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SPECIAL ISSUE REVIEW

The role of long-distance mobile metabolites in the plant stress response and signaling

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SUMMARY

Plants developed sophisticated mechanisms to perceive environmental stimuli and generate appropriate signals to maintain optimal growth and stress responses. A fascinating strategy employed by plants is the use of long-distance mobile signals which can trigger local and distant responses across the entire plant. Some metabolites play a central role as long-distance mobile signals allowing plants to communicate across tissues and mount robust stress responses. In this review, we summarize the current knowledge regarding the various long-distance mobile metabolites and their functions in stress response and signaling pathways. We also raise questions with respect to how we can identify new mobile metabolites and engineer them to improve plant health and resilience.

Keywords: long-distance signaling, metabolites, nicotine, 1-aminocyclopropane-1-carboxylic acid, *N*-hydroxy-pipecolic acid, glucosinolates, azelaic acid, stress response, mobile metabolites.

INTRODUCTION

As sessile organisms, plants grow in a complex and dynamic environment that not only provides energy and nutrients for their growth but also frequently imposes harm and constraints on growth and development. To survive these diverse stresses, plants evolved sophisticated systems to monitor and integrate these stresses or stimuli into proper signaling and respond appropriately. Despite lacking a central nervous system, plants can still perceive local or specific stimuli, respond by activating signal transduction networks, and broadcast this information to distant tissues or the entire plant, which is known as long-distance communication (Lough & Lucas, 2006). To accomplish this communication, signaling molecules are translocated as 'broadcasters' to systemic parts (Huber & Bauerle, 2016). These 'broadcasters' include electrical signals, ion fluxes, reactive oxygen species (ROS), microRNA, mRNA, hormones, peptides, and proteins (Choi et al., 2016; Takahashi & Shinozaki, 2019; Yoshida et al., 2021). As early as the 1920s, plant scientists discovered that electrical changes were associated with some rapid responses such as leaflet movements in *Mimosa* (Scorza & Dornelas, 2011). Electrical signals could be transmitted from damaged leaves to undamaged ones through long-distance signaling (Hedrich et al., 2016). Several mRNAs that encode transcription factors have been identified as mobile signals that move from leaves to roots or stolons via the phloem to affect tuber formation (Hannapel et al., 2013; Hannapel & Banerjee, 2017). The FLOWERING LOCUS T (FT) protein is a long-distance signal that is synthesized in the leaves, moves to the shoot apical meristem, and acts as a florigen during flowering (Jaeger & Wigge, 2007). Jasmonates (JAs) are produced in response to tissue damage caused by pathogens and insect herbivores, resulting in enhanced systemically induced immunity in both above- and belowground tissues (Wasternack & Hause, 2013). The C-TERMINALLY ENCODED PEPTIDES (CEPs) CLE-ROOT SIGNAL 1 (CLE-RS1) and CLE-RS2 have been identified in leguminous plants as long-distance translocation signals in the xylem in response to root nodulation (Okamoto et al., 2013). In addition to classical hormones, metabolites play vital roles in long-distance communication in plants.

Small molecules, frequently referred to as metabolites, mediate various aspects of plant growth and development, including seed germination, fruit ripening, and senescence (Alexander & Grierson, 2002; Bharath et al., 2021; Huang et al., 2017; Pieterse et al., 2009). They also play ecological roles in defense against a variety of

abiotic stresses, such as ultraviolet (UV) light, oxidative stress, and drought, as well as biotic stresses, including viruses, pathogens, and herbivores (Massalha et al., 2017; Tholl & Aharoni, 2014). These molecules can be synthesized relatively easily within plant cells and frequently reach high concentrations. Due to the distinct spatial and temporal expression pattern of biosynthe genes, as well as the confinement of substrates, energy and resource utilization, and the safeguarding of cellular integrity, a subset of metabolites is exclusively synthesized within particular cell types, tissues, and organs. This restricted synthesis of metabolites is essential for maintaining proper cellular function and physiological equilibrium. Moreover, these metabolites can be easily dissolved and travel because of their hydrophilic chemical properties and low molecular weight. Thus, as some specific metabolites are also required in other organs and tissues, they are transported throughout the plant to achieve long-distance communication or function as mobile signals. This review will focus solely on metabolites that act as signals in stress responses. Based on the site of biosynthesis and the direction of translocation, metabolites can be classified into three different groups; shoot to root, shoot to shoot, and multiple directions (Figure 1). In this review, we address the following questions: (i) How and where are long-distance translocated metabolites synthesized? (ii) How do metabolites travel long distances to other parts of plants (Table 1)? (iii) What are the roles of these long-distance mobile metabolites? (iv) And what methods can be employed to identify novel long-distance mobile metabolites?

ROOT-TO-SHOOT MOBILITY OF METABOLITES

Roots are vital organs that also act as envoys sensing physical and biological information in the soil, converting it into complex signals, and transmitting it to shoots or other parts of the plant to optimize plant performance under specific conditions. Among these complex signals, metabolites derived from roots represent a significant group that can be translocated over long distances from roots to shoots and assist the plant in adapting to the environment. So far, most hormones were identified to be translocated as root-to-shoot signals. For example, cytokinins synthesized in the root are translocated to shoots and play important roles in shoot growth (Ko et al., 2014). Strigolactones (SL) are transported from roots to shoots to influence shoot branching in rice (*Oryza sativa*) (Xie et al., 2015). Root-derived abscisic acid (ABA) travels to shoot guard cells to prevent water loss (Zhang et al., 2021). In addition to these hormones, other metabolites have been identified as 'bottom-up' root-to-shoot mobile signals, particularly in response to certain stresses. This section will concentrate on some root-to-shoot translocated mobile metabolites.

