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REVIEW PAPER

Nitrogen metabolism meets phytopathology

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Abstract

Nitrogen (N) is essential for life and is a major limiting factor of plant growth. Because soils frequently lack sufficient N, large quantities of inorganic N fertilizers are added to soils for crop production. However, nitrate, urea, and ammonium are a major source of global pollution, because much of the N that is not taken up by plants enters streams, groundwater, and lakes, where it affects algal production and causes an imbalance in aquatic food webs. Many agronomical data indicate that the higher use of N fertilizers during the green revolution had an impact on the incidence of crop diseases. In contrast, examples in which a decrease in N fertilization increases disease severity are also reported, indicating that there is a complex relationship linking N uptake and metabolism and the disease infection processes. Thus, although it is clear that N availability affects disease, the underlying mechanisms remain unclear. The aim of this review is to describe current knowledge of the mechanisms that link plant N status to the plant's response to pathogen infection and to the virulence and nutritional status of phytopathogens.

Key words: Disease, metabolome, nitrogen, pathogen, resistance, transcriptome.

Introduction

Plant pathogens include oomycetes, fungi, bacteria, and viruses. Pathogens have developed different strategies to invade, feed on, and grow in the plant. Biotrophic pathogens need living plant tissue for growth and reproduction. In the case of hemibiotrophic pathogen attacks, the plant tissue will die in late stages of the infection process. Necrotrophic pathogens kill the plant tissue in early stages of the infection and are considered to feed on dead plant tissue. All viruses need living plant tissue to perform their life cycle, whereas bacteria, oomycetes, and fungi can be biotrophic or necrotrophic, depending on the species. The mechanisms of infection of viruses are very distinct from those of bacteria, oomycetes, and fungi, which share common features, and viruses trigger plants defences that are specific to these pathogens (Mandadi and Scholthof, 2013). The present review focuses on the interactions of plants with bacteria, oomycetes, and fungi and the importance of nitrogen (N) metabolism in the outcome of these interactions.

To resist pathogen attack, plants possess pre-formed defences such as cell walls, epidermal cuticles, and bark. Plants can also activate, both at the site of infection and systemically, an arsenal of inducible defences that includes massive transcriptional reprogramming, production of reactive oxygen species (ROS), reinforcements of the cell wall, such as accumulation of hydroxyproline-rich glycoproteins (HRGPs) and callose deposition, and synthesis of antimicrobial secondary metabolites and pathogenesis-related (PR) proteins involved in resistance (Glazebrook *et al.*, 2005; Bellincampi *et al.*, 2014). The molecular mechanisms underlying activation of plant defence responses are extremely complex and depend on the major defence hormones salicylic acid (SA) and jasmonic acid (JA); however, the phytohormones ethylene, gibberellins, auxins, abscisic acid (ABA), cytokinins, and brassinosteroids are also known to act as modulators of the immune response (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012).

Recognition of pathogens by plants

Pathogen recognition by plants leading to defence activation occurs mainly at two levels (Jones and Dangl, 2006). The first line of inducible plant defence is formed by pattern recognition receptors (PRRs). These cell surface receptors recognize microbe- or pathogen-associated molecular patterns (MAMP/PAMPs) that are generally highly conserved molecules within a class of microbes and generally have an essential function in microbial fitness or survival, although counter-examples exist (Thomma *et al.*, 2011). Well-studied examples of PAMPs are bacterial flagellin and fungal chitin. Upon detection of PAMPs, PRRs activate an innate immune response called PAMP-triggered immunity (PTI). Successful pathogens are able to overcome PTI by secreting effectors that suppress PTI responses. Many phytopathogenic bacteria inject type three effectors (T3Es) directly into the host cytoplasm through their type three secretion system (T3SS), while many oomycetes and fungi secrete effectors in the intercellular space that can then be taken up by plant cells using specific recognition motifs (Kale and Tyler, 2011). During evolution, plants have responded to these effectors through the development of cytoplasmic resistance (R) proteins that recognize (the presence or activity of) single effectors and activate effector-triggered immunity (ETI). In general, ETI is considered to occur more rapidly and to be stronger than PTI; however, transcriptome analysis showed that the sets of defence genes induced during ETI and PTI are largely overlapping, the main difference between the two responses being in the timing and the intensity of the responses (Tao *et al.*, 2003). When the outcome of a plant–pathogen interaction is disease, the interaction is considered compatible, whereas when the outcome is resistance, it is considered incompatible.

The production of effectors by pathogens to suppress plant defence is a widespread virulence strategy. However, other virulence strategies exist. In particular, necrotrophic pathogens can produce pectin-degrading enzymes as well as cell death-inducing toxins, both of which can play major roles in virulence. For example, the virulence of the bacterial phytopathogens *Pectobacterium carotovorum* and *Dickeya dadantii* is strongly dependent on the production of pectin-degrading enzymes, whereas the virulence of the necrotrophic fungus *Botrytis cinerea* depends on both pectin-degrading enzymes and toxins (Choquer *et al.*, 2007; Davidsson *et al.*, 2013). The action of cell wall-degrading enzymes (CWDEs) leads to the production of pectin-derived oligogalacturonides (OGs). These host-derived molecules are called danger-associated molecular patterns (DAMPs) in reference to the MAMP/PAMPs cited above. Apart from pectin-derived OGs, AtPep1, a peptide derived from a cytoplasmic plant protein PROPEP1, has also been identified as a DAMP in plants. Plants have the capacity to detect the effect of infection via the detection of these host-derived molecules, and DAMP-induced defence shares many features with ETI and PTI (Mengiste, 2012).

The signalling pathways that result from pathogen attack and lead to defence activation have been widely studied, and many molecular players in the signalling pathways involved have been identified. Interestingly, increasing evidence points to a strong convergence between signalling pathways involved in the response to biotic and abiotic stress (Fujita *et al.*, 2006). Indeed, many signalling components, including mitogen-activated protein (MAP) kinase and transcription factors, have now been shown to be involved in the response to biotic and abiotic stress. Moreover, recent data indicate that the response of plants to a combination of stresses is not a simple addition of the responses to the individual stresses (Prasch and Sonnewald, 2013). On the contrary, the plant's response to stress combinations at the transcriptional level is for the most part not predictable from the response to individual stresses (Rasmussen *et al.*, 2013).

Plant metabolic status and pathogen infection are mutually inter-related

Superimposed on defence suppression, pathogens reconfigure host metabolism, and phytopathogen infection has a strong impact on both primary and secondary metabolism in plants (Ward *et al.*, 2010). These changes in primary plant metabolism notably can affect plant growth and development, but also lead to crop yield losses even when plant–pathogen interactions do not lead to disease and/or cell death (Berger *et al.*, 2007). There are three main aspects to the impact of pathogen infection on primary metabolism (Berger *et al.*, 2007): (i) defence is cost-intensive; (ii) the pathogen often tries to manipulate plant metabolism to its advantage creating a withdrawal of nutrients such as sugars and amino acids; and (iii) the development of chlorotic and necrotic areas following infection decreases photosynthethic activity and certainly also other chloroplast metabolism pathways locally.

