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PLANT-MICROBE-INSECT INTERACTIONS

Cytokinins as key regulators in plant–microbe–insect interactions: connecting plant growth and defence

David Giron*[,1], Enric Frago[2], Gaëlle Glevarec[3], Corné M. J. Pieterse[4] and Marcel Dicke[5]

[1] *Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS – Université François-Rabelais, Tours, France;* [2] *Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS UK;* [3] *Biomolécules et Biotechnologies Végétales, EA 2106 Université François-Rabelais, Tours, France;* [4] *Plant-Microbe Interactions, Department of Biology, Utrecht University, Padualaan 8, Utrecht, 3584 CH the Netherlands; and* [5] *Laboratory of Entomology, Wageningen University, P.O. Box 8031, Wageningen, NL-6700 EH the Netherlands*

Summary

1. Plant hormones play important roles in regulating plant growth and defence by mediating developmental processes and signalling networks involved in plant responses to a wide range of parasitic and mutualistic biotic interactions.

2. Plants are known to rapidly respond to pathogen and herbivore attack by reconfiguring their metabolism to reduce pathogen/herbivore food acquisition. This involves the production of defensive plant secondary compounds, but also an alteration of the plant primary metabolism to fuel the energetic requirements of the direct defence.

3. Cytokinins are plant hormones that play a key role in plant morphology, plant defence, leaf senescence and source–sink relationships. They are involved in numerous plant–biotic interactions.

4. These phytohormones may have been the target of arthropods and pathogens over the course of the evolutionary arms race between plants and their biotic partners to hijack the plant metabolism, control its physiology and/or morphology and successfully invade the plant. In the case of arthropods, cytokinin-induced phenotypes can be mediated by their bacterial symbionts, giving rise to intricate plant–microbe–insect interactions.

5. Cytokinin-mediated effects strongly impact not only plant growth and defence but also the whole community of insect and pathogen species sharing the same plant by facilitating or preventing plant invasion. This suggests that cytokinins (CKs) are key regulators of the plant growth-defence trade-off and highlights the complexity of the finely balanced responses that plants use while facing both invaders and mutualists.

Key-words: cytokinins, defence modulation, growth-defence trade-off, nutritional resources allocation, plant signalling, symbiosis

Introduction

Plants constitute key nutritional resources for many organisms on Earth and therefore interact with multiple biotic partners ranging from antagonists to mutualists. Plants are under selection pressure to enhance interactions with mutualists while reducing interactions with antagonists. As allocation strategies are constrained by a limiting pool of nutrients, plants are faced with a trade-off between allocation of resources to growth versus defence (Herms & Mattson 1992). In this context, it is interesting to see that plant growth and defence can be regulated by similar phytohormones (Fig. 1).

Various community members can contribute to plant growth, while others attack plants and need to be countered by effective defences that may be induced by the attackers. Interactions of plants with members of their ecological community, once perceived by the plant, can lead to a profound metabolic reconfiguration of the plant physiology, which favours beneficial organisms and deters antagonists like pathogens or herbivores (e.g. Kessler & Baldwin 2002; Pieterse & Dicke 2007; Schwachtje & Baldwin 2008). For instance, under pathogen infection and

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*Correspondence author: E-mail: david.giron@univ-tours.fr

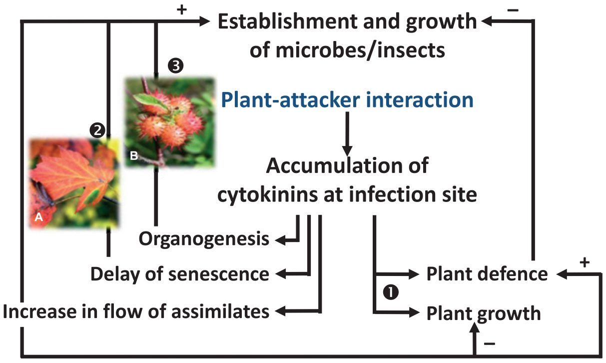

Fig. 1. The cytokinin-mediated growth/defence trade-off. Insect or pathogen attack is usually followed by an increase of cytokinins (CKs) at the infection site. These can lead to a profound reconfiguration of the plant primary and secondary metabolism associated with plant-induced defence. As allocation strategies are constrained by a limited pool of nutrients, plants are faced with a growth-defence trade-off (1). For example, modification of resource allocation can decrease energetic supply for plant growth but can fuel the plant defence machinery. Cytokinin-mediated effects can increase plant defence against biotic invaders but also facilitate the establishment and growth of microbes and insects by delaying leaf senescence (2) and providing them with nutritional supply or by inducing the creation of new organs (such as galls (3) and nodules) used as shelter against biotic and abiotic factors. Small captions: (A) Insect-induced green island. (B) insect-induced gall. © D. Giron. + *stimulating effects*/– *reducing effects*.

herbivore attack, plants mount a defensive response, which targets specifically the biotic invader. A key step in this defence process is the recognition of the biotic partner and the activation of a signalling network that will regulate, both locally and systemically, the biochemical reconfiguration of the plant (e.g. Pieterse *et al.* 2009). In recent years, the improvement of molecular techniques and the use of key model plants such as *Arabidopsis* or *Nicotiana* have allowed for a tremendous increase in our understanding of the signalling cascades involved. The plant hormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) have rapidly emerged as key response regulators leading to specific defence gene activation (Kessler & Baldwin 2002; Pieterse & Dicke 2007; Erb *et al.* 2008; Schwachtje & Baldwin 2008; Pieterse *et al.* 2012). Their involvement in plant defences and their consequences for plant survival and fitness has been investigated under laboratory conditions as well as field conditions where plants were exposed to natural ecological communities (Baldwin 1998; Thaler 1999; Kessler, Halitschke & Baldwin 2004). Thus, there is a thorough understanding of the role of the three main phytohormones involved in the regulation of plants defences, plant physiology and plant ecology. Linking research on subcellular mechanisms with studies on the ecological functions provides profound understanding of ecological interactions (Dicke & Baldwin 2010).