Root-to-shoot mobile alkaloids

Nicotine, a renowned alkaloid, was identified as a root-derived specialized metabolite involved in tobacco plant defense against insect herbivory as early as 1941 (Dawson, 1941; Steppuhn et al., 2004). It is found in young leaf tissue, stems, and flowers, suggesting that it is a mobile metabolite translocated from roots to shoots (Zenkner et al., 2019). Nicotine biosynthesis involves multiple enzymatic reactions. Nicotine contains two nitrogen-containing rings, the pyrrolidine and pyridine rings, which are derived from two distinct pathways (Zenkner et al., 2019). Pyrrolidine ring biosynthesis begins with arginine and proceeds through reactions catalyzed by arginine decarboxylase (ADC), ornithine decarboxylase (ODC), *S*-adenosyl-L-methionine decarboxylase (SAMDC), *S*-adenosyl-L-methionine synthetase (SAMS), putrescine *N*-methyltransferase (PMT), and *N*-methyl putrescine oxidase (MPO) to form 4-methylaminobutanal (Zenkner et al., 2019). Pyridine ring biosynthesis begins with aspartic acid and proceeds through reactions catalyzed by aspartate oxidase (AO), quinolinate synthase (QS), and quinolinic acid phosphoribosyl transferase (QPT) to produce nicotinic acid (Zenkner et al., 2019). Another two enzymes, an isoflavone reductase-like enzyme (A622) and a berberine bridge enzyme-like enzyme (BBL), were proposed to catalyze nicotine formation from nicotinic acid and the 4-methylaminobutanal cation (DeBoer et al., 2009; Kajikawa et al., 2011). Moreover, nicotine can also be metabolized by nicotine *N*-demethylase (NND), an enzyme from the cytochrome P450 family, to produce nornicotine (Lewis et al., 2010). The expression of nicotine biosynthesis genes and nicotine levels in the roots can be strongly induced by shoot-derived biotic and abiotic stresses, such as herbivore feeding (Baldwin, 1988), pathogen infection (Vogel-Adghough et al., 2013), mechanical wounding (Shi et al., 2006), salt (Chen et al., 2016), topping (Shi et al., 2006), and jasmonic acid (Shoji et al., 2008), but suppressed by auxin (Shi et al., 2006), SL (Li et al., 2020), and gibberellin acid (Parups, 1959). Exogenous application of methyl jasmonate in tobacco BY-2 cells or cultured roots can activate expression of a series of nicotine biosynthesis genes and enhance nicotine levels (Cane et al., 2005; Imanishi et al., 1998). However, this enhancement could be dramatically reduced by ethylene treatment (Winz & Baldwin, 2001), suggesting antagonistic actions between ethylene and JA in nicotine biosynthesis. Likewise, CORONATINE INSENSITIVE1 (COI1), JASMONATE-ZIM DOMAIN (JAZ), and MYELOCYTOMATOSIS (MYC2) proteins, which are key regulators of the JA signaling pathway, are required for JA-mediated nicotine biosynthesis (Shoji et al., 2008). Mechanical wounding and shoot apex excision can increase JA levels, resulting in enhanced nicotine production, yet this induction is affected by auxin, which is a negative regulator of nicotine biosynthesis in tobacco plant roots (Shi et al., 2006). SL signaling tunes nicotine biosynthesis by tailoring the interplay between JA and auxin to mediate herbivore resistance of stems (Li et al., 2020).

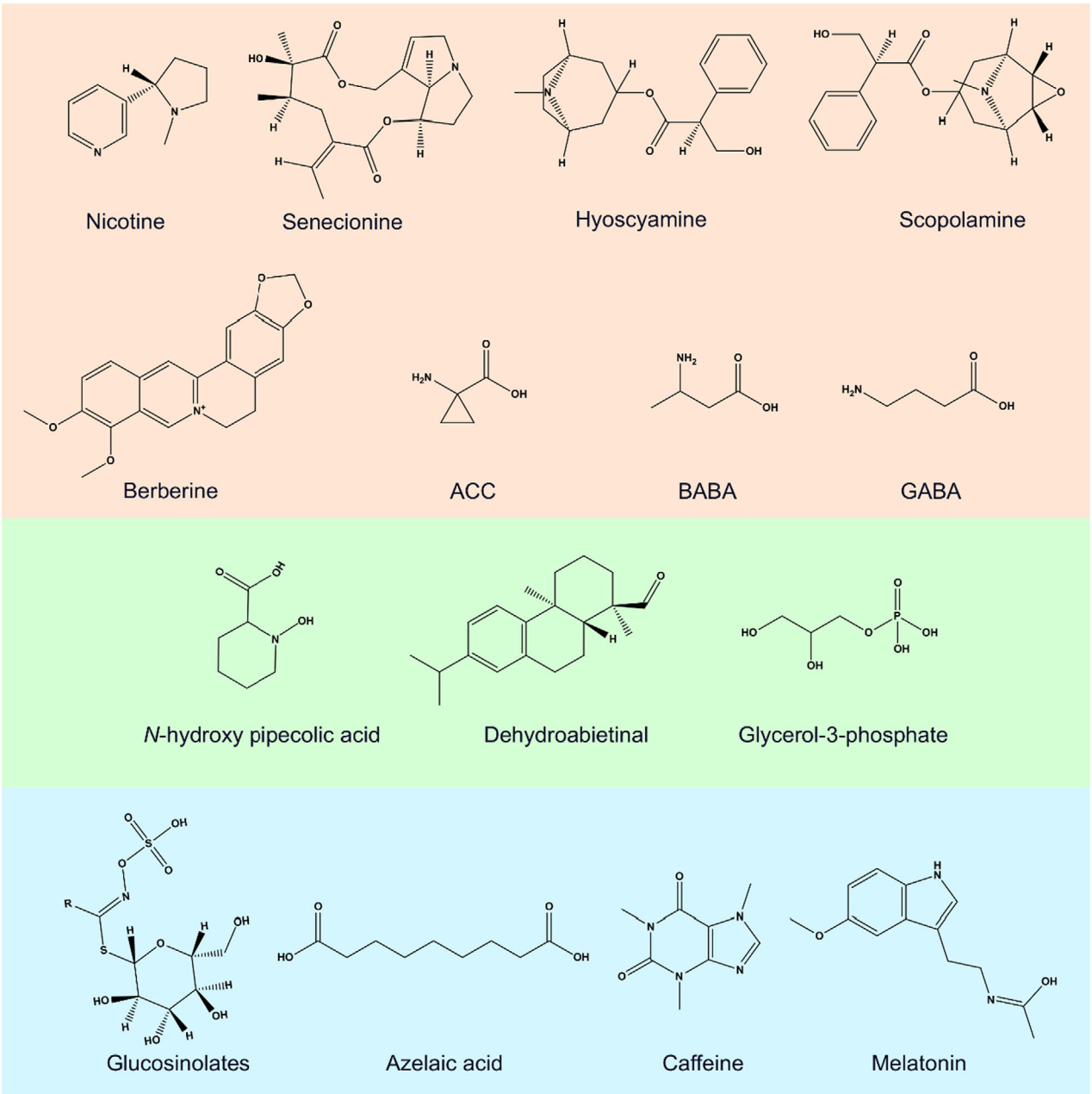

Figure 1. Structural information and transport direction classes of small molecules involved in long-distance communication. ACC, 1-aminocyclopropane-1-carboxylic acid; BABA, β-aminobutyric acid; GABA, γ-aminobutyrate acid. Pink, green, and blue represent root-to-shoot, shoot-to-shoot, and multiple transport directions, respectively.

Constitutive overexpression of *PYRABACTIN RESISTANCE-LIKE4* (*NtPYL4*), which encodes an ABA receptor, reduces the transcript levels of nicotine biosynthesis genes, resulting in lower nicotine levels in the roots (Lackman et al., 2011).