Infection with both compatible and incompatible pathogens leads to a local decrease in chlorophyll fluorescence (Swarbrick *et al.*, 2006). As with defence activation (Tao *et al.*, 2003), the main difference between the reduction of chlorophyll fluorescence in response to these two types of pathogens lies in the kinetics, incompatible interactions leading to a more rapid decrease in chlorophyll fluorescence (Swarbrick *et al.*, 2006). In some cases, but not all, the observed decrease in chlorophyll fluorescence is correlated with a decrease in associated gene expression (Berger *et al.*, 2007). Finally, several data indicate that as a result of infection, reduction of the rate of photosynthesis is a fast and rapid process, while down-regulation of photosynthethic gene expression is a slower process. These changes in primary metabolism following pathogen infection lead to a reduction in photosynthetic assimilate availability which transform the originally source tissue into a sink tissue. One manifestation of this is an increase in cell wall invertases that cleave apoplastic sucrose into glucose and fructose, thereafter transported into the cell (Baker *et al.*, 2012). Repression of photosynthesis and induction of sink metabolism seem to be a general response to pathogen infection (Berger *et al.*, 2007). However, the effect of infection on the accumulation of macromolecules such as sugars depends on the pathogen (Berger *et al.*, 2007).

Several recent studies have focused on large-scale analysis of metabolic changes following plant infection by pathogens [reviewed in Balmer *et al.* (2013) for cereals]. One challenge in plant pathology is to be able to distinguish between defence-associated metabolites and disease-associated metabolites. Metabolic modifications associated with basal defence are rapidly activated, whereas disease-associated modifications, which require transcription and translation of virulence factors by the pathogen, occur at later time points. In response to the virulent bacterial phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000, *Arabidopsis* leaves display significant modifications starting from 8 h post-infection (Ward *et al.*, 2010). Many metabolite modifications detected by gas chromatography–mass spectrometry (GC-MS) were of low intensity, reflecting wide and subtle remodelling of the plant's metabolome following infection (Ward *et al.*, 2010). However, the accumulation of a number of metabolites displayed important changes following infection such as the phytoalexin camalexin, a non-pathogen-specific antimicrobial compound, and stigmasterol, which is known to reduce membrane permeability (Wang *et al.*, 2012). The amount of only one amino acid, aspartate, was found to be reduced in response to *P. syringae* DC3000 infection; the other amino acids were either not affected or accumulated to a higher extent than in non-infected *Arabidopsis* leaves, such as the three aromatic amino acids involved in the biosynthesis of the defence-associated secondary metabolites such as flavonoids (Ward *et al.*, 2010). In *Arabidopsis* leaves infected by the necrotrophic fungus *Alternaria brassicicola*, the amount of a large number of metabolites was also significantly altered (Botanga *et al.*, 2012). Several modifications were identical to those observed in response to *P. syringae*, such as stigmasterol and trehalose accumulation. Accumulation of the three aromatic amino acids also occurred in response to *A. brassicicola* in *Arabidopsis*, but the overall pattern of amino acid and sugar modifications retained specificities in response to each pathogen, indicating both common and specific metabolic patterns in response to different pathogens. This feature was confirmed by our GC-MS analysis of the non-host metabolic responses occurring in *Arabidopsis* Col-0 leaves, 24 h after inoculation with the phytopathogenic bacterium *Erwinia amylovora* (A. Launy and M. Fagard, unpublished). *E. amylovora* induces modifications shared with *P. syringae* (sucrose, valine, and isoleucine accumulation), *A. brassicicola* (homoserine and lysine accumulation), and both pathogens [stigmasterol, tyrosine, and γ-aminobutyric acid (GABA) accumulation, aspartate decrease] but also shows specific features such as a decrease in methionine content. Thus, metabolic reprogramming seems to be a general feature of plant–pathogen interactions and is a consequence of both defence (through the accumulation of secondary metabolites for example) and the requirement for pathogens to acquire nutrients, in particular carbon sources through sugar accumulation and N sources through amino acid accumulation (see below), which in turn can affect defence activation (Rojas *et al.*, 2014).

The metabolic state of the plant during the infection process, which is in part controlled through transcriptional regulation of N metabolism enzymes (Ward *et al.*, 2010), might thus be important for the interaction. It was shown that N-related gene expression can be affected by pathogen infection (see 'Impact of amino acid metabolism and recycling on plant–pathogen interactions'). For example, the cytosolic glutamine synthetase (GS1) isoform-encoding genes have been shown to be induced following infection by pathogens in tobacco (Pageau *et al.*, 2006), and we found in *Arabidopsis* that *GLN1.1* is strongly induced following *E. amylovora* infection (Fig. 1). In order to determine if the modification of N-related gene expression is specific for certain types of plant–pathogen interactions, we retrieved transcriptome data for *Arabidopsis* N metabolism genes (Masclaux-Daubresse *et al.*, 2010) from public databases (Fig. 1). Interestingly, most of the N metabolism genes modulated in response to infection by pathogens show a consistent pattern in response to different pathogens (either induced or repressed by several pathogens). A smaller proportion of genes displayed a variable pattern of modulation, both induced and repressed by pathogens, with no obvious pattern linked to either pathogen life style or compatibility of the interaction, perhaps as a result of specific manipulation by pathogens. No obvious pattern of modulation could be associated with the gene functions, apart from the fact that the chloroplastic GS (*GS2*) is repressed during most of the interactions associated with cell death (hemibiotrophs and incompatible interactions leading to resistance). *GS2* repression emphasizes the correlation between the chloroplastic decay leading to chlorosis and the cell death process occurring in these interactions. On the other hand, the cytoplasmic GS-encoding genes (*GLN1.1*–*GLN1.5*) show variable patterns of modulation in response to pathogens, which may also suggest specific manipulation by pathogens. Also, consistent with GABA accumulation in response to pathogens, GABA metabolism genes are mostly induced by infection (Fig. 1; see 'Impact of amino acid metabolism and recycling on plant–pathogen interactions'). Several members of the NRT2 family of high-affinity nitrate transporters are also strongly induced in response to pathogens, consistent with previous reports (see 'Nitrate uptake and plant–pathogen interactions'). Concerning pathogen life style (biotrophic, hemibiotrophic, or necrotrophic) and type of interactions (compatible or incompatible), it appears that there is a gradient showing an increase of the number of N metabolism genes up- or down-regulated during the infection. Indeed, in response to necrotrophic pathogens and pathogens triggering resistance, more N metabolism genes are up- or down-regulated than in response to biotrophic pathogens, suggesting that many of the observed gene modulations are associated with cell death and/or plant defence. Overall, N metabolism genes are strongly affected by pathogen infection, probably as a result of both defence activation and attempted pathogen manipulation of host metabolism for nutritional purposes.

