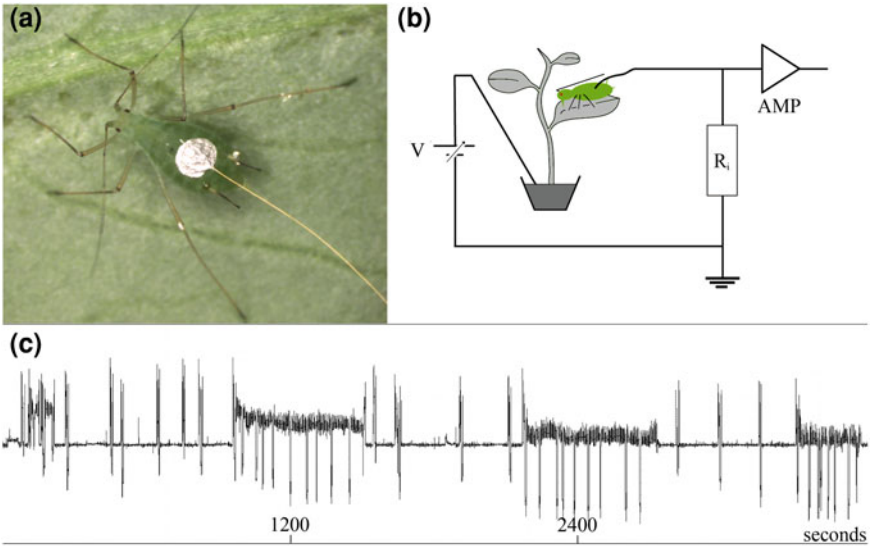


Fig. 4 Aphid behavior observed using the electrical penetration graph (EPG) technique. By attaching a thin gold wire with conductive silver glue to the dorsal abdomen (**a**), the aphid can be integrated in a direct current electrical circuit (**b**). The plant is integrated by inserting an electrode into the soil. The applied voltage is adjustable (V). The aphid and plant together represent a variable resistor. The input resistor (R_i) of the EPG amplifier has a value of 1 GΩ, about the mean value of the aphid. The measured signal is amplified 50-fold (Amp) and is recorded with a computer. (**c**) A 1-hour overview of an EPG recording. Specific waveforms in the EPG reveal information about stylet movement, salivary secretion, and ingestion, for example

main phases of plant-associated aphid behavior involve plant chemicals. The first phase is host plant identification by color and odor [155, 156]. This behavior can be studied with choice experiments using dual-choice chambers by which intact plants (resistant and susceptible) are offered to aphids [157]. Olfactometers, which can be designed as Y-track or four-arm models, are used as an additional tool to study the influence of plant repellents on aphids and the attraction of parasitoids *in vitro*, e.g. [141, 158].

In the second phase, aphids briefly penetrate epidermal and mesophyll cells and test the suitability of the plant as a potential host by taking a small sample of cell sap [159, 160]. The third phase is comparable to the second, but the ingested solution is sieve tube sap [16]. Varying artificial feeding setups, such as choice chambers [17, 161] or flow-through chambers [7], can be used to study aphid stylet orientation inside the plant as well as the influence of intracellular chemical and physical variations on feeding behavior. The electrical penetration graph (EPG) technique (Fig. 4) integrates the aphid and plant into an AC and/or DC electrical circuit [162, 163, 13, 14, 164], allowing feeding behavior inside the plant to be observed. The aphid and plant represent variable resistance in the electrical circuit that, in accordance with Ohm's law, influences the continuously recorded voltage [165]. Changes in resistance induced by the secretion of saliva or the uptake of

nutrients result in complex wave patterns that have been correlated with different patterns of behavior [21]. The EPG technique is a powerful tool to test the resistance of plants against piercing–sucking insects [166, 167] and to determine the site of resistance in the plant, such as in the epidermis, cortex, or phloem [168].

4 Future Perspectives for GM Plants

The first GM plants produced in 1983 by Fraley et al. [169]. It contained antibiotic resistance genes without any specific use in agriculture, but subsequent development focused on herbicide resistance [170] and pest resistance [171]. Although such first-generation GM crops with altered input traits remain the most widely grown, more recent developments include GM plants modified for output traits, such as β-carotene production in Golden Rice [172], and GM plants producing added-value compounds such as vaccines and antibodies [173]. New approaches in agro-biotechnology include RNAi, the expression of antimicrobial peptides, and the production of repellents for the control of aphids. The basis of this new generation of GM crops is the availability of more biological information and genome sequences from a higher number of pest organisms to facilitate target selection. Because these new approaches address physiological processes and basic modes of intraspecific and interspecific interactions among pests, their symbionts and their hosts, the development of resistant or tolerant pest populations appears unlikely. This is the basis of a new trend towards the development of tailor-made GM crops that can withstand one or several selected prominent pests in a respective habitat.

GM crops are currently grown on 160 million hectares [174], which represents 11.6 % of the total arable land area [175]. The five most important countries for production of GM crops are the USA, Brazil, Argentina, India, and Canada, and the four most prominent crops are soybean, corn, cotton, and rapeseed. The uptake of GM agriculture in developed and developing countries is expected to increase further, following a trend observed since the first GM crops were commercialized [174]. The consumer attitude towards GM agriculture differs between countries, with high acceptance in USA and Asia and a more cautious view in Europe [176]. It can be assumed that next-generation GM crops, developed according to knowledge-based principles, will increase overall acceptance and the economic potential of such crops. However, this will require better communication with the general public [177].

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