High levels of nicotine in roots can exert significant feedback inhibition on its production by suppressing the expression of its biosynthesis genes (Wang et al., 2015). Thus, low levels of nicotine in roots are critical for its continuous biosynthesis. To avoid cytotoxicity of high nicotine levels and ensure continuous biosynthesis in roots, it is transported to other tissues and compartmentalized in the vacuoles (Shoji et al., 2009). Several transporters have been identified to be involved in these two processes. The tonoplast-localized MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE)-type transporters NtMATE1 and NtMATE2 are proton antiporters that sequester nicotine in the vacuole of nicotine-producing roots (Shoji et al., 2009). JASMONATE-INDUCIBLE ALKALOID TRANSPORTER (NtJAT1)

Table 1 Summary of the biosynthesis, destination, and transport mechanisms for long-distance mobile metabolites presented in this study

Metabolite	Biosynthesis	Destination	Mechanism of transport	References
Nicotine	Root	Root, leaf, stem, flower, vacuole	MATE1/2, JAT1/2, NUP1	Shoji et al. (2009); Morita et al. (2009); Shitan et al. (2014); Hildreth et al. (2011)
Senecionine	Root	Root, stem, leaf, flower		Hartmann et al. (1989)
Berberine	Lateral root	Rhizome, vacuole	ABCB1, ABCB2, MATE1	Fujiwara et al. (1993); Shitan et al. (2003); Shitan et al. (2013)
Tropane alkaloids	Root	Aerial parts		
NHP	Local leaf	Systemic leaf		Chen et al. (2018); Cai et al. (2021); Mohnike et al. (2021)
Glucosinolate	Leaf, root, inflorescence, stems	All tissues, S-cell, vacuole,	GTR1/2	Nour-Eldin et al. (2012)
ACC	All tissues		LHT1/2	Shin et al. (2015); Choi et al. (2019)
Azelaic acid	Leaf, root,	Systemic leaf, systemic root, root		Jung et al. (2009); Cecchini, Jung, et al. (2015); Cecchini, Steffes, et al. (2015); Korenblum et al. (2020)
Caffeine	Leaf	Leaf, seed, vacuole	PUP	Baumann and Wanner (1972); Suzuki et al. (1992); and Suzuki (2004); Zhang et al. (2022)

NHP, *N*-hydroxy-pipecolic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; MATE1/2, MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE)-type transporters 1 and 2; JAT1/2, JASMONATE-INDUCIBLE ALKALOID TRANSPORTERS 1 and 2; NUP1, NICOTINE UPTAKE PERMEASE 1; ABCB1/2, MULTIDRUG RESISTANCE (MDR)-type ATP-BINDING CASSETTE (ABC) transporters 1 and 2; GTR1/2, GLUCOSINOLATE TRANSPORTERS 1 and 2; LHT1/2, LYSINE HISTIDINE TRANSPORTERS 1 and 2; PUB, PURINE PERMEASE.

and NtJAT2 function in nicotine accumulation in the vacuole (Morita et al., 2009; Shitan et al., 2014). NtJAT1 has been implicated in vacuolar nicotine sequestration in different tissues, whereas NtJAT2 mediates nicotine sequestration in leaves following herbivore attack (Morita et al., 2009; Shitan et al., 2014). The plasma membrane-localized NICOTINE UPTAKE PERMEASE 1 (NUP1) was identified as the transporter required for nicotine uptake from the apoplast to avoid the loss of secretion into the rhizosphere (Hildreth et al., 2011). Following biosynthesis in roots, nicotine is transported to the aboveground tissues and accumulates in the vacuoles, and this results in low nicotine levels in the roots (Zenkner et al., 2019). Grafting experiments showed that nicotine travels up through the xylem along with the transpiration stream (Shi et al., 2006). However, the transporters involved in loading nicotine into the xylem in roots and unloading nicotine into leaf cells remain unknown to date. Thus, further research is required to decipher the mechanism of nicotine translocation from roots to shoots. Although nicotine was reported multiple times to exert antibacterial, antifungal, anti-insect, and antiparasitic effects (Pavia et al., 2000; Weber et al., 2019), it is currently unknown whether it can activate plant defense systems.

Besides nicotine, other alkaloids have also been reported to be long-distance mobile metabolites. Senecionine *N*-oxide (Sen) was first reported as a root-to-shoot long-distance mobile metabolite (Hartmann et al., 1989). It is the main component of the polyamine-derived pyrrolizidine alkaloids in *Senecio vulgaris* (50–60%). Sen is distributed in all tissues, including root, stem, leaf, and flowers, especially in the inflorescences, which account for 90% of the total Sen (Hartmann et al., 1989). Feeding experiments showed that labeled Sen can be transported into the shoot and mainly into the inflorescences (Hartmann et al., 1989). However, detached *S. vulgaris* shoots cannot synthesize Sen, suggesting that Sen biosynthesis occurs in the roots (Hartmann et al., 1989). The long-distance transport of Sen is primarily accomplished via the phloem (Hartmann et al., 1989). Despite the fact that long-distance Sen translocation is well documented, transporters involved in Sen translocation remain unknown, as well as its exact physiological role.

Berberine, an alkaloid found in a variety of plants, is a metabolite with antibacterial and antifungal properties. It showed great potential to inhibit the growth of *Monilinia fructicola* (Hou et al., 2010; Pei et al., 2019). Moreover, berberine inhibits the activity of cutinase, which is a plant cell-degrading enzyme secreted by pathogens (Li et al., 2021). The berberine biosynthesis pathway has been well characterized, beginning with the amino acid L-tyrosine. Berberine is found in the leaves, stems, and lateral roots of *Coptis Japonica*, but predominantly accumulates in the main roots (Otani et al., 2005). However, (*S*)-scoulerine 9-*O*-methyltransferase (SMT), a key enzyme in berberine biosynthesis, is specifically present and active in lateral root tissue (Fujiwara et al., 1993). This suggests that following its biosynthesis, berberine is transported from lateral roots and highly accumulates in the rhizome. The plasma membrane-localized MULTIDRUG RESISTANCE

(MDR)-type ATP-BINDING CASSETTE (ABC) transporter CjABCB1 is primarily expressed in the rhizome xylem and mediates the transport of berberine from the lateral root to the rhizome (Shitan et al., 2003). Likewise, CjABCB2 acts as an influx pump for berberine translocation to the rhizome (Shitan et al., 2013). To avoid negative effects of high levels of berberine in the cytosol, the rhizome-expressed and tonoplast-localized CjMATE1 transports berberine into vacuoles for storage in rhizomes (Takanashi et al., 2017). Berberine may accumulate in rhizomes to protect them from pathogens in *C. japonica*. Although the berberine biosynthesis pathway has been revealed, only two transcription factors, CjWRKY1 and CjbHLH1, have been found to regulate its biosynthesis in *C. japonica* cells. These regulatory proteins bind to different *cis*-elements in the promoters of biosynthesis genes to enhance berberine biosynthesis (Kato et al., 2007; Yamada et al., 2016).

Tropane alkaloids such as hyoscyamine and scopolamine have been proposed as long-distance translocated metabolites that play important roles in plant defense against insect herbivores (Krug & Proksch, 1993; Wink & Theile, 2002). According to grafting experiments, tropane alkaloids are primarily biosynthesized in the roots and translocated to the aerial parts of the plants through the xylem (Kohnen-Johannsen & Kayser, 2019). Further research revealed that the biosynthesis enzymes responsible for tropane alkaloid production are located specifically in the root pericycle and the expression of tropane alkaloid biosynthesis genes (*pmt*, *tr-1*, and *cyp80f1*) is higher in the roots (Kohnen et al., 2018).