Nitrogen supply and disease

Many agronomical studies indicate that N fertilizer application affects plant disease (reviewed in Huber and Watson,

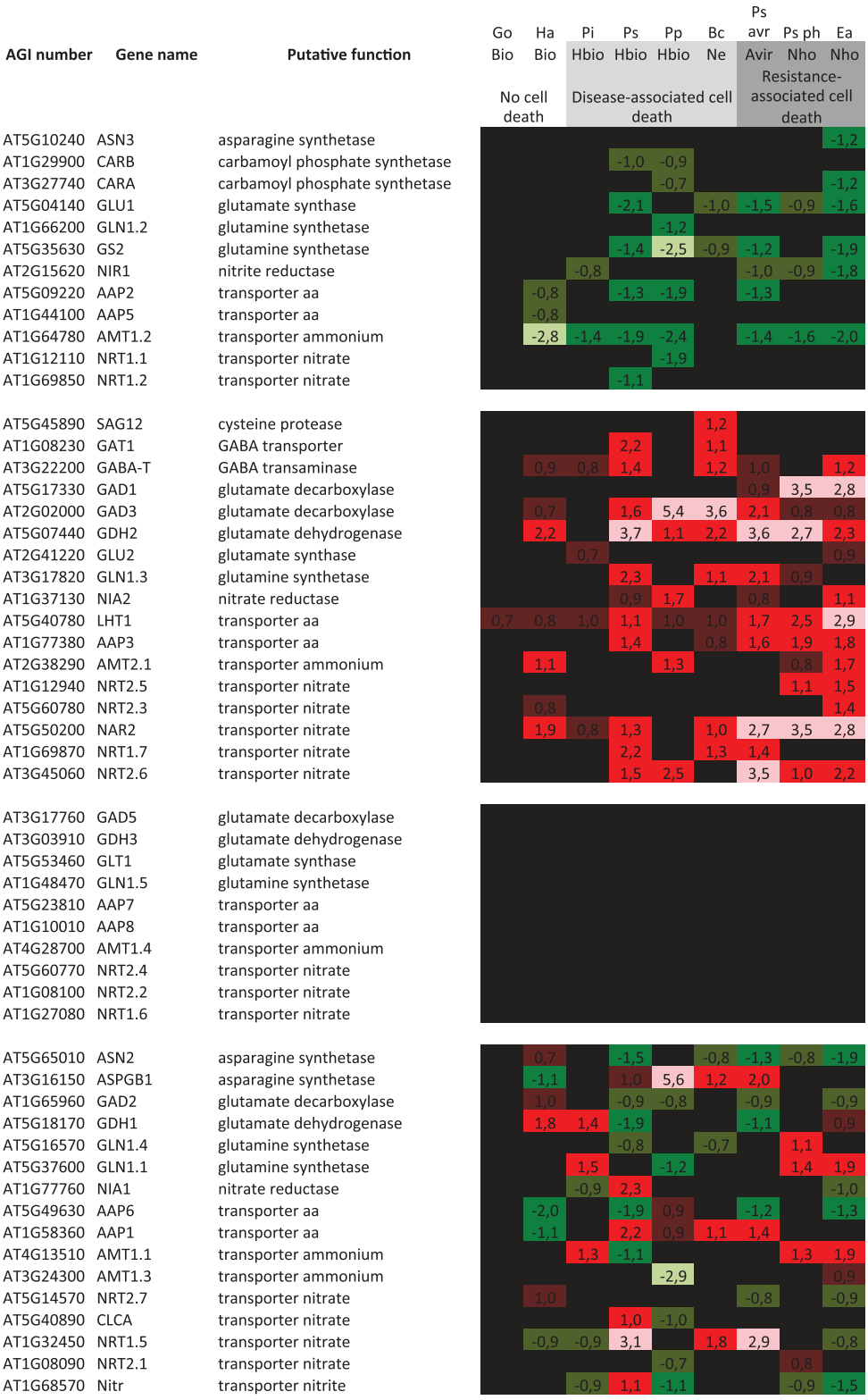

Fig. 1. Expression of selected N metabolism genes in response to biotic stress. Values represent the log2 (ratio) of the fold modulation of *Arabidopsis* genes in response to infection compared with mock treatment. Data were collected from the Genevestigator database (Zimmermann *et al.*, 2004) and correspond to Affymetrix analysis except for the Ea data that we generated by CATMA analysis (Moreau *et al.*, 2012) and are present in the catDB database (Gagnot *et al.*, 2008). Log ratios above 0.8 or below –0.8 are presented using a colour code according to the intensity of the modulation. The data set corresponds to a 24 h post-inoculation (hpi) time point except for Ha (12 hpi), Bc (18 hpi), and Pp (30 hpi). We selected a panel of compatible interactions leading to disease (Go, Ha, Pi, Ps, Bc, Pp) and incompatible interactions leading to resistance (Ps avr, Ps ph, Ea). We also selected representatives of the different pathogen life styles: biotrophic (Bio), hemibiotrophic (Hbio), necrotrophic (Ne), avirulent (Avir), and non-host (Nho) pathogens. Go, *Golovinomyces orontii*; Ha, *Hyaloperonospora arabidopsidis*; Pi, *Phytophthora infestans*; Ps, *Pseudomonas syringae* pv. *tomato* DC3000; Ps avr, *Pseudomonas syringae* pv. *tomato* avrRpm1; Ps, *Pseudomonas syringae* pv. *phaseolicola*; Ea, *Erwinia amylovora*; Pp, *Phytophthora parasitica*; Bc, *Botrytis cinerea*.

1974; Walters and Bingham, 2007; Dordas, 2008). However, these studies led to contradictory conclusions, probably in part because of different requirements for growth and sensitivity to defence metabolites of pathogens. It has also been suggested that obligate and biotrophic pathogens might have a different sensitivity to N fertilizers compared with necrotrophic pathogens. Indeed, N fertilizers generally increase the susceptibility of plants to biotrophs, whereas they generally decrease the susceptibility of plants to necrotrophs (Snoeijers *et al.*, 2000; Dordas, 2008; Ballini *et al.*, 2013). However, this is clearly not the whole story. For example, the impact of N supply on the susceptibility of plants to the necrotrophic fungus *B. cinerea* depends on the virulence of the strain (Lecompte *et al.*, 2010), which could in part explain the contradictions observed in the literature as to the effect of N fertilization on disease. We analysed the impact of growth in N limitation conditions on the susceptibility of *Arabidopsis* Col-0 plants to the necrotrophic phytobacterium *E. amylovora* and to the necrotrophic fungus *B. cinerea*, two interactions during which cell death is thought to play an important role (Govrin and Levine, 2000). We found that N limitation reduced the resistance of *Arabidopsis* to *E. amylovora* (Fig. 2A; M. Farjad and M. Fagard, unpublished data), which is consistent with our previous results indicating that *Arabidopsis* resistance against *E. amylovora* is an active process (Moreau *et al.*, 2012) and suggesting that this defence process is strongly affected by N supply. On the other hand, N limitation reduced the susceptibility of *Arabidopsis* to *B. cinerea* (Fig. 2B; J. Courtial and M. C. Soulié, unpublished data). Altogether, current knowledge indicates that the impact of N supply on plant disease is complex and thus requires in-depth scientific investigation.