Other phytohormones, such as abscisic acid (ABA), gibberellins (GBs), auxins and cytokinins (hereafter CKs) have more recently emerged as important defence regulators as well (Robert-Seilaniantz *et al.* 2007; Robert-Seilaniantz, Grant & Jones 2011). Because the role of CKs in the modulation of plant defences is relatively anonymous to date, the aim of the current review is (i) to highlight CKs as key regulatory molecules inducing profound morphological and/or biochemical reconfiguration of plants when challenged by various biotic partners. We will also seek (ii) to emphasize that such regulators can be the target of both insects and microbes to disrupt the plant defensive response and/or to withdraw plant resources for their own benefit. (iii) The evolutionary origin of CKs in plants and their associated biotic partners as well as the ecological implications of these interactions will also be addressed. It is now clear that CKs can be synthesised by both microbes and plants, and emerging data strongly suggest that insects may indirectly produce such regulators thanks to their association with endosymbiotic bacteria (Table 1). This could give rise to intricate plant–microbe–insect interactions. The ability to perceive, interpret and manipulate plant signals likely provides insect herbivores and plant pathogens with novel adaptive capacities and allows them to expand their ecological niche. But, CK-mediated effects on plant physiology can have dramatic consequences for plant resource allocation which can impact not only plant growth and defence but also the whole community of species sharing the same plant.

A pivotal role for cytokinins in plant defence against pathogens and insects

Cytokinins are a group of plant hormones that promote cell division and play a major role in the regulation of various biological processes associated with active growth, metabolism and plant development (Mok & Mok 2001; Sakakibara 2006). They are also known to play a role in the synthesis and maintenance of chlorophyll and are known to influence chloroplast development and metabolism. As such, CKs have long been known to delay senescence (Mothes & Engelbrecht 1963; Gan & Amasino 1995). CKs also impact plant nutrient translocation by converting source tissues into active sinks (Mok & Mok 2001). Finally, they are also known to play a role in integrating diverse environmental stress responses (Hare, Cress & Van Staden 1997). For instance, an increase in CK concentration is commonly observed after insect or pathogen attack, suggesting that these molecules play a pivotal role in the profound reconfiguration of the plant primary and secondary metabolism associated with plant-induced defence (Fig. 1).

CYTOKININS AS KEY FACTORS DELAYING PLANT SENESCENCE AND MEDIATING SOURCE–SINK RELATIONSHIPS

The observation that radioactively labelled nutrients are preferentially transported and accumulated in CK-treated tissues suggests that the hormone creates a new source–sink relationship, thus causing nutrient mobilization (Mothes & Engelbrecht 1961). Further support for a role of CKs in nutrient translocation comes from data that link CK and invertase activity. Indeed, several studies showed that extracellular invertases were induced in various plants by biologically relevant concentrations of CKs (Ehness & Roitsch 1997; Godt & Roitsch 1997). The latter work also found that hexose transporters were co-induced with extracellular invertase by CKs. The coordinated upregulation by CKs of the two functionally linked key enzymes of an apoplasmic phloem unloading pathway may account for the transport of nutrients to CK-treated tissue and the accumulation of nutrients at infection sites first highlighted by Mothes and coworkers in the 1960s. Lara *et al.* (2004) found that extracellular invertase is also an essential component of the CK-mediated delay in senescence, suggesting that CK accumulation results in nutrient mobilization and delayed senescence by increasing the activity of extracellular invertase (Walters & McRoberts 2006; Walters, McRoberts & Fitt 2008).

Such accumulation of nutrients at the infection site can provide the increased demand for energy and carbon skeletons to sustain the plant defence machinery and/or to directly act as plant defensive compounds (Schwachtje & Baldwin 2008; Kerchev *et al.* 2012).

CYTOKININS AS KEY REGULATORS PRIMING PLANTS AND INDUCING THE PRODUCTION OF PLANT SECONDARY METABOLITES

Mechanical wounding and herbivory have been shown to increase CK concentration that increases plant resistance to insects by stimulating wound-inducible gene expression and by inducing the accumulation of insecticidal compounds (Smigocki *et al.* 1993; Hui *et al.* 2003; Dervinis *et al.* 2010). Elevated CK concentrations also cause higher inducibility of a plant endogenous cytochrome P450 gene involved in the synthesis of a variety of secondary plant metabolites (Dervinis *et al.* 2010). It was also suggested that increased CK concentration may contribute to tissue repair by stimulating cell division (Crane & Ross 1986).