New role of 1-aminocyclopropane-1-carboxylic acid, a long-distance mobile metabolite

1-Aminocyclopropane-1-carboxylic acid (ACC) serves as the direct precursor of the hormone ethylene (Kende, 1993). ACC biosynthesis begins with methionine and proceeds through two enzymatic steps. Methionine is first converted to *S*-adenosyl-L-methionine (SAM) by SAM synthase, which can then be converted to ACC by ACC synthase (ACS) (Kende, 1993). ACC is further modified by ACC oxidase (ACO) to produce ethylene (Kende, 1993). ACC can also be converted into three different derivatives, all of which play important roles in regulating ACC levels in plants (Amrhein, 1981). ACC-*N*-malonyl transferase catalyzes the biosynthesis of *N*-malonyl-ACC (MACC) (Amrhein, 1981), while γ-glutamyl transpeptidase can metabolize ACC to form γ-glutamyl-ACC (GACC) (Martin et al., 1995). JASMONIC ACID RESISTANCE1 (JAR1) is the enzyme involved in jasmonoyl-ACC (JA-ACC) biosynthesis (Staswick & Tiryaki, 2004).

ACC has been shown in numerous studies to act as a signaling molecule independent of ethylene biosynthesis, affecting plant development and growth, as well as responding to a variety of stimuli, including biotic and abiotic stresses (Polko & Kieber, 2019; Yoon & Kieber, 2013). Moreover, it was also reported as a long-distance transport metabolite. ACC is synthesized in the anaerobic roots and transported to the shoot via the xylem, resulting in enhanced ethylene production in the shoot (Bradford & Yang, 1980). Furthermore, applying labeled ACC to a single leaflet causes the radioactivity to accumulate in other leaves and roots (Amrhein et al., 1982). In addition, ACC has been proposed to be transported between different organs and cell types. Externally applied radiolabeled ACC to the top of the central column of *Cymbidium* flowers can be rapidly translocated to all the other flower parts, where it can be immediately converted into ethylene (Woltering, 1990). Moreover, spatial differences in *ACS* and *ACO* expression are observed in different organs and cell types (Vanderstraeten & Van Der Straeten, 2017), implying that ACC transport occurs between organs and cell types. ACC was initially thought to be transported from root to shoot via the xylem (Bradford & Yang, 1980). However, further evidence suggests that phloem is involved in its long-distance transport as well. Foliar application of radiolabeled ACC to cotton (*Gossypium hirsutum*) plant leaves results in rapid export and distribution in the shoot (Morris & Larcombe, 1995), which is consistent with phloem translocation. Similarly, labeled ACC is found in the phloem exudate (PEX) collected from labeled ACC-treated leaves (Morris & Larcombe, 1995). Moreover, grafting experiments with wild-type plants and the *acs8x* mutant (possessing lower ACC levels) revealed that a combination of an *acs8x* scion and a wild-type rootstock graft results in a partial reversion of the *acs8x* phenotype due to extremely low levels of ACC (Vanderstraeten et al., 2022). This indicates that roots can synthesize ACC and transport it to the *acs8x* scion via phloem, affecting rosette development.

Since ACC can be transported both within the cell and throughout the plant, the identification of ACC transporters is crucial for understanding its action. ACC uptake experiments in soybean (*Glycine max*) leaf disks and tomato (*Solanum lycopersicum*) pericarp sections showed that ACC could be transported by an amino acid transport system (Lurssen, 1981; Saftner & Baker, 1987). Arabidopsis *ACC-resistant* (*are2*) was later identified as a potential ACC transporter mutant with reduced exogenous ACC uptake (Shin et al., 2015). The *are2* mutant, which is allelic to *lysine histidine transporter1* (*lht1-5*), is insensitive to ACC but displays a normal triple response when exposed to ethylene (Shin et al., 2015). LHT1 was previously identified as a transporter of some amino acids in plant roots (Hirner et al., 2006), giving credence to the theory that ACC shares a transporter system with other amino acids. Radiolabeled ACC uptake was dramatically reduced in *are2* protoplast uptake experiments (Shin et al., 2015), suggesting that LHT1 functions as the transporter for ACC uptake. Furthermore, the incomplete reduction of ACC uptake suggests the presence of additional transporters for ACC uptake. Complementation of the Arabidopsis ACC-insensitive line *lht1* prompted the discovery of a second ACC transporter (LHT2) (Choi et al., 2019). Electrophysiological analysis of

Xenopus oocytes expressing *LHT1* and *LHT2* confirmed that both LHT1 and LHT2 exhibit comparable ACC transport activity (Choi et al., 2019). However, the *lht2* mutant did not show a reproductive defect phenotype like the *acs8x* mutant and other *LHT* genes exhibited similar expression patterns to *LHT2* in flowers, suggesting that other ACC transporters may be involved in ACC transport (Choi et al., 2019). Apart from ACC uptake transporters, the other transporters responsible for ACC long-distance translocation and vacuole compartmentalization have yet to be identified.

Mobile or immobile? β-Aminobutyric acid and γ-aminobutyrate acid

β-Aminobutyric acid (BABA) is known as a plant defense priming activator that induces resistance to a wide range of biotic and abiotic stresses. Chemically synthesized BABA has been found to be delivered to a variety of tissues. External application of BABA to the root system results in BABA accumulation and enhances resistance in the uppermost leaves (Cohen & Gisi, 1994). Treatment of lower leaves with ^{14}C-BABA provided similar results (Cohen & Gisi, 1994). A recent study reported that LHT1 acts as the key transporter for BABA cellular uptake and systemic distribution (Tao et al., 2022). However, it has been found that endogenous BABA only accumulates in local tissues following pathogen infection (Balmer et al., 2019). Additionally, γ-aminobutyrate (GABA), an isoform of BABA, was also reported to be a mobile metabolite involved in the response to multiple stresses. Exogenous GABA treatment increases endogenous GABA levels in *Caragana intermedia* (Shi et al., 2010), *Stellaria longipes* (Kathiresan et al., 1998), *Citrus sinensis* (Hijaz et al., 2018), maize (*Zea mays*) (Wang et al., 2017), and sunflower (*Helianthus annuus*) (Kathiresan et al., 1997). Moreover, D_6-labeled GABA can be transported from roots to the phloem cortex, inner stem, and leaves, which is unaffected by girdling, implying that D_6-labeled GABA is transported to the upper parts via the xylem (Hijaz & Killiny, 2020). Following both mechanical wounding and *Spodoptera littoralis* feeding, a significant increase in GABA levels was observed in both local and systemic leaves of *pollen-pistil incompatibility* 2 (*pop2-5*) plants, a loss-of-function mutant in the GABA catabolism gene *GABA TRANSAMINASE* (Scholz et al., 2017). However, D_2-labeled GABA could not be identified in unwounded leaves of *pop2-5* following external application of D_2-labeled GABA on wounded leaves (Scholz et al., 2017). Therefore, whether BABA and GABA are mobile molecules has to be validated further.