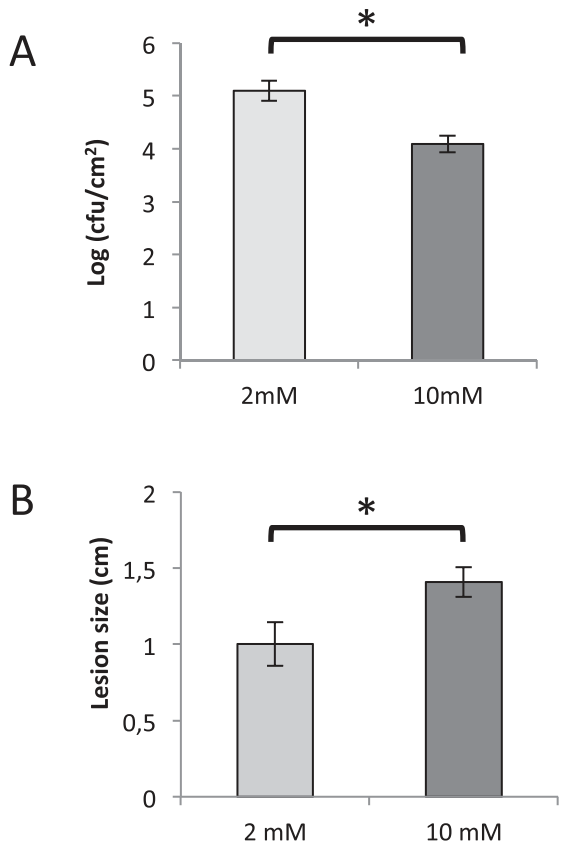

Fig. 2. Effect of N limitation on the susceptibility of *Arabidopsis* to pathogens. *Arabidopsis* Col-0 plants were grown in sand on limiting nitrate (2 mM NO_3^-) or non-limiting nitrate (10 mM NO_3^-). As described previously, the 2 mM condition was limiting for plant growth (Lemaître *et al.*, 2008) as the rosette biomass of 5-week-old plants grown at 2 mM NO_3^- was significantly lower than that of plants grown at 10 mM NO_3^- (not shown). Leaves of 5-week-old plants were infected with the bacterial phytopathogen *E. amylovora* (A; strain CFBP1430) or the necrotrophic fungus *B. cinerea* (B; strain B0510) as described previously (Degrave *et al.*, 2013; Morcx *et al.*, 2013). *E. amylovora* bacterial growth is shown 24 h post-inoculation, and *B. cinerea*-induced lesion size is shown 3 d post-infection. Asterisks indicate a significant statistical difference between the two conditions according to Student's *t*-test (*P*-value <0.05). The experiments were each repeated at least three times with similar results, and a representative experiment is shown.

Effect of N supply on defence

One way in which N supply may affect plant–pathogen interactions is through an impact on plant defence production. Contradictory generalities are found in the literature concerning the impact of nutritional status on plant defence production: on the one hand, it is generally thought that there is a trade-off between growth and defence (Walters and Heil, 2007), but on the other hand, some authors have suggested that low nutrition weakens plants and is thus not favourable for defence (Snoeijers *et al.*, 2000). The growth–differentiation balance hypothesis (GDBH) and the carbon nutrient balance hypothesis (CNBH) both predict that under limiting growth conditions, available resources would be allocated to higher defence production (Massad *et al.*, 2012). This prediction was partly confirmed in the legume *Pentaclethra macroloba* for which the authors found a trade-off between growth (biomass) and flavan production (phenolic defence metabolites) (Massad *et al.*, 2012). However, the authors found that saponins (terpenoid defence metabolites) increased with biomass, suggesting that the trade-off between defence and growth may not be a general feature of plant–pathogen interactions as is often believed. The monitoring of defence-associated enzymatic activities such as chitinase, chitosanase, and peroxidase in *Arabidopsis* plants showed that all three basal activities were reduced in low N (Dietrich *et al.*, 2004). In addition, the induction level of these enzymes following treatment with BION®, a chemical elicitor of plant defence (Dietrich *et al.*, 2004), was reduced in low N. It has been shown recently that the form of N available to plants can also affect plant defence (see 'The role of NO').

Although it is clear that N limitation has an impact on plant defence, it is still difficult to obtain a general picture of this effect (Dietrich *et al.*, 2004). Furthermore, the molecular mechanisms underlying this control of defence by N limitation are globally unknown. One of the few interesting candidates is the RING-type ubiquitin E3 ligase BHA1/NLA, which is involved in regulating both SA accumulation and plant adaptation to N limitation (Yaeno and Iba, 2008). These authors suggest that BAH1/NLA could play a role in the regulation of SA levels under conditions of N starvation.

Effect of N supply on pathogen virulence

N supply can impact plant–pathogen interactions through an effect on pathogen virulence. The perception of N nutritional status by pathogens can contribute to the signals controlling activation of virulence factors and metabolic adaptation (reviewed by Snoeijers *et al.*, 2000). Several infection processes are activated under N starvation *in vitro*. For instance, the development of the filamentous form of the basidiomycete *Ustilago maydis*, which is a prerequisite for plant infection, is stimulated by N starvation (Horst *et al.*, 2012). Expression of some bacterial *hrp* genes, encoding T3SS and T3Es, is up-regulated in minimal medium *in vitro* and repressed by ammonium or the amino acids asparagine and histidine (Wei *et al.*, 1992). Expression of effector proteins involved in virulence from fungi or oomycetes can be up-regulated *in vitro* under N starvation (Bolton and Thomma, 2008). The preferred N source ammonium was found to repress the capacity of the fungal species *Fusarium oxysporum*, *Fusarium graminearum*, and *Magnaporthae oryzae* to penetrate cellophane membrane, which is a well-established virulence factor (Lopez-Berges *et al.*, 2010). Soft rot pathogens secrete CWDEs, leading to tissue disorganization, cell death and cell content release. Interestingly, the production of CWDEs of the soft rot bacterium *P. carotovorum* is up-regulated *in vivo* in chicory heads under high N (Schober and Vermeulen, 1999). Likewise, *D. dadantii* production of pectinases is repressed under low ammonium (Hugouvieux-Cotte-Pattat *et al.*, 1992). The presence of ammonium probably represents favourable nutritional conditions that do not require expression of virulence factors.

How do pathogens sense the level of N and translate this into a fine-tuning of virulence factor expression? In several fungal species the transcription factor AreA/Nit2 belonging to the GATA family of transcription factors is a global N regulator that activates the expression of genes encoding proteins involved in the transport and catabolism of secondary N sources (Bolton and Thomma, 2008). The pathogenicity of *areA*-deficient mutants from several phytopathogenic fungal species is affected, indicating the requirement for a functional N perception for full pathogenesis (Bolton and Thomma, 2008). Interestingly, N starvation-induced filamentous growth of *U. maydis* is largely dependent on functional AreA/Nit2 (Horst *et al.*, 2012). In addition, the production of the mycotoxin fumonisine B1 by the maize pathogen *F. verticilloides* is also dependent of the AreA regulator (Kim and Woloshuk, 2008).

Recently, further investigation of the link between N signalling and virulence factors showed that the TOR kinase, which regulates eukaryotic cell growth in response to nutrient availability and in particular N (De Virgilio and Loewith, 2006; Rohde *et al.*, 2008), and the bZIP MeaB protein, which acts as a negative regulator of the N catabolic response (Wong *et al.*, 2008), are both involved in the repression of virulence functions in *F. oxysporum*. Indeed, *F. oxysporum* repression of cellophane penetration by the favourable N source ammonium requires TOR and MaeB (Lopez-Berges *et al.*, 2010).

In Gram-negative bacteria, the global regulators of N sensing are the sigma 54 factor rpoN and the two-component system formed by NtrB–NtrC (Weiss *et al.*, 2002). The NtrB protein is anchored at the plasma membrane and senses environmental stimuli. In *Escherichia coli*, N metabolism regulation is in part controlled by the catabolic repressor protein CRP (cAMP receptor protein; van Heeswijk *et al.*, 2013). Knowing that genes encoding CWDEs in several *Pectobacterium* and *Dickeya* species are positively regulated by CRP (Reverchon *et al.*, 1997; Matsumoto *et al.*, 2003), it would be worth investigating the cross-talk between CRP and N-sensing systems in regulation of CWDE-encoding genes. All these reports provide evidence that phytopathogens integrate signals concerning the source and availability of N to fine-tune the expression of their virulence functions. However, the exact underlying signalling mechanisms still remain to be elucidated.