More recently, it has been shown that CKs prime plant responses to wounding by conditioning CK-treated plants for a more rapid or higher-magnitude response to a subsequent insect attack (Dervinis *et al.* 2010). Priming is a physiological process by which a plant displays either faster or stronger, or both, activation of the various cellular defence responses that are induced following attack by either pathogens or insects or in response to abiotic stress

Table 1. Examples of organisms known to influence plant cytokinin levels through direct and/or indirect synthesis

Species	Interaction with cytokinins	References
Plant–pathogenic bacteria		
Examples: *Agrobacterium rhizogenes, Agrobacterium tumefasciens, Agrobacterium vitis, Erwinia herbicola, Pseudomonas Solanacearum, Pseudomonas syringae* and *Rhodococcus fascians*.	Direct synthesis (genes for CK synthesis in plasmids)	Jameson 2000; Kakimoto 2003; Frébort *et al.* 2011
Plant–mutualistic bacteria		
Examples: *Sinorhizobium meliloti* and *Mesorhizobium loti*.	Direct synthesis	Lohar *et al.* 2004; Frugier *et al.* 2008
Plant–phytopathogenic viruses		
Examples: Geminiviruses, Begomoviruses (Cabbage leaf curl virus and Tomato golden mosaic virus), Curtoviruses (Spinach curly top virus) and Potexviruses (White clover mosaic potexvirus).	Indirect synthesis (modulation of plant-derived CKs)	Jameson 2000; Baliji, Lacatus & Sunter 2010
Plant–pathogenic fungi		
Examples: Hemibiotrophic fungi (*Helminthosporium teres, Plasmodiophora brassicae, Pyrenopeziza brassicae, Rhodococcus fascians,* and *Venturia inaequalis)* and biotrophic fungi (*Blumeria graminis, Cladosporium fulvum, Fusarium moniliforme, Puccinia tricina* and *Uromyces fabae*).	Direct synthesis + eventually indirect synthesis (modulation of plant-derived CKs)	Cooper & Ashby 1998; Jameson 2000; Walters & McRoberts 2006; Walters, McRoberts & Fitt 2008; Pertry *et al.* 2009
Plant–mutualistic fungi		
Examples: Arbuscular mycorrhiza (*Glomus intraradices*) and ectomycorrhiza (*Lactarius piperatus, Rhizopogon roseolus* and *Thelephora terrestris*).	Direct synthesis and/or indirect synthesis (modulation of plant-derived CKs)	Barker & Tagu 2000; Jameson 2000; Walters, McRoberts & Fitt 2008
Plant–gallinginsects/Plant–leaf-mining insects		
Examples: *Ectodemia argentipedella, E. argyropeza, Stigmella argyropeza, Stigmella argentipedella* (Lepidoptera: Nepticulidae), *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae), *Pontania proxima* (Hymenoptera: Tenthredinidae), *Hartigola annulipes* (Diptera: Cecidomyiidae), *Eurosta solidaginis* (Diptera: Tephritidae) and *Pachypsylla* spp. (Homoptera: Psyllidae).	Direct synthesis potentially mediated by bacterial endosymbionts	Engelbrecht, Orban & Heese 1969; Engelbrecht 1971; Elzen 1983; Mapes & Davies 2001; Giron *et al.* 2007; Kaiser *et al.* 2010

(Conrath *et al.* 2006; Frost *et al.* 2008a). The majority of previous work on defence priming in plant–insect interactions has focussed on volatile compounds as priming signals highlighting plant-to-plant communication (Engelberth *et al.* 2004; Frost *et al.* 2008b). However, it was also suggested that nonvolatile molecules transported through the plant vasculature are used to prime plants systemically (Frost *et al.* 2007; Heil & Silva Bueno 2007; Erb *et al.* 2008).

Studies indicate that CKs also enhance plant resistance to pathogens (more specifically pathogens that do not secrete CKs). Indeed, elevated CK levels can modulate SA signalling and enhance resistance against viruses and bacteria through an increased expression of SA-related defence genes (Jameson 2000).

Cytokinins as a tool for invading the host plant

Besides their role in plant defence against pathogens and insects, CKs have also been shown to play a central role in plant colonization and exploitation by various plant-associated organisms including both antagonists and mutualists. In such interactions, CKs can be the specific target of biotic invaders to withdraw plant resources for their own benefit. A clear example of this is the implication of CKs in the formation, after pathogen or insect attack, of often spectacular green and metabolically active areas in otherwise yellow senescent leaves known as ‘green islands’. In these islands, nutrients are redirected towards the infection site where host cell death is delayed. As important molecules implicated in plant defence, CKs can also be used to disrupt defensive responses. There has been considerable debate about the likely origin of CKs in infected leaves, as it is not yet clear whether they are produced by the pathogen/insect or by the plant. Indeed, pathogens and herbivorous insects potentially influence the levels of phytohormones by inducing plant genes involved in CKs biosynthesis, degradation or response, but they can also produce and secrete relevant phytohormones themselves (Jameson 2000; Farnsworth 2004; Robert-Seilaniantz *et al.* 2007; Walters, McRoberts & Fitt 2008; see also Table 1).

PLANTS AND ANTAGONIST MICROBES

Phytopathogenic bacteria

Recent investigations provide support for the role of CKs as key regulators of plant defence response against non-CK-producing plant-pathogenic bacteria such as *Pseudomonas syringae* by suppressing bacterially induced hypersensitive response symptoms and by increasing antioxidative enzyme levels (Barna, Smigocki & Baker 2008; Choi *et al.* 2010). However, the synthesis of CKs as a mechanism for successful invasion of plant tissues seems to be widespread in phytopathogenic bacteria, which commonly carry genes for CK synthesis in plasmids. CK production is exclusive to pathogenic strains with examples among gram-negative and gram-positive phytopathogenic bacteria (Jameson 2000). The best characterized system is the crown gall formation induced by *Agrobacterium tumefaciens*. In this bacterium, biosynthetic genes for CK [*isopentenyl transferase* (*ipt*)] and auxin production are carried by bacterial plasmids, which have been integrated into the plant genome (Jameson 2000). Similar *ipt* genes have been found in other bacteria of the same genus (i.e. *A. vitis* and *A. rhizogenes*), but also in other plant-pathogenic bacteria (reviewed in Kakimoto 2003; Frébort *et al.* 2011). For example, pathogenicity of *Rhodococcus fascians* that is responsible for leafy gall diseases of numerous host plants is also associated with the presence of a large linear plasmid on which a gene with homology to the *ipt* gene (known as *fas1*) has been localized (Jameson 2000). This bacterium secretes six CKs that synergistically redirect the developmental programme of the plant to stimulate proliferation of young shoot tissue, which expands the niche the bacteria can colonize and live in (Frébort *et al.* 2011). CK biosynthetic genes located on plasmids have also been reported in other gall-inducing bacteria such as *P. syringae* and *Erwinia herbicola* (Jameson 2000; Frébort *et al.* 2011).