MOBILE METABOLITES INVOLVED IN SYSTEMIC ACQUIRED RESISTANCE

Long-distance communication between aboveground organs is critical for the plant defense response. Systemic acquired resistance (SAR) is a well-known defense mechanism that is activated by local defense responses following pathogen attack, resulting in priming and enhanced resistance to pathogens in systemic tissues (Durrant & Dong, 2004). SAR can provide long-lasting and broad-spectrum resistance to pathogens, such as bacteria, fungi, viruses, and oomycetes (Durrant & Dong, 2004). SAR has been extensively studied and its mechanism of action is becoming increasingly clear (Kim & Lim, 2023). According to grafting experiments, SAR activation in distal scion leaves requires mobile signal(s) from primary infected stock leaves, and these mobile signal(s) are dependent on the phloem (Jenns & Kuć, 1979; Tuzun & Kuć, 1985). Experiments with phloem sap-enriched PEX further indicated that the phloem is responsible for long-distance translocation of SAR signal(s) (Carella et al., 2016). Proteins (Maldonado et al., 2002), miRNA (Natarajan et al., 2018), and phased small RNA (Shine et al., 2022) have been identified as signaling molecules involved in the SAR response. Apart from these, plant-derived metabolites such as salicylic acid (Vlot et al., 2009), methyl salicylate (Park et al., 2007), azelaic acid (AzA) (Jung et al., 2009), glycerol-3-phosphate (G3P) (Chanda et al., 2011), dehydroabietinal (DA), pipecolic acid (Pip), and its derivative *N*-hydroxy-pipecolic acid (NHP) (Chen et al., 2018; Hartmann et al., 2018; Návarová et al., 2012) have been associated with long-distance communication and SAR signal amplification. Among these metabolites, NHP has become a research hotpot due to its critical role in SAR establishment (Hartmann et al., 2018).

N-hydroxy-pipecolic acid, a recently recognized mobile factor in SAR

The NHP biosynthesis pathway has been investigated in several plant species (Holmes et al., 2019). Three enzymes are responsible for NHP biosynthesis, including AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1) (Návarová et al., 2012), SAR-DEFICIENT 4 (SARD4) (Ding et al., 2016), and FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) (Hartmann et al., 2018). Lysine is first transformed into ε-amino-α-keto caproic acid by ALD1, followed by cyclization to generate Delta1-piperidine-2-carboxylic acid (P2C) (Návarová et al., 2012), which is then reduced by SARD4 to yield Pip in the chloroplast (Ding et al., 2016). The ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) transporter next transfers Pip to the cytosol (Rekhter et al., 2019), where it is hydroxylated by FMO1 to produce NHP (Hartmann et al., 2018). The transcript abundance of genes that encode NHP biosynthesis enzymes is low in healthy plants but is systemically increased following pathogen attack, leading to the accumulation of large amounts of NHP in both local and systemic leaves of Arabidopsis (Chen et al., 2018; Ding et al., 2016; Hartmann et al., 2018; Mishina & Zeier, 2006; Song et al., 2004). The SAR response is substantially weaker in NHP biosynthesis mutants (Ding et al., 2016; Mishina & Zeier, 2006; Song et al., 2004). Exogenous application of NHP is sufficient to override the

SAR deficiencies that are present in NHP biosynthesis mutants (*ald1* and *fmo1*) (Hartmann et al., 2018). In addition, transient expression of Arabidopsis NHP biosynthesis genes in tomato leaves results in a stronger SAR response in distal leaves and enhances resistance to pathogens (Holmes et al., 2019). Moreover, external application of NHP in a variety of monocots and dicots also enhances the resistance to diverse diseases (Schnake et al., 2020), indicating that NHP has a consistent function in SAR establishment.

Several studies have suggested that NHP functions as a mobile signal. Firstly, NHP was found to accumulate in both local and systemic leaves following local infection (Hartmann et al., 2018). Secondly, experiments revealed that NHP could be identified in the distal leaves of the NHP-deficient *fmo1* mutant after local NHP infiltration (Cai et al., 2021). Identical results were also obtained when deuterated NHP (D_9-NHP) was infiltrated into local leaves of *fmo1* (Mohnike et al., 2021). Furthermore, upon pathogen attack, NHP accumulates in systemic leaves and petiole sap of *Cucumis sativus* (Schnake et al., 2020). The identification of NHP in the phloem raises the question of how NHP enters and exits the phloem. Although previous research suggested that the generation or transportation of mobile signals for SAR activation requires the presence of NHP, the contrary was also reported. Kachroo and Kachroo showed that Pip and NHP could not be detected in PEX derived from infected wild-type Arabidopsis leaves (Kachroo & Kachroo, 2020). Moreover, in a subsequent study they found that PEX collected from *ald1* mutant plants, which are deficient in Pip and NHP, was still able to activate a SAR response in Arabidopsis (Shine et al., 2022). This finding implies that generation or transport of Pip or NHP might not be necessary for SAR activation. Interestingly, despite being regarded as a Pip- or NHP-deficient mutant, according to multiple studies the levels of Pip in the PEX of the *ald1* mutant were found to be similar to those in wild-type plants (Cecchini, Jung, et al., 2015; Shine et al., 2022). Furthermore, our results also showed that NHP glycoside (NHPG) could be detected in *ald1* plants (Cai et al., 2021), suggesting that the *ald1* mutant accumulates low levels of Pip or NHP. This might explain why PEX collected from the *ald1* mutant can still trigger a SAR response in wild-type plants.

To date, at least five NHP derivatives have been found in Arabidopsis leaves following pathogen infection, including NHPG, NHP glucose ester (NHPGE), NHP dihexose, NHP methyl ester (MeNHP), and NHPG malonic acid (Mohnike et al., 2022) (Figure 2). Whether these derivatives are mobile signal metabolites remains to be determined. Pathogen infection can systemically stimulate the production of NHPG and NHP dihexose (Chen et al., 2018; Hartmann & Zeier, 2018). Moreover, NHP and NHPG were found in the distal leaves of *fmo1* after NHP penetration into local leaves (Cai et al., 2021; Mohnike et al., 2021). These findings suggests that NHP or NHPG may migrate to systemic leaves. Our team and three other groups discovered that UDP-DEPENDENT GLYCOSYL TRANSFERASE 76B1 (UGT76B1) is the enzyme that catalyzes the glycosylation of NHP to produce NHPG (Bauer et al., 2021; Cai et al., 2021; Holmes et al., 2021; Mohnike et al., 2021). *ugt76b1* mutants accumulate more NHP and other NHP-derived metabolites, but much less NHPG, resulting in enhanced disease resistance and a stronger SAR response (Cai et al., 2021; Mohnike et al., 2021). However, this enhanced resistance can be eliminated by blocking NHP biosynthesis (Bauer et al., 2021; Mohnike et al., 2021). Similar effects were also found in tomato leaves transiently overexpressing *UGT76B1* (Holmes et al., 2021). Furthermore, when we transiently overexpressed the entire NHP biosynthesis pathway in *Nicotiana benthamiana* leaves, extremely high levels of NHP accumulated, resulting in severe cell death, whereas leaves producing NHPG suppressed NHP-induced cell death (Cai et al., 2021). These findings imply that the bioactive molecule for SAR activation is NHP or another NHP-derived metabolite rather than NHPG. NHPGE is another NHP-derived metabolite that systemically accumulates in Arabidopsis (Bauer et al., 2021). Further studies, such as those involving grafting and discovery of the transporters responsible for NHP translocation, are required to determine unambiguously which metabolite is the SAR mobile signal (Figure 2).