Nitrate uptake and plant–pathogen interactions

The NRT2 gene family belongs to the major facilitator superfamily (MFS) of transporters. In *Arabidopsis*, the NRT2 family comprises seven genes, among which four have been characterized as high-affinity nitrate transporters. NRT2.1 is the main high-affinity nitrate transporter in *Arabidopsis* roots under low nitrate availability (Orsel *et al.*, 2004; Li *et al.*, 2007). Regarding the higher plant NRT2s characterized to date, no substrate other than nitrate has been identified so far. However, several NRT2 proteins from *Chlamydomonas reinhardtii* and *Hansenula polymorpha* are nitrate/nitrite bispecific transporters (Machin *et al.*, 2004; Fernandez and Galvan, 2007). In addition to the nitrate transport function, nitrate transporters have recently been evidenced to be involved in nitrate sensing and act as so-called transceptors (transporter and receptor) (Ho *et al.*, 2009; Gojon *et al.*, 2011). Of all the NRT2 family members, to date only AtNRT2.1 has been shown to modify lateral root development independent of nitrate transport and thus to act as a transceptor (Little *et al.*, 2005). Recently two NRT2 genes have been identified as players in plant defence responses: the *Arabidopsis* mutants *nrt2.1* and *nrt2.6* are modified in the responses to *P. syringae* and to *E. amylovora*, respectively (Camanes *et al.*, 2012*b*; Dechorgnat *et al.*, 2012).

NRT2.1 represses reponses to biotrophic pathogens, probably favouring abiotic stress resistance by safeguading energy (Camanes *et al.*, 2012*a*, *b*). The reduced *P. syringae* susceptibiliy of the *nrt2.1* mutant was attributed to a faster response of the SA-dependent signalling pathway and a reduced sensitivity to the bacterial toxin coronatin. The ABA and JA signalling pathways were also modified, but probably as a result of SA priming. In an SA-deficient background the *nrt2.1* mutant was as susceptible as the wild type. In addition, the metabolic status of the *nrt2.1* mutant prior to infection might contribute to the reduced susceptibility. Indeed, the mutant accumulated higher levels of aromatic amino acids and phenylpropanoids (Camanes *et al.*, 2012*a*). Thus, priming, not

only by SA, but also by other secondary metabolites, seems to contribute to the reduced susceptibility of *nrt2.1* mutants to *P. syringae*. Transcriptomic analysis supported these results, as genes involved in SA and aromatic acid synthesis were differentially expressed between mutant and wild-type plants. In addition several ribosomal proteins are overexpressed in the mutant, which might be another factor contributing to resistance to bacterial infection (Camanes *et al.*, 2012*a*). No evidence for the mechanism of the altered coronatin sensitivity has been found yet, but a bacterial strain deficient for coronatin triggered clearly similar responses in wild-type and *nrt2.1* mutants (Camanes *et al.*, 2012*a*, *b*).

NRT2.6 has been shown to participate to the response to the necrotrophic bacterium *E. amylovora*. The *Arabidopsis nrt2.6* mutant was more sensitive to *E. amylovora* than wild-type plants. The hypersensitivity of the mutant was correlated with a reduced ROS accumulation, whereas no difference in the transcriptional response to the pathogen nor in other cellular responses such as callose synthesis and NO production has been evidenced (Dechorgnat *et al.*, 2012). The link between NRT2.6 and ROS production is not restricted to pathogen attack, but was also revealed after treatment with the redox-active methyl viologen herbicide. However, to date, no evidence for a function of NRT2.6 in nitrate transport has been obtained, and the molecular link between NRT2.6 and ROS production needs further investigation.

Altogether, these data indicate that nitrate sensing or nitrate transport has an important impact on defence reactions and suggest that the SA defence pathway, ROS production, and metabolite status play important roles in the cross-talk between N availability and biotic stress.

The role of NO

NO is a highly reactive gas with high diffusion rates across membranes. Several molecules can derive from NO and are collectively called reactive nitrogen species (RNS) that comprise the radical NO·, its nitrosonium (NO^+), and ni-troxyl ions (NO^-). When NO is produced in conjunction with ROS, such as during plant–pathogen interactions, NO can react with the superoxide anion $O_2^{\cdot -}$ to generate peroxynitrite ($ONOO^-$). In animals, the generation of NO under infectious conditions is mainly due to an inducible nitric oxide synthase (iNOS), which catalyses the NADPH-dependent oxidation of L-arginine to L-citrulline and NO (Stuehr *et al.*, 2004). In plants several data suggest the existence of such an enzymatic activity, but we still do not know the enzyme involved in this process (Besson-Bard *et al.*, 2008; Bellin *et al.*, 2013). Production of NO in plants has been suggested to depend on several routes that can be divided into oxidative and reductive routes. Oxidative routes include the enzymatic activities polyamine and hydroxylamine oxidation (Tun *et al.*, 2006; Ruemer *et al.*, 2009). Accordingly, a copper amine oxidase was proposed to be involved in NO production *in planta* in response to ABA (Wimalasekera *et al.*, 2011). Reductive routes consist of nitrite reduction via mitochondrial electron transfer systems (Modolo *et al.*, 2005; Planchet *et al.*, 2005; Gupta and Igamberdiev, 2011), a root-specific nitrite:NO-reductase (Ni-NOR), the peroxisomal xanthine oxidoreductase enzyme (Stohr *et al.*, 2001), and nitrate reductase (NR) (Rockel *et al.*, 2002; Moche *et al.*, 2010). NO is involved in several physiological processes in plants, including germination, development, stomatal closure, and immunity where it was shown to be involved in the hypersensitive response (HR) and during compatible interactions (Delledonne *et al.*, 1998, 2001; Durner *et al.*, 1998). The role of NO in plant–pathogen interactions has been reviewed recently (Bellin *et al.*, 2013). Here, we will only focus on aspects that link N nutrition and metabolism to NO production in plant–pathogen interactions.

In the context of plant–pathogen interactions, it seems that NR is an important source of NO. Modolo *et al.* (2005) were the first to show that NR activity is the major source of NO during the pathogenic interaction *Arabidopsis–P. syringae*. Other reports showed that NR participates in NO accumulation in plant–pathogen interactions (Asai and Yoshioka, 2009; Perchepied *et al.*, 2010) or in response to elicitors (Yamamoto-Katou *et al.*, 2006). However, decreased HR in *Arabidopsis* plants treated with *P. syringae* pv. *maculicola* in NR-deficient plants was correlated to a lack of L-arginine and NO_2, two important endogenous substrates for NO synthesis (Modolo *et al.*, 2006). Conversely, it was later shown that the increased susceptibility to *P. syringae* of the *NR*-deficient plants was independent of amino acid accumulation and was more likely to be due to a reduced ability of these mutants to synthesize NO (Oliveira *et al.*, 2009). Interestingly, the activity of *NIA2*-encoded *Arabidopsis* NR enzyme was shown to be up-regulated through phosphorylation by the MAP kinase MPK6 (Wang *et al.*, 2010), involved in biotic stress responses (Pitzschke *et al.*, 2009); however, the role of this regulation during plant–pathogen interactions remains to be investigated.