Phytopathogenic viruses

Viruses also have the ability to interfere with endogenous plant hormone levels and can induce a wide range of symptoms including abnormal growth forms such as the formation of galls and general tissue distortions or alterations of photosynthesis like chlorosis or the formation of green islands (Jameson 2000; Walters, McRoberts & Fitt 2008). As hormones involved in plant defence, CKs can interfere with virus proliferation, but the relationship between CK content and virus replication remains often unclear and the underlying mechanisms poorly understood. CKs enhance the resistance of *Arabidopsis* against the *tobacco mosaic virus*, of *Phaseolus vulgaris* against the *White clover mosaic virus* and of tobacco against a broad spectrum of plant viruses through an increased activity of a pathogenesis-related protein (Sano *et al.* 1996; Synkova, Semoradova & Burketova 2004; Choi *et al.* 2010). In contrast, a clear example of the role of CKs in viral phytopathogenicity comes from studies on Gemini-, Begomo- and Curto-viruses. These viruses have evolved multiple strategies to provide a cellular environment favourable to viral amplification, including an increase in CK levels and an increase in the pool of bioactive CKs (by preventing their phosphorylation) (Baliji, Lacatus & Sunter 2010). Both factors promote cell proliferation that viruses rely on for replication of their DNA genome. Additional support for a role of CKs in viral pathogenesis is provided by observations that exogenous application of CKs results in an increase in the kinetics of viral DNA accumulation and reduces the mean latent period for symptom appearance of both *Tomato golden mosaic virus* and *Spinach curly top virus* (Baliji, Lacatus & Sunter 2010). Viral infection can also down-regulate plant defences through decreasing the proportion of CKs in active form prior to virus replication (Jameson 2000). In addition, application of exogenous CKs or transgenic plants over-expressing CKs has been shown to delay senescence, which would be advantageous to virus survival as senescence eventually leads to leaf death and mobilization of nutrients to other parts of the plant (Li, Hagen & Guilfoyle 1992).

Phytopathogenic fungi

Infection by fungi is often associated with delayed senescence, nutrient accumulation at infection sites and growth malformations, suggesting specific interactions with the host plant cell cycle, photosynthesis and nutrient allocation (Walters & McRoberts 2006). It is now clearly established that ectomycorrhizal and arbuscular mycorrhizal, biotrophic and hemibiotrophic fungi have the ability to produce CKs, while necrotrophic fungi lack this capacity (Jameson 2000; Walters & McRoberts 2006; Walters, McRoberts & Fitt 2008). This suggests a correlation between obligate parasitism and CK production.

Evidence for the role of CKs in symptoms induced by fungi comes from data showing (i) increased CK concentrations in infected tissues; (ii) the ability of infected tissues to grow *in vitro* on hormone-free medium, whereas healthy tissues require CKs (but also auxins); (iii) the fact that CKs are found in spores, mycelial extracts and culture filtrates in several fungal species; and (iv) direct evidence for biosynthesis of CKs by phytopathogenic fungi based on radioactive isotope studies (Jameson 2000; Walters & McRoberts 2006). Additionally, (as with some bacteria) the hemibiotrophic actinomycete *R. fascians* has been shown to have a functional *ipt* gene and to produce CKs, which are recognized by CK receptors in *Arabidopsis* (Pertry *et al.* 2009). As with some viruses, plant infection by some phytopathogenic fungi is also associated with spectacular green islands, which have been found to correlate with the ability to produce CKs (Walters & McRoberts 2006). There is, however, conflicting evidence about the ability of different fungi to produce CKs. For example, green islands have been commonly observed in obligate biotrophic and some species of hemibiotrophic fungal pathogens as a strategy to redirect plant nutrients towards the infection site and to suppress plant cell death. By contrast, obligate necrotrophic and other species of hemibiotrophic fungal pathogens induce localized green areas, in which the pathogen cells are alive but the host plant cells are dead or dying. These green islands are induced by toxins produced by these fungal pathogens (Walters, McRoberts & Fitt 2008). Another question that still needs to be clarified is whether fungi could manipulate plant physiology to the extent that the plant releases its own CKs. Cooper & Ashby (1998), for instance, found that β-glucosidases produced by several fungal pathogens release active CKs from stored plant CK-glucosides.

PLANTS AND MUTUALISTIC MICROBES

Mutualistic bacteria

Not only bacterial pathogens but also bacterial mutualists form galls and evidence suggests that CKs are produced by nodulating rhizobia (Frugier *et al.* 2008). The Nod factor signalling pathway is known to be directly involved in nodule formation in these species, but CKs are also essential and likely act downstream of this pathway. Indeed, (i) CKs can mimic some of the morphogenetic effects of the *Nod* factor (Cooper & Long 1994), (ii) positive correlations between plant CK concentration and nodulation have been reported in several legume species (Lorteau, Ferguson & Guinel 2001), (iii) CKs rapidly appear at the infection site (Lohar *et al.* 2004), (iv) CK signalling genes are up-regulated after rhizobial inoculation (Frugier *et al.* 2008), (v) reduced CK accumulation and/or perception blocks nodulation (Murray *et al.* 2007), and finally (vi) gain-of-function mutants at the *LHK1* CK receptor develop nodules spontaneously in the absence of rhizobia demonstrating that CK signalling is necessary and sufficient to induce cortical cell divisions and nodule organogenesis (Tirichine *et al.* 2007). An interesting feature is that, despite a lack of direct evidence linking rhizobial CK production and nodulation, bacterial CK and/or CK-like compounds seem to be important for *Nod* factor-independent nodulation in some legumes, suggesting a combination of plant and bacterial-mediated processes in the formation of nodules. All together, these results point to CKs as the key differentiation signal for nodule organogenesis and genes homologous to the *A. tumefaciens ipt* gene have been recorded in the rhizobial bacteria *Sinorhizobium meliloti* and *Mesorhizobium loti*.