MOBILE METABOLITES TRANSLOCATED IN MULTIPLE DIRECTIONS

Long-distance translocation of glucosinolates

Glucosinolates (GSLs) are prominent specialized metabolites produced by many Brassicaceae plants, including *Arabidopsis*, cabbage (*Brassica oleracea* var. *capitata*), cauliflower (*Brassica oleracea* var. *botrytis*), mustard (*Brassica rapa*), and horseradish (*Armoracia rusticana*) (Ishida et al., 2014). They play important roles in plant growth, development, and interaction with the environment. GSLs are classified into three types based on their amino acid precursors (aliphatic, indolic, and aromatic) (Nguyen et al., 2020). Aliphatic GSLs are derived from methionine, alanine, valine, leucine, or glutamate; indolic GSLs are synthesized from tryptophan; and aromatic GSLs are derived from phenylalanine or tyrosine (Nguyen et al., 2020). GSL biosynthesis is comprised of three stages: amino acid chain elongation, core structure synthesis, and secondary side chain modifications (Nguyen et al., 2020). Generally, GSLs can be found in all tissues of Brassicaceae plants. However, the composition and quantity of GSLs vary significantly between plant species, varieties, tissues, and growth stages (Bhandari et al., 2015). Arabidopsis leaves accumulate high concentrations of aliphatic GSLs, whereas the roots contain predominantly indolic GSLs (Brown et al., 2003; Petersen et al., 2002). GSL levels decrease with leaf age, being almost absent during senescence (Brown et al., 2003). In addition, abiotic stresses, such as salt, drought, heat shock, heavy metal, and UV stresses, also affect the levels and composition of GSLs (Chowdhury, 2022), implying that they may play

important roles in the responses to abiotic stresses. GSL levels rise in response to biotic stresses, such as oomycete infection (Schlaeppi et al., 2010), bacterial infection (Clay et al., 2009), fungal infection (Sanchez-Vallet et al., 2010), herbivory, and insect attack (Textor & Gershenzon, 2009). When plants are attacked by pathogens or herbivores, GSL biosynthesis genes are significantly induced, resulting in enhanced production of GSLs and enhanced resistance (Tsunoda et al., 2018). Plants with reduced levels of GSLs are more susceptible to pathogen infection and herbivory (Brader et al., 2006; Clay et al., 2009; Textor & Gershenzon, 2009).

Numerous observations indicate that GSLs are long-distance mobile metabolites. For example, leaves are the primary tissue for benzyl GSL biosynthesis in *Tropaeolum majus* (Lykkesfeldt & Moller, 1993). However, high levels of benzyl GSLs are found in other tissues, particularly in the developing seed (Lykkesfeldt & Moller, 1993), implying that benzyl GSLs are transported from leaves to other tissues. Evidence from reciprocal crosses between lines with different GSL profiles showed that GSL levels in seeds of *Brassica napus* and *Arabidopsis* are determined by the maternal genotype (Kliebenstein et al., 2007; Kondra & Stefansson, 1970; Magrath & Mithen, 1993), suggesting that GSLs can be transferred from maternal tissue into the developing seeds. Moreover, application of radiolabeled GSLs to *B. napus* rosette leaves confirmed that GSLs can move into the embryo of the seeds (Brudenell et al., 1999). Similarly, infiltration of radiolabeled *p*-hydroxybenzylglucosinolate (*p*-OHBG) to local leaves of Arabidopsis results in radioactivity in various tissues, including roots, systemic leaves, stem, flowers, and siliques (Chen et al., 2001). In addition, grafting experiments with wild-type plants and a long-chain aliphatic GSL-deficient mutant showed that GSLs could be transported from leaves to seeds (Ellerbrock et al., 2007). Several lines of evidence suggest that long-distance transportation of GSLs occurs in the phloem. Firstly, GSLs possess appropriate physicochemical properties for phloem mobility (Brudenell et al., 1999). Based on results from aphid feeding experiments on black mustard (*Brassica nigra*), high levels of the GSLs sinigrin (more than 10 mM) were found in the phloem sap of young leaves, but much lower levels were found in mature, pre-senescent, and senescent leaves (Merritt, 1996). Moreover, intact *p*-OHBG was detected in PEX collected from cut petioles of *Arabidopsis* leaves (Chen et al., 2001). Long-distance mobility of GSLs requires crossing of multiple membrane barriers to depart from biosynthesis tissues and enter systemic ones. Therefore, identification and characterization of the essential transporters involved in long-distance translocation is becoming

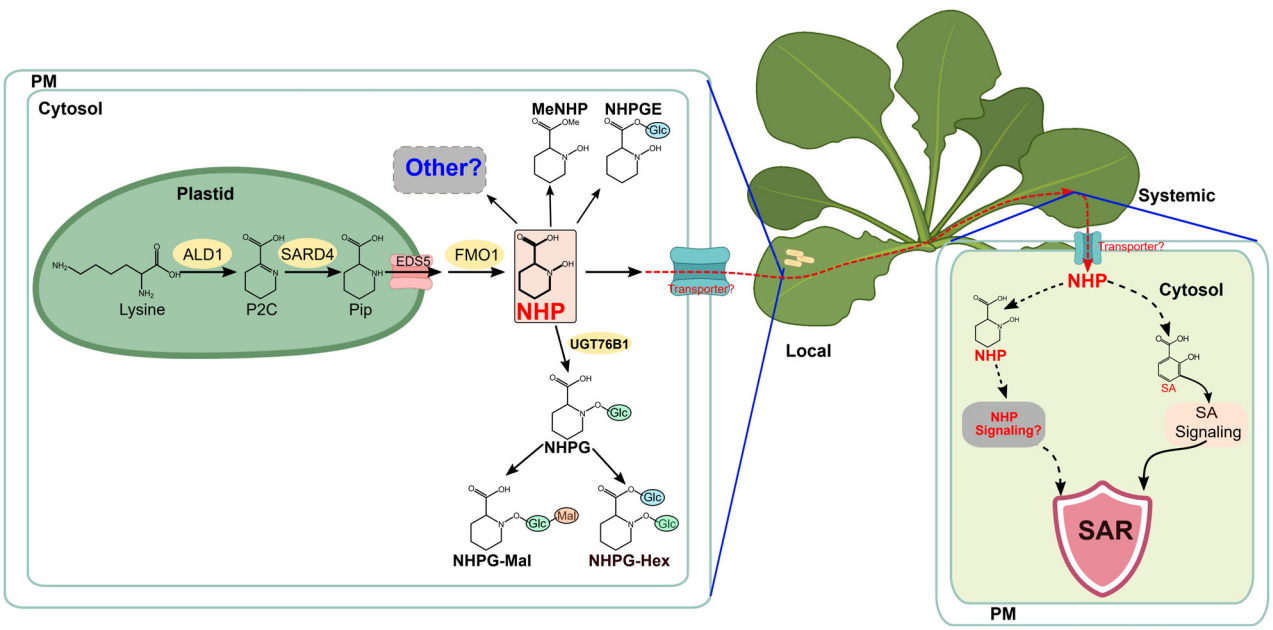

Figure 2. The networks of *N*-hydroxy-pipecolic acid (NHP) biosynthesis, transcriptional regulation, and its signaling.
NHP biosynthesis starts from lysine and is catalyzed by the aminotransferase AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1), the reductase SAR-DEFICIENT4 (SARD4), and FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1). ALD1 removes the amino group from lysine, resulting in ε-amino-α-keto caproic acid, which spontaneously cyclizes into Delta1-piperidine-2-carboxylic acid (P2C). SARD4 catalyzes P2C into pipecolic acid (Pip), which is then exported into the cytosol by ENHANCED DISEASE SUSCEPTIBILITY (EDS5). FMO1 hydroxylates Pip to produce NHP. UDP-DEPENDENT GLYCOSYL TRANSFERASE 76B1 (UGT76B1) is the glycosyltransferase that converts NHP to NHP glycoside (NHPG). In addition, unknown enzymes can convert NHP and NHPG into NHP glucose ester (NHPGE), methyl NHP (MeNHP), NHPG hexose (NHPG-Hex), NHPG malonic acid (NHPG-Mal), and other derivatives. Pathogen infection induces the biosynthesis of NHP, which is further transported into systemic leaves by unknown mechanisms or transporters. Moreover, NHP is perceived by unknown receptors and stimulates its signaling pathway, resulting in an enhanced SAR response and elevated salicylic acid (SA) production in systemic leaves. PM, plasma membrane.