Interestingly the nutrition of the plant can have an effect on NO production. Tobacco grown with nitrate was found to produce more NO than tobacco grown on ammonium when plants were inoculated with the pathogenic bacterium *P. syringae* pv. *tabaci* or the incompatible bacterium *P. syringae* pv. *phaseolicola* (Gupta *et al.*, 2013). The authors showed that NO accumulation was associated with increased resistance to the pathogens. Conversely, in soybean cotyledons, no difference was observed in NO production whether the plants were grown with nitrate or ammonium (Galatro *et al.*, 2013). Pathogens can contribute to the scavenging of NO. For instance, the flavohaemoglobin HmpX from the pathogenic bacterium *D. dadantii* was shown to contribute to the reduction of NO during HR (Boccara *et al.*, 2005). Thus NO is a pivotal element in plant–pathogen interactions, and its production and turnover are strongly linked to N metabolism.

Impact of amino acid metabolism and recycling on plant–pathogen interactions

Presumptions that amino acid metabolism can impact plant–pathogen interactions had been raised by the observations

that amino acid contents and relative concentrations are modified during plant disease or in response to pathogen attack. When peach tree leaves are infected by the Eastern X-disease pathogen, they accumulate proline (McKee, 1972). Kumar and Prasad (1992) observed that the whole amino acid pool increases in crucifers when infected by compatible or incompatible *Xanthomonas campestris* pv. *campestris*. Aubergines (eggplants) suffering little leaf disease, caused by *Candidatus* Phytoplasma asteris, accumulate specifically lysine and asparagine, whereas histidine and arginine are depleted (Das and Mitra, 1993). Reports of changes in amino acid composition during plant–pathogen interaction are numerous, and the more recent metabolomic technologies also confirm that amino acid metabolism is greatly modified during plant–pathogen interactions. However, none of these observations provided insight into the role of such amino acid modifications in the outcome of the interaction.

Relationships between amino acid accumulation and pathogen diet preferences suggested, however, that the co-evolutionary conflict between plant and pathogen has led, in compatible interactions, to pathogen adaptation to the host nutritional resources. As such it is interesting to note that GABA, which is the favourite N source of *Cladosporium fulvum*, accumulates in tomato leaves specifically during compatible interactions (Solomon and Oliver, 2001, 2002). Similarly, glutamine, which is preferentially used among all amino acids by *Colletotrichum lindemuthianum*, accumulates to high levels in French bean leaves during compatible interactions (leading to disease), but not in leaves that are infected by avirulent strains, which trigger resistance (Tavernier *et al.*, 2007). Nutritional specialization of the *P. syringae* pv. *tomato* for GABA, aspartate, glutamate, and glutamine also suggests in this case a trophic adaptation of this pathogen to its host N resources. Paradoxically, in the plant–pathogen interactions reported above, it can be observed that the amino acids preferentially used by pathogens are often those that accumulate specifically during disease. As it cannot be assumed that plants lay the table for the meal of their pathogens, it can be considered that pathogens have adapted to use amino acids that accumulate in their host under stress conditions. However, the possibility that pathogens manipulate plant metabolism to pump amino acids should also be considered.

It has been known for a long time that amino acids such as GABA and proline have a role in plant tolerance to abiotic stresses (Snedden and Fromm, 1999; Sharma and Dietz, 2006). GABA and proline could protect cells against oxidative stress and osmotic stress, and regulate cytosolic pH (Bouche and Fromm, 2004; Szabados and Savoure, 2010). Together with arginine, they are also N storage amino acids known to accumulate during stress and to give back N and energy during recovery periods. GABA, proline, and arginine are direct products from glutamate metabolism (Fig. 3). The GABA shunt is a way to control the C:N status and to replenish the tricarboxylic acid (TCA) cycle with carbon to sustain mitochondrial respiration (Bolton, 2009; Michaeli *et al.*, 2011). Proline is known to accumulate to very high levels under drought conditions (Verslues and Juenger, 2011); however, its role in drought resistance is unclear, and, as for GABA, it seems that the proline pool accumulated during the stress period is useful during the recovery time to provide new glutamate pools and energy (Szabados and Savoure, 2010).

Interestingly, GABA, proline, and arginine, as well as glutamine and asparagine are known in several plants to be involved in N recycling, remobilization, and translocation pathways. Their role during natural leaf senescence and sink/source N remobilization has been reported in several reports (for a review, see Masclaux-Daubresse *et al.*, 2010). We know that during leaf senescence chloroplast dismantling releases a large pool of amino acids from the degradation of stromal and photosynthetic apparatus proteins. Amino acids that are not directly loaded to the phloem sap for translocation are used to support mitochondrial respiration through the catabolism of GABA (GABA shunt) and glutamate (through glutamate dehydrogenase, GDH) in the mitochondria and to form asparagine and glutamine as the result of the condensation of ammonium on aspartate and glutamate molecules (Fig. 3). The synthetized glutamine and asparagine are then uploaded to the phloem sap. Induced expression of several of the senescence-associated N remobilization enzymes was observed during plant–pathogen interactions (Buchanan-Wollaston, 1997; Pérez-Garcia *et al.*, 1998*a*, *b*; AbuQamar *et al.*, 2006; Pageau *et al.*, 2006; Tavernier *et al.*, 2007). In response to pathogen attack and to a large variety of stresses such as drought, salt stress, or heavy metal, and more globally in response to oxidative stress, plants induce N remobilization processes in order to translocate and safeguard nutrients in their non-infected tissues (Chaffei *et al.*, 2004; Olea *et al.*, 2004). It is thus possible that pathogens adapted to their host then take advantage of recycling metabolism for their own benefit.

Conversely, some evidence suggests that induction of N remobilization genes in response to plant pathogens is modulated in parallel to defence genes. Tavernier *et al.* (2007) showed that the expression of the GS1-encoding gene (*GS1-a*) paralleled the expression of the *PAL3* and *CHS* defence genes, suggesting a role for GS1 in plant defence. Similarly, the pepper asparagine synthetase 1 (*CaAS1*) gene showed exactly the same expression pattern as the defence marker gene *CaBPR1* during compatible and incompatible interactions with *X. campestris* pv. *vesicatoria* strains (Hwang *et al.*, 2011). The pepper *CaAlaAT1* alanine aminotransferase gene was also induced in senescing leaves, in response to SA and ethylene but not JA, and was enhanced in the incompatible interaction with *Tobacco mosaic virus* (TMV-Po). In response to three different *P. syringae* strains, the tobacco *GS1* gene was also only found to be induced during incompatible interactions. Therefore it is likely that amino acid metabolism and plant–pathogen interactions have a more complex connection than simply a trophic relationship, and it is possible that N enzymes participate in plant defence signalling.