Mutualistic fungi

Mycorrhizal symbioses are mutualistic interactions between plant roots and fungi where the microbe and the plant exchange water and mineral nutrients for photosynthates. Mycorrhizae can also benefit plants by increasing resistance against biotic (pathogens and herbivores) and abiotic stresses (Pineda *et al.* 2010; Kiers *et al.* 2011; Zamioudis & Pieterse 2012). Phytohormones play a primary role in physiological and morphological alterations of roots and CK accumulation throughout the plant is specifically enhanced by symbiosis (Barker & Tagu 2000). Although clear examples of CK production by mycorrhizae are known (Jameson 2000), it is not always clear whether the CKs are of fungal or plant origin, or both. Irrespective of their origins, hosts and fungal symbionts exchange several rhizospheric signals that are responsible for important morphological and physiological changes in both hyphae and roots and CKs appear as multifunctional regulators (Barker & Tagu 2000). As a result of CK accumulation, we can observe increased root growth (reduced apical growth, elongation of the colonized section and increased lateral root induction), which is likely to enhance colonization, and altered expression of plant defence response genes. The altered hormone balance was also suggested to be directly responsible for suppressed expression of PR protein, chitinase, endoglucanase and chalcone isomerase defence genes, but underlying mechanisms remain unclear (Barker & Tagu 2000).

PLANTS AND ANTAGONIST HERBIVORES

Gall insects and leaf-mining insects

Cytokinins have been found to mediate plant resistance against insects most likely through the production of secondary metabolites. This has been reported in different species like the tobacco hornworm *Manduca sexta*, the gypsy moth *Lymantria dispar* or the green peach aphid *Myzus persicae* whereby these responses might deter insect feeding, delay larval development or reduce weight gain by insect larvae (Dervinis *et al*. 2010). Endophagous arthropods (i.e. living within plant tissues), such as galling or leaf-mining insects, are characterized by a very intimate association with their host plant, leading to an active manipulation of the plant morphology and/or physiology. This includes the differentiation of additional tissues to feed on, the upregulation of protein and/or sugar synthesis *in situ*, the induction of green islands, and/or the modification of source–sink relationships leading to nutrient translocation towards the insect's feeding site (Stone & Schönrogge 2003; Giron *et al*. 2007; Schwachtje & Baldwin 2008). Already in the 1970's, several authors suggested that CKs could be the underlying cause for green island formation in insects based on increased levels of CKs in infected tissues. This was first described in mining microlepidopterans (Engelbrecht, Orban & Heese 1969; 1971) and later in the green islands surrounding the galls of phytophagous hymenopterans, psyllid homopterans and cecidomiids as well as tephritid dipterans (Engelbrecht 1971; Elzen 1983; Mapes & Davies 2001). In the *Malus domestica*/*Phyllonorycter blancardella* leaf-mining system, for example, increased levels of several CKs (zeatin, isopentenyladenine and ispentenyl adenosine) are found in infected tissues. This could explain the 'stay-green' phenotype of mined areas, while other regions of the same leaf are turning yellow, as well as the net accumulation of nutrients in the mined tissues in a specific pattern matching the energetic requirements of the developing larvae (Giron *et al*. 2007; D. Giron unpublished data). A similar increase in isopentenyladenine was found in galls induced by larvae of *E. solidaginis,* but also in plant–bacteria interactions (Jameson 2000; Mapes & Davies 2001; Sakakibara 2006).

Given the evidence for CK production by gall-forming bacteria and green island inducing fungi, various workers suggested that localized delay of senescence and other phenomena associated with green islands on insect-infected leaves might be the result of release of CKs by the insects (Walters, McRoberts & Fitt 2008; Kaiser *et al*. 2010). Engelbrecht, Orban & Heese (1969) already suggested that larvae were able to produce CKs because high CK levels were found in larval frass and in gastrointestinal tracts and labial glands of leaf-mining larvae (Engelbrecht 1971). Plant growth hormones such as CKs have also been found in a wide range of galling insect species (reviewed by Elzen 1983), primarily in insect secretions or glands associated with oviposition and initiation of galls, suggesting again that insect larvae may act as a source of CKs (Elzen 1983). The finding of CKs inside larvae that had been otherwise emptied from vegetal remains – and in similar proportions to those found in mined tissues – strongly suggests that some (if not all) extra CKs in the *Malus domestica* leaves infected by *P. blancardella* larvae could originate from the insect (D. Giron unpublished data).