increasingly important. GLUCOSINOLATE TRANSPORTER 1 (GTR1) and GTR2, two plasma membrane-localized nitrate/peptide transporters, were identified as high-affinity and proton-dependent GSL-specific transporters (Nour-Eldin et al., 2012). The *gtr1 gtr2* double mutant fails to accumulate GSLs in seeds, instead accumulating excess GSLs in parental tissues, such as leaves and silique walls (Nour-Eldin et al., 2012), implying that GTR1 and GTR2 are required for long-distance GSL transport. They are localized in phloem companion cells and involved in phloem loading (Nour-Eldin et al., 2012). GTR2 was proposed to play an important role in apoplastic phloem loading of GSLs as it is primarily expressed in leaf veins, whereas GTR1 may participate in GSL distribution in the leaf as it is found in adjacent mesophyll cells (Nour-Eldin et al., 2012). Apart from these, GTR1 and GTR2 have also been reported to have important roles in GSL distribution in the root. A metabolomic study of specific root cell types revealed that aliphatic GSLs mainly accumulate in the cortex layer, whereas indolic GSLs are predominantly found in the columella (Moussaieff et al., 2013). Accordingly, GTR1 and GTR2 are highly expressed in cortex cells and the root vasculature (Andersen et al., 2013). Feeding experiments with rosette leaves showed that transport of GSLs to roots is abolished in the *gtr1 gtr2* double mutant (Andersen et al., 2013), suggesting that GTR1 and GTR2 are required for GSL transport from rosettes to roots. Following external application of GSLs to roots, the majority of GSLs accumulated in wild-type and *gtr1 gtr2* rosettes, implying the distribution of GSLs from roots to rosettes is GTR1- and GTR2-independent (Andersen et al., 2013). Root xylem sap analysis revealed that long-chain aliphatic GSLs are present at much higher levels in the xylem sap of the *gtr1 gtr2* double mutant as compared to wild type (Madsen et al., 2014). Aliphatic GSL levels are drastically reduced in the roots and strongly increased in the leaves of the *gtr1 gtr2* double mutant (Madsen et al., 2014). These results suggest that xylem is involved in the transport of GSLs from roots to shoots and contributes to the overaccumulation of GSLs in rosette leaves.

Short-distance translocation of glucosinolates

Apart from long-distance translocation, GSLs were also reported to be short-distance mobile metabolites. GSLs were observed to accumulate along the midrib and margins (Madsen et al., 2014). However, given that a significant increase in GSL accumulation in the margins was observed in *gtr1 gtr2*, it is likely that other GTR-dependent mechanisms affect GSL allocation to leaf margin cells (Madsen et al., 2014). Jørgensen et al. (2015) proposed a model for GSL distribution in mature leaves, in which GSLs are synthesized in the cytosol of cells adjacent to the vasculature and exported to various leaf cells via different transporters. A GTR1- and GTR2-dependent mechanism is involved in the uptake from the apoplast of GSL biosynthesis cells and their transport into phloem cells for export out of the leaf, storage cells, and vasculature-adjacent mesophyll cells (Jørgensen et al., 2015). Alternatively, GSLs might remain in the cytosol and travel to the leaf margin via plasmodesmata (PDs) and the symplastic route (Jørgensen et al., 2015). Moreover, all three classes of GSLs and their catabolites can be found in the root exudates of Arabidopsis and other plants of the Brassicaceae family (Xu et al., 2017). The levels of GSLs, particularly long-chain aliphatic ones, are strongly reduced in the root exudates of the *gtr1 gtr2* double mutant, suggesting that GTR1 and GTR2 are also required for root exudation of GSLs (Xu et al., 2017).

GSLs are produced in a variety of tissues, including leaves, roots, and inflorescence stems, and are then stored in the vacuoles of S-cells, which are characterized by very high sulfur content (Koroleva et al., 2010). S-cells are thought to be a major sink, accumulating extremely high concentrations of GSLs (>130 mM) (Koroleva et al., 2010). Upon attack by insects or wounding, S-cells rupture and release large amounts of GSLs through the action of myrosinases, producing compounds that are toxic to insects (Lv et al., 2022). Since GSL biosynthesis genes are not expressed in S-cells (Nintemann et al., 2018), the accumulation of GSLs in this cell type must be due to transport from other cell types. It appears that the levels of GSLs in S-cells derived from the stem of the *gtr1 gtr2* double mutant are significantly lower than those in wild-type ones (Xu et al., 2019), indicating that GTR1 and GTR2 are required for GSL accumulation in S-cells. However, GTR1 and GTR2, like GSL biosynthesis genes, are merely expressed in the phloem parenchyma and starch sheath surrounding S-cells (Xu et al., 2019). Thus, two routes were proposed for transport of GSLs into S-cells. In the first route, GSLs are transported through a symplastic pathway from biosynthesis cells that are adjacent to S-cells, such as starch sheath cells (Xu et al., 2019). In the second route, GSLs can be exported from nearby biosynthesis cells (parenchyma cells) and later imported by GTR to a symplastic domain that is connected to S-cells (Xu et al., 2019). The results of these studies demonstrate that GTRs are also essential for short-distance movement of GSLs. Given the importance of GTRs in GSL allocation and long-distance transport, they are suitable targets for generating crop plants with low GSL levels in seeds and high levels in leaves. *GTR2* knockdown *Brassica juncea* plants exhibit a significant reduction in GSL levels in seeds, but higher GSL accumulation in leaves and pods, resulting in enhanced resistance to *Spodoptera litura* (Nambiar et al., 2021).

The study of GSLs provides an excellent example of characterization of long-distance metabolite transport. However, several additional critical questions regarding GSL transport have been raised. GSLs are known to be produced in the cytosol but stored in the vacuoles. More research is required to determine how GSLs enter the vacuoles and whether other transporters are involved in this process. Furthermore, we still do not understand how GSLs are exported from the biosynthesis domains into the

apoplast and what is the physiological role of these export processes.