The role of amino acid metabolism in the control of plant–pathogen interactions is highlighted by recent studies that used mutants affected in amino acid metabolism enzymes or transporters. Hwang *et al.* (2011) showed that disease symptoms were higher in *CaAS1*-silenced pepper leaves infected by *X. campestris* pv. *vesicatoria* than in controls

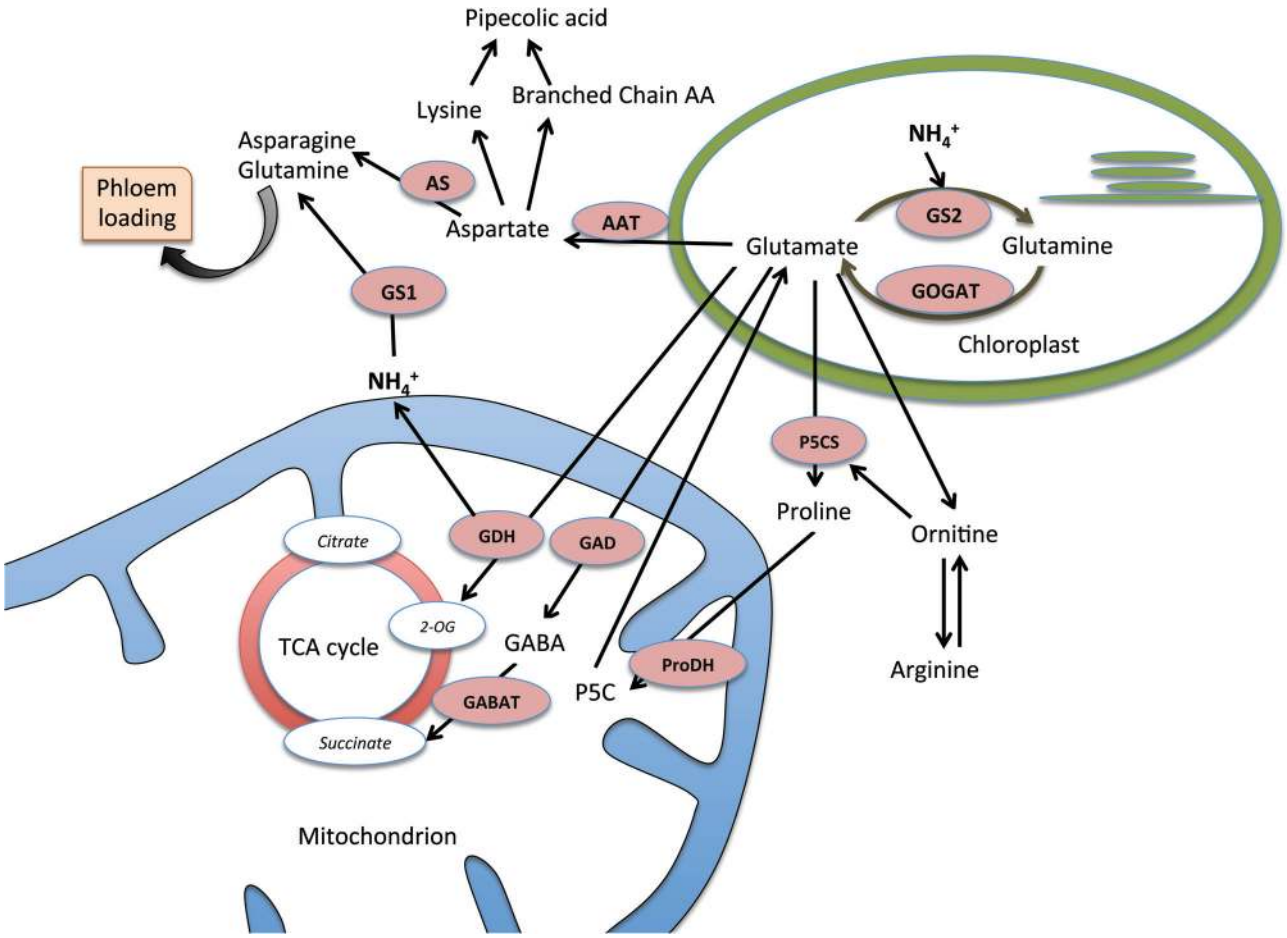

Fig. 3. Schematic representation of amino acid metabolism in plants. The major routes of amino acid metabolism and recycling are presented. Asparagine synthetase (AS), aspartate amino transferase (AAT), cytosolic glutamine synthetase (GS1), glutamate dehydrogenase (GDH), glutamate decarboxylase (GAD), GABA transaminase (GABAT), proline dehydrogenase (ProDH), 1-pyrroline-5-carboxylate synthetase (P5CS), chloroplastic glutamine synthetase (GS2), and glutamate synthase (GOGAT) are represented in pink ovals. In the tricarboxylic acid cycle (TCA), α-ketoglutarate is represented as 2-OG. Adapted from Avila-Ospina L, Moison M, Yoshimoto K, Masclaux-Daubresse C. 2014. Autophagy, plant senescence, and nutrient recycling. *Journal of Experimental Botany* **65,** 3799–3812. With permission from the Society for Experimental Biology.

and that *Arabidopsis* plants overexpressing *CaAS1* exhibited enhanced resistance to *P. syringae* pv. *tomato* DC3000 and *Hyaloperonospora arabidopsidis*. Consistently, asparagine levels were associated with early defence responses such as electrolyte leakage and ROS accumulation. It remains unclear, however, how the conversion of aspartate to asparagine can modulate plant defence. Recently Seifi *et al.* (2014) showed that asparagine synthetase plays a role in the immune response of tomato to *B. cinerea* infection and that asparagine might also promote *B. cinerea* pathogenesis. Further evidence comes from the infection by *B. cinerea* of *Arabidopsis* plants overexpressing aspartate aminotransferase (AAT). AAT catalyses transamination between glutamate and oxaloacetate to produce aspartate and α-ketoglutarate. The overexpression of the *Asp2* gene encoding AAT and known to be induced in *Arabidopsis* by several biotic stresses led to larger lesions spreading after *B. cinerea* infection and to changes in amino acid composition (Brauc *et al.*, 2011). More recently, the alterations in the GABA shunt, GS/GOGAT cycle, and phenylpropanoid pathways observed at transcriptional, enzymatic, and metabolic levels in the ABA-deficient *sitiens* mutant of tomato (*Solanum lycopersicum*) suggested that glutamine and GABA could be important for the resistance of tomato to *B. cinerea* and for the rapid epidermal HR and phenylpropanoid pathway-derived cell wall fortification. Microarrays and further studies had revealed that in addition to genes involved in plant defence (*PR1*) and cell wall biogenesis, several genes, including AAT, glutamate decarboxylase, GABA transaminase (*GABAT*), and *GS1* were significantly up-regulated in the resistant tomato cultivars a few hours post-inoculation (Asselbergh *et al.*, 2007). As *GABAT* and *GS1* genes showed higher expression levels in the *sitiens* mutants, gene silencing was performed on these genes using virus-induced gene silencing (VIGS) technology on both wild-type and *sitiens* genotypes. While no effect of *GABAT* and *GS1* silencing can be observed at the symptom level on the wild type after *B. cinerea* infection, both the *GABAT*- and *GS1*-silenced *sitiens* mutants exhibited significantly higher susceptibility phenotypes compared with the *sitiens* controls transformed with empty vectors (Seifi *et al.*, 2013*a*). The authors thus proposed a model in which the overactivation of nutrient recycling through GS1 and the replenishment of the TCA cycle through the GABA shunt maintain cell viability in the areas surrounding invaded tissue. Cell survival