Origin of cytokinins: phylogenetic espionage, manipulation and insect endosymbionts

Due to the regulatory role of CKs on plant morphology, plant defence, leaf senescence and source–sink relationships (causing nutrient mobilization towards the infection site), it is not surprising that these phytohormones have been a privileged target of arthropods and pathogens over the course of the evolutionary arms race between plants and their biotic partners. The ability to perceive, interpret and manipulate plant signals is likely to provide insect herbivores or plant pathogens with novel adaptive capacities enabling them to invade new ecological niches (Schultz 2002; Schultz & Appel 2004; Kaiser *et al*. 2010). Indeed, many signalling molecules involved in plant response to insects and pathogens are phytohormones and a high number of them are similar across kingdoms. Such similarities set the ground for possible exploitation of signalling pathways by one participant for its own benefit. Cyanobacteria, for example, are able to synthesize several plant hormones including CKs, auxins, GBs, ABA, ET and JA. In the particular case of CKs, genome-wide studies suggest that their metabolism in plants has evolved from these bacteria that contain the minimal genetic apparatus needed for the biosynthesis and metabolic control of the hormone but with a different signal-transduction machinery (Frébort *et al*. 2011). It is plausible that CK-producing plant-associated partners manipulate CK signalling to their own benefit by promoting the host division cycle (potentially leading to the formation of new plant organs such as galls/nodules), nutrient mobilization and leaf longevity (leading, for example, to green-island phenotypes), processes that are prerequisite for their successful development on plants (Jameson 2000; Choi *et al*. 2010). As mentioned above, this is well known in plant-associated bacteria especially in *Agrobacterium,* which induces spectacular galls through CK signalling (Robert-Seilaniantz *et al*. 2007). Similarly, phytophagous insects and other plant-exploiting organisms may have taken advantage from their close association with endosymbiotic bacteria to hijack the plant metabolism by acquiring the ability to control the regulation of plant CK levels.

CYTOKININ-INDUCED PHENOTYPES CAN BE MEDIATED BY INSECT BACTERIAL SYMBIONTS

Many invertebrates are known to have intimate relationships with bacterial symbionts and molecular studies on the evolution and ecological function of these symbionts are revealing diverse ecological and evolutionary effects on insect hosts (Frago, Dicke & Godfray 2012). As research progresses, it has become apparent that many phenotypes traditionally associated with insects are now attributed to their symbionts living in tight association. This might explain why insects represent over 75% of all animal species and have successfully invaded very diverse habitats, illustrating their remarkably adaptive capacities. These insect symbionts have contributed to insect adaptation mainly by providing novel metabolic capacity, enabling better exploitation of nutritional resources but also providing novel defence mechanisms and affecting reproductive strategies (Janson *et al.* 2008). Nutritional provisioning has been proposed to be one of the major routes leading to symbiosis between micro-organisms and many insects. These symbionts can enable their hosts to develop on limited nutrient sources such as blood in the case of blood-feeding insects, or plant sap and wood in the case of herbivore–plant interactions.

Given that bacteria are key partners of many plant–insect interactions and that microbes can produce CKs, it was recently investigated whether insect symbionts could be implicated in host plant manipulation and CK-induced phenotypes (Barr *et al.* 2010; Kaiser *et al.* 2010). The discovery that *Wolbachia* is involved in the formation of green islands induced by the caterpillars of *P. blancardella* suggests that insect bacterial symbionts can be involved in insect herbivore–plant interactions by a different mechanism than nutrient synthesis. This might involve, for example, manipulation of host plant physiology through manipulation of CK levels. Interestingly, a decrease of plant defences in mined and galled tissues is also frequently observed and recent data also suggest a primary role of insect microbial symbionts in the down-regulation of maize defence against the root-feeding insect *Diabrotica virgifera virgifera* (Barr *et al.* 2010). Whether or not CKs are involved in this interaction and in the reduction of plant defences remains to be elucidated.

For leaf miners, the full molecular mechanisms at the basis of green-island induction and in particular the origin of CKs within the green mine are still unknown, but several hypotheses can be advanced to explain the implication of symbiotic bacteria in the green-island phenotype. Symbiotic bacteria might (i) directly synthesize CKs or (ii) enable the insect to synthesize/secrete CKs, or even (iii) produce regulators of plant CK gene expression. The first two hypotheses are supported by data on insect CK composition (D. Giron unpublished data) and previously obtained results by Engelbrecht, Orban & Heese (1969) who show that large quantities of CKs are present in the labial glands of several leaf miners. Furthermore, the ability of bacteria to produce CKs has already been demonstrated in a variety of plant-bacteria interactions such as the bacterial pathogens *Agrobacterium* spp. or the symbiotic plant interaction involving nodulating bacteria. Additionally, forms of CKs involved in plant–insect interactions closely match those used by bacteria to manipulate the plant physiology (Jameson 2000; Sakakibara 2006; Giron *et al.* 2007; Kaiser *et al.* 2010).

Plant tissues offer ample opportunity for herbivores and microbes to interact. One possible first step for the establishment of these associations could be through insects vectoring plant pathogens which might become insect mutualists when the insect benefits from feeding on a diseased plant (Frago, Dicke & Godfray 2012). In the case of CK-producing microbes, the benefits for the vector might not only arise through localized increase in plant nutritional quality as in the case of the green islands or galls but also through systemic manipulation of plant defence. The origin of CKs, as shared ancestral traits among plants and their biotic partners, still remains poorly understood. Apart from being acquired through symbiotic associations, CKs might also have evolved as the result of adaptive convergence or they can even have been acquired through horizontal gene transfer. Using antibiotic curing or genome-wide sequencing techniques, it would be very interesting to explore in depth through which route specific taxons of plant invaders have acquired these traits.