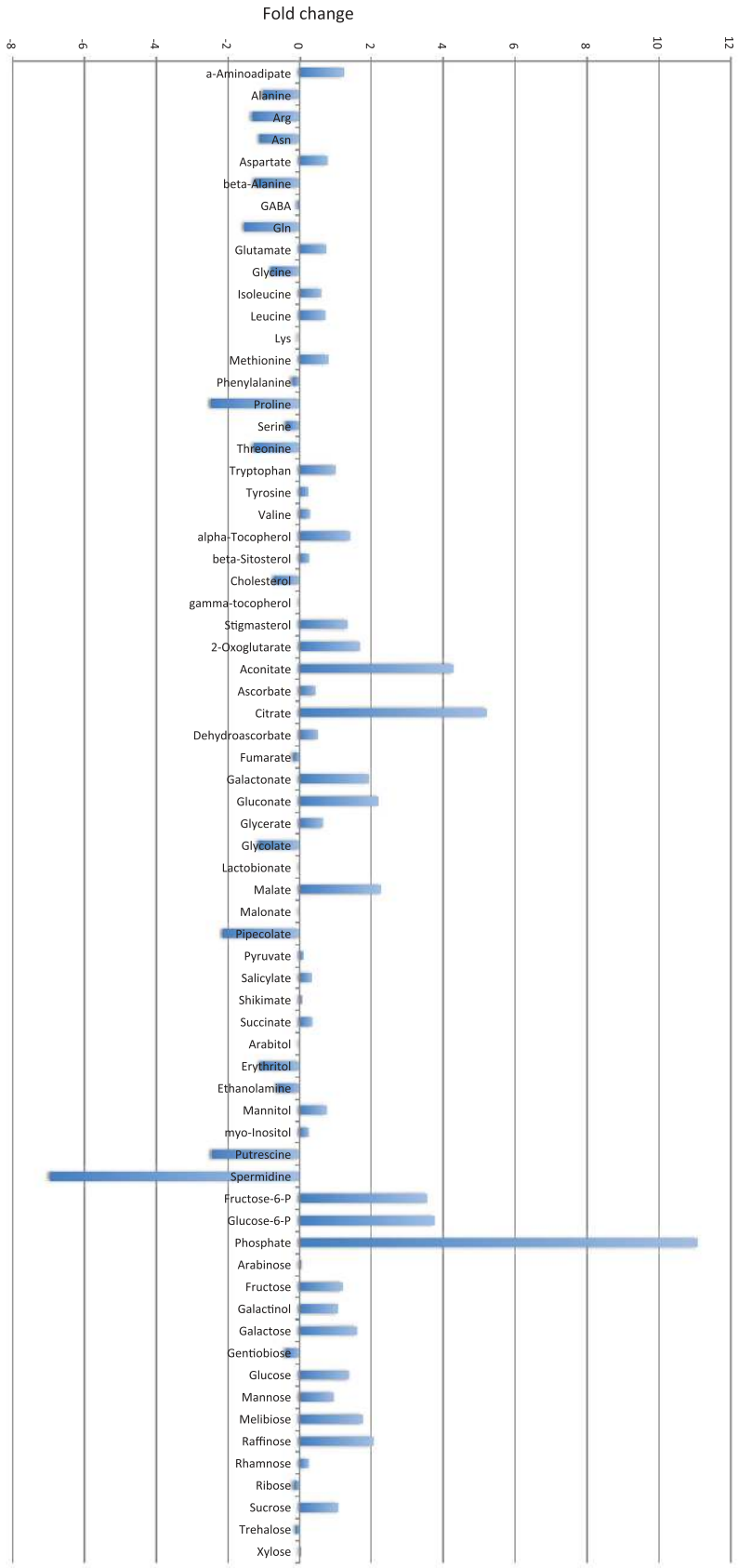

Fig. 4. Metabolomic analysis of *Arabidopsis* plants grown in limiting or non-limiting N conditions. Metabolite profiling of *Arabidopsis* plants grown in limiting or non-limiting nitrate (Lemaitre *et al.*, 2008) was analysed by GC-MS. The results are shown as the ratio of accumulation of each metabolite between the two conditions (n=3 plant repeats; only significant differences according to Student's t-test, P<0.05 are shown). Positive values correspond to metabolites that accumulate more in high nitrate conditions (10 mM NO_3^-), while negative values correspond to values that accumulate more in low nitrate conditions (2 mM NO_3^-).

in these areas then allows efficient defence-associated HR, cell wall fortification, and the down-regulation of the pathogen-induced senescence and associated deleterious effects. Maintaining mitochondrial respiration and nutrient recycling that participate in cell viability would in that case result in the resistance to a necrotroph such as *B. cinerea*. The importance of GS activity in resistance is further supported by the fact that both bacterial and fungal toxins are known to repress GS activity directly (reviewed in Seifi *et al.*, 2013*b*).

In their report, Liu *et al.* (2010) show that knocking out a single amino acid transporter is sufficient to confer resistance of *Arabidopsis* against a large spectrum of pathogens. *LHT1* (Lysine Histidine Transporter 1) disruption in *Arabidopsis* enhanced disease resistance to *P. syringae* pv. *tomato* DC3000, *Colletotrichum higginsianum*, and *Erysiphe cichoracearum*. Disruption of *LHT1* decreased the concentrations of almost all amino acids, with a stronger effect on glutamine, alanine, and proline concentrations in leaves. This metabolic effect is consistent with the previous finding that LHT1 is mainly involved in alanine and glutamine uptake (Svennerstam *et al.*, 2007). Using both exogenous glutamine administration onto leaves and glutamine synthetase inhibitor treatments, Liu *et al.* (2010) correlated glutamine deficiency or replenishment with the level of activation of defence responses. Interestingly, the *lht1* mutation-conferred phenotypes were SA dependent and associated with an altered redox status. The authors thus suggested that N metabolism, and more precisely glutamine metabolism, could modulate plant defence responses moderating cellular redox status as well as secondary metabolite pathways.

As shown in this review, the links between N assimilation, amino acid metabolism, and plant defence molecules are numerous. As glutamate is the first amino acid to be synthesized from inorganic N assimilation, all other amino acids are obtained from it, as well as the many molecules involved in plant defence that are dependent on amino acid metabolism. It is well known, for example, that phytoalexin, anthocyanin, and SA pathways are derived from the aromatic amino acid phenylalanine. Antioxidants such as glutathione derive from cysteine, vitamin E, also called α-tocopherol, is derived from tyrosine, and ethylene is derived from methionine. Recently, Navarova *et al.* (2012) reported that the non-protein pipecolic amino acid, which is a lysine catabolite, is an endogenous mediator of defence that plays a role in plant immunity. They showed that pipecolate accumulates after plant infection in inoculated leaves, in the distal leaves, and in the petiole exudates. Defects in defence priming and systemic acquired resistance were observed in pipecolate-deficient *Arabidopsis* mutants, as well as depletion of SA and camalexin molecules.

All these studies thus provide good evidence that amino acid anabolic and catabolic pathways are important for plant immunity.

Conclusion

The question that remains to be fully explored is the generic character of the observations reported above. One way to answer the question is certainly to combine transcriptomic and metabolic studies on a large set of pathosystems.

Another way is to determine how N availability can modulate plant response. We already know from previous studies that amino acid content is different in leaves of plants grown under N-rich and N-limiting conditions (Lemaître *et al.*, 2008). In a recent study, we have also found that other molecules vary between the two conditions (Fig. 4; C. Masclaux-Daubresse and G. Clément unpublished). Determining whether metabolite changes influence plant resistance or susceptibility and if pathogen aggressiveness is better if some molecules are present or absent in the plant organs it infects are other ways to investigate this difficult question. In addition, both systems biology and natural variation studies should provide new evidence to highlight the role of plant metabolites in the feature of plant–pathogen interactions as well as the impact of plant nutrition on plant defence and pathogen virulence.

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