Ecological implications of cytokinin-mediated effects

CASCADING EFFECTS IN THE FOOD WEB

The importance of CKs for plant physiological alterations (such as green island formation) and for regulation of altered organogenesis (such as rhizobial nodulation and gall formation) have recently gained much interest. Data collected on numerous biological models suggest potentially converging mechanisms for plant–microbe and plant–endophagous insect symbioses and point towards a key regulatory role of CKs in many biotic interactions. As stated earlier in this review, CKs might benefit insect herbivores and plant pathogens via their effects on extracellular invertase which can stimulate the cell cycle and therefore delay senescence, induce sink metabolism and provide nutrients. Additionally, the implication of CKs in signalling processes underlying plant growth and development, nutrient translocation and plant defences can positively impact the plants (through endogenous synthesis but also indirectly *via* their associated symbionts), but also sometimes their pathogens and herbivores. These interactions can have ecological consequences still to be unravelled and that can scale up to plant-associated communities (Fig. 2). The well-studied case of the green islands, for example,

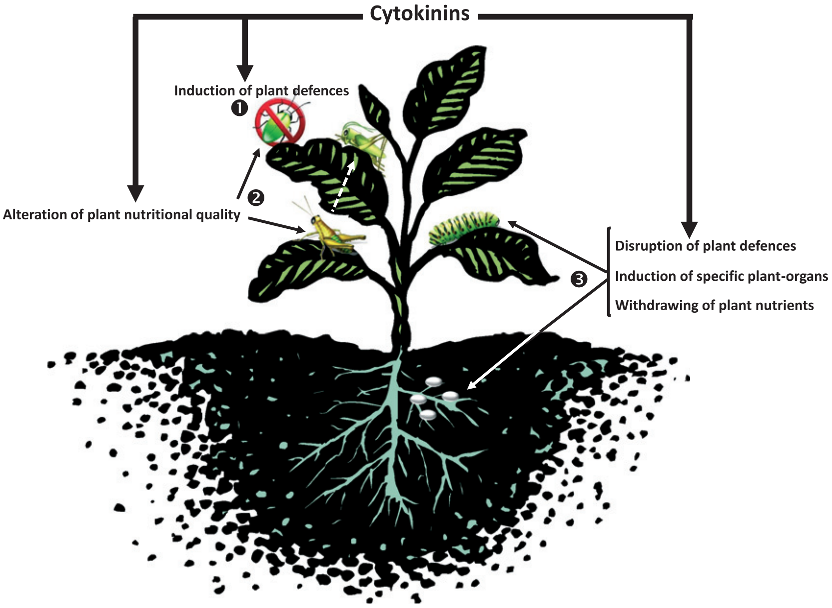

Fig. 2. The cytokinin-mediated facilitation/defence trade-off. Cytokinins are involved in plant defence against pathogens and herbivores (1) and as such can positively impact plant fitness. They can also alter plant nutrient allocation leading to strong modifications of plant quality for plant biotic invaders (2). This can impact directly or indirectly the whole community of herbivores/pathogens and either increase or decrease plant fitness. Finally, cytokinins (CKs) can also be used by these biotic invaders to disrupt the plant defence machinery, to induce specific plant organs that could be used as shelter, but also to withdraw plant nutrients for their own benefits (3). This can facilitate the plant colonization by plant mutualists such as nodulating rhizobacteria but also by plant antagonists such as herbivorous insects and plant pathogens. As key regulatory molecules in plants, cytokinins can impact the whole community of organisms associated with plants.

suggests that the use of CKs to manipulate plant physiology might be widespread. Green islands appear as islands only because the leaf is turning yellow, but they might represent events, which also occur unnoticed earlier in the season. Green islands can therefore be the only visible part of a more extensive phenomenon among insects as well as plant pathogens. As in the case of the leaf miner *P. blancardella*, the green island provides a precious food supply for the insect (Giron *et al.* 2007; Kaiser *et al.* 2010), which enables the insect to win a race against time and hence potentially enable the completion of a supplementary generation (Engelbrecht, Orban & Heese 1969; Giron *et al.* 2007). This can offer competitive advantages over other species but also affect interactions with natural enemies if it provides a window of low natural enemy pressure (Godfray 1994). Additionally, recent studies have revealed how organisms sharing the same plant can be interconnected through indirect interactions mediated by changes in plant quality (Ohgushi 2005; Utsumi, Ando & Miki 2010). After colonization by phytopathogenic microbes, for example, induced changes in plant defensive chemistry can affect herbivores feeding on the same plant, and vice-versa (Stout, Thaler & Thomma 2006). Through changes in plant quality, early season herbivores can also affect the whole community of insects feeding on these same plants later in the season (Poelman *et al.* 2008). In the context of plant-mediated indirect effects, a deeper understanding of CKs and their role in the regulation of complex source–sink relationships will help to understand the structure and function of plant-based food webs.

Although considered as separated modules, plant roots and shoots share the limited plant resources available. Plant-associated insects and microbes might benefit from the use of CKs to increase local abundance of resources on the tissues they inhabit. These interactions, however, might be asymmetric depending on whether they occur below- or above-ground given that biochemical signals transmitted from roots may serve as active regulators of many physiological processes affecting growth and development of both shoots and roots (Aiken & Smucker 1996; Pineda *et al.* 2010). In the particular case of CKs, their synthesis in the roots and translocation to other plant modules can be used by plants to integrate environmental signals contributing to the development of a coordinated plant defensive and growth response both below- and above-ground (van Dam 2009). As such, CKs may play a central role in plant-mediated interactions between above- and below-ground plant-associated communities Van Dam & Heil (2011). In this context, a recent example suggests that the endosymbiotic bacterium *Wolbachia,* which is associated with the western corn rootworm, *Diabrotica virgifera,* plays an important role in down-regulating beetle-induced plant responses in maize (Barr *et al.* 2010). Although these changes were found not to affect beetle fitness the manipulation of induced plant defence using CKs cannot be ruled out as endosymbiotic bacteria in the same genus are known to be capable of synthesizing CKs (Kaiser *et al.* 2010). If this is the case, the *Diabrotica virgifera* – *Wolbachia* system would be an ideal system to test belowground-aboveground interactions mediated by plant-associated partners using CKs to manipulate plant defences. Although the local effects of CK production by insect herbivores or gall-forming organisms is partially understood in some systems, it remains to be uncovered how these effects influence nearby tissues, and the whole plant physiology.

THE GROWTH-DEFENCE TRADE-OFF IN PLANTS

Plants must grow fast enough to compete against other plants but also need to allocate enough resources to chemical and structural defences necessary for their survival in the presence of herbivores and phytopathogens. As primary and secondary metabolic pathways share common precursors, allocation strategies are constrained by a limiting available pool of nutrients which underlies the evolutionary trade-off between growth and defence in plants (Herms & Mattson 1992). CKs, by affecting cell division, growth, nutrient translocation, retardation of senescence, and plant defence undoubtedly play an important role in the growth-defence trade-off (Fig. 1). The dual role of CKs in plant growth on the one hand and on induced plant defence on the other hand suggests that an appropriate plant adaptive response to environmental stresses relies on a finely tuned and complex regulation of CKs (Hare, Cress & Van Staden 1997). In this context, not only CK quantities in various plant tissues are important but also the type of CKs (which may differ if they are originating from the plant or from the biotic invaders), the nature of the target cells and the developmental state of the plant, and the hormonal balance between CKs and other phytohormones (Hare, Cress & Van Staden 1997; Farnsworth 2004; Frébort *et al.* 2011).

THE FACILITATION/DEFENCE TRADE-OFF IN PLANTS

Additionally, while CKs are actively involved in plant defence against pathogens and herbivores, they can also be used by these biotic invaders to disrupt the plant defence machinery, to induce specific plant organs that could be used as shelters and protection against biotic and abiotic factors, but also to withdraw plant nutrients for their own benefits (Figs 1 and 2). These contrasting effects of CKs in plants are well illustrated, for example, by conflicting data on viruses where viral infection success can be facilitated either by an increase or a decrease of CK activity and quantity in infected tissues (Hare, Cress & Van Staden 1997; Jameson 2000). This CK-mediated facilitation of plant exploitation by biotic invaders can have profound cascading effects both in plants and through the entire plant-associated community. For instance, plant association with beneficial organisms such as nodule-inducing bacteria and symbiotic mycorrhizae is highly dependent on CKs. The main advantage of these plant symbionts is in improving plant nutrient uptake, especially P and N, in exchange for carbon (Kiers *et al.* 2011). If these costs are low relative to the benefits gained from improved nutrient uptake, then the symbiosis is advantageous for the host plant, the fungi/bacteria can be considered as mutualistic symbionts, and this results in higher plant growth and competitive ability. By promoting or preventing the development of biotic invaders (e.g. through their action on plant defence or on plant nutrient allocation), CKs can impact photosynthetic capacities, and nutrient levels in plants which, in turn, can shift the balance between costs and benefits in the plant–symbiont relationship. For example, if CKs facilitate the development of herbivores consuming photosynthetic organs and depleting plant reserves of photosynthates, this can erase the selective advantage of plant symbionts and is expected to lead to their elimination.

Future perspectives

Understanding the cellular and molecular dialogue between plants, microbes and insects, which involves shared hormonal signals, and studying ecological and evolutionary implications can greatly improve our understanding of the interactions of plants with microbes and insects. Such information is important to understand the options for each partner to adopt an adaptive response to its biotic environment and the possible implication and origin of key universal regulatory molecules shared by many plant–biotic interactions.

It is now becoming clear that signalling networks that are activated by plants in response to parasitic, herbivorous and beneficial organisms interact either positively or negatively. The crosstalk mediating these signalling network interactions may explain the specificity of the responses but also highlight the complexity of the finely balanced responses that plants use while facing both invaders and mutualists. The *interplay* between CKs and other signalling phytohormones and their dynamics in plant–biotic interactions remains to be investigated, especially regarding the fact that CKs might act upstream of JA biosynthesis and expression of wound- and herbivory-induced genes, and that auxin and CKs have been known for a long time to act either synergistically or antagonistically to control several significant plant developmental processes. Such investigations may help to unravel the different successive steps of interactions and to understand whether CKs can really be considered as key regulatory molecules acting universally in plant–biotic interactions. The *origin* of CKs in these interactions remains also to be clearly investigated and the possible role of endosymbionts for insects deserves in depth exploration. The *dynamics* of the CK signal also remain poorly understood. Whether this is a transient or a continuous signal or a feed-forward mechanism involving secondary signalling molecules such as sugars, and how this signal evolves over the course of the infection and/or pest development (regarding successive larval stages with different requirements and/or feeding habit for example) needs further investigations and may open new avenues for the control of plant pathogens and pests. Moreover, in addition to mechanical and chemical processes, various electric and hydraulic perturbations of the plant may also contribute to the plant's response. They usually are early events occurring before herbivore attack and pathogen attack–related gene expression that are responsible for cascades of events and signal transductions, eventually leading to indirect and direct plant responses. How these perturbations impact production and action of CKs (and other phytohormones) in plants remains to be established. Finally, CKs are a large group of different molecules that can act not only for the benefits of the plant (e.g. stimulation of plant defences) but also for the benefits of the invader (e.g. reduction of the plant defences). An accurate *characterization* of CKs involved in plant–biotic interactions may not only help to better understand their plant or pathogen/herbivore origin but also to clearly identify their function in the plant, their role in the interaction and their ecological consequences for the whole plant-associated communities.

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