Azelaic acid is not merely involved in SAR

AzA was initially identified as a mobile signal involved in SAR in Arabidopsis leaves (Jung et al., 2009). It accumulates to high levels in the PEX of pathogen-infected Arabidopsis (Jung et al., 2009). Local application of AzA in Arabidopsis confers both local and systemic resistance to *Pseudomonas syringae* (Jung et al., 2009). Additionally, labeled AzA could be detected in systemic leaves after injection into local leaves (Jung et al., 2009). Moreover, bacterial infection causes an increase in the accumulation of AzA in Arabidopsis vascular sap, which also confers both local and systemic resistance to pathogens (Jung et al., 2009). AzA is a C9 dicarboxylic organic acid. A study by Wang et al. (2014) showed that leaves supplied exogenously with several unsaturated C18 fatty acids displayed a significant increase in AzA levels, suggesting that C18:1, C18:2, and/or C18:3 are likely AzA precursors. All three fatty acids contain a double bond between carbon atoms 9 and 10, and cleavage of this double bound results in the formation of a C9 compound, 9-oxononanoic acid (ONA, a monocarboxylic acid), which is further oxidized by aldehyde dehydrogenase (ADH) and eventually converted to AzA (Zoeller et al., 2012). C18 fatty acids are typically derived from monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which account for approximately 80% of total plant membrane lipids (Gao et al., 2014). Interestingly, loss of function of *MGDG SYNTHASE* (*MGD*) and *DGDG SYNTHASE* (*DGD*), which encode enzymes responsible for MGDG and DGDG biosynthesis, results in lower levels of AzA following pathogen infection, indicating that MGDG and DGDG are likely the *in vivo* precursors for AzA biosynthesis (Gao et al., 2014).

As a mobile signal, AzA can be transported systemically from local tissues. However, the mechanism of AzA transport is still unknown. Transport assays with isotope-labeled AzA showed that roughly 7% of AzA moves from local to systemic tissues, and AzA dimethyl ester is found in systemic leaves (Best et al., 2012; Yu et al., 2013). AzA accumulates in the PEX of tobacco mosaic virus (TMV)-infected tobacco leaves (Nagy et al., 2017). Following pathogen infection, increased levels of AzA are detected exclusively in the PEX, which serves as an indicator of both symplastic and apoplastic signals. Conversely, no significant rise in AzA levels was observed in the apoplastic fluid, which solely reflects apoplastic signals (Lim et al., 2016). This strongly implies that AzA enters the phloem via the symplastic route. PDs and the PD-localized proteins AZELAIC ACID INDUCED 1 (AZI1), PLASMODESMATA LOCALIZING PROTEIN 1 (PDLP1), and PDLP5 have been reported to regulate AzA transport from local to systemic tissues (Lim et al., 2016). AzA transiently and significantly induces expression of *AZI1*, which encodes a predicted lipid transfer protein (Jung et al., 2009). Application of AzA to the *azi1* mutant can enhance resistance in local leaves, but fails to activate a SAR response (Jung et al., 2009). AZI1 and its ortholog EARLY ARABIDOPSIS ALUMINUM INDUCED 1 (EARLI1) are localized in PDs/endoplasmic reticulum (ER), chloroplast outer envelopes, and membrane contact sites, all of which are involved in AzA transport (Cecchini et al., 2021; Cecchini, Steffes, et al., 2015; Lim et al., 2016; Yu et al., 2013). Plants with *AZI1* or *EARLI1* mutations fail to trigger a SAR response and show reduced AzA transport to systemic leaves (Cecchini, Steffes, et al., 2015). Moreover, the movement of ^{14}C-AzA to systemic tissues and its uptake in a leaf disk assay were also significantly reduced in *azi1* and *earli1* mutants (Cecchini, Steffes, et al., 2015). Thus, AZI1 and EARLI1 are most likely required for AzA-induced SAR establishment and AzA transport. In addition, both *pdlp1* and *pdlp5* mutants can still generate SAR signals in local leaves but fail to induce a SAR response in systemic ones (Lim et al., 2016). PDLP1 interacts with AZI1 and is required for the stability and intracellular partitioning of AZI1 (Lim et al., 2016). Overexpression of *PDLP5* decreases PD permeability, resulting in a significant reduction in AzA levels in the PEX following pathogen attack (Lim et al., 2016). The results of these studies suggest that PDLPs affect PD gating- and AZI1-mediated AzA symplastic transport. In addition, AZI1 can also interact with another PD-localized protein, DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1), whose mutant fails to activate SAR in response to AzA (Yu et al., 2013). Systemic AzA movement and uptake of labeled AzA in leaf disk assays are not affected in the *dir1* mutant (Cecchini, Steffes, et al., 2015), suggesting that DIR1 is not necessary for AzA transport. Moreover, pathogen-induced AzA may be transported to belowground tissues (Cecchini, Steffes, et al., 2015). On the other hand, root application of AzA can trigger systemic disease resistance in aerial tissues, which is fully dependent on EARLI1 and partially dependent on AZI1 (Cecchini et al., 2019). However, enhanced resistance in aerial tissues is not due to AzA movement from roots to shoots, as labeled AzA cannot be detected in shoots after root application (Cecchini et al., 2019). This suggests that root-applied AzA may activate other signals (AzA derivatives or other mobile signals) that can enhance immunity in aerial tissue. AzA can also be found in roots (Mukhtarova et al., 2011), yet it is currently not known whether it is biosynthesized there or transported from other tissues. AzA has been associated with systemic signaling in tomato roots as part of a newly discovered mechanism termed systemically induced root exudation of metabolites (SIREM) (Korenblum et al., 2022). The AzA derivative AzA dihexose was identified in tomato systemic roots following treatment of local roots with tomato rhizosphere microbiome in a split root assay (Korenblum et al., 2020). AzA when externally applied to local roots triggered AzA dihexose accumulation in systemic tissues as well as AzA secretion in systemic roots (Korenblum et al., 2020). In addition, local application of AzA significantly affects the metabolic profiles of shoots and systemic root exudates

(Korenblum et al., 2020). As AzA can be taken up by the roots but cannot move to the shoots, AzA dihexose could be the mobile signal that is transported to systemic tissues. However, the enzymes responsible for AzA dihexose biosynthesis and hydrolysis are still unknown. More research is required to understand how plants perceive and transport AzA-related signals, as well as the process and role of AzA secretion in systemic roots.

Caffeine and melatonin are not only mobile in the human body but also in plants

Caffeine is a purine alkaloid found in over 80 plant species (Gramza-Michałowska, 2014). It is a prominent constituent of tea, especially in young leaves (Ashihara & Suzuki, 2004; Suzuki et al., 1992). The biosynthesis pathway of caffeine has been well documented, yet the location of caffeine biosynthesis varies with species. Most key enzymes of caffeine biosynthesis were found in the chloroplast of tea plants (Ashihara et al., 2013), whereas caffeine was reported to be synthesized in the cytoplasm (Kumar et al., 2007; Ogawa et al., 2001). Caffeine has been shown to be toxic to a variety of pathogens and herbivores. For example, exogenously applied caffeine can directly inhibit the growth of *Aspergillus ocbraceus* and the production of its toxic metabolite ochratoxin A (Akbar et al., 2016). External application of caffeine can effectively repel slugs and kill snails and other insects (Hollingsworth et al., 2002). Caffeine has been reported to function as a defense inducer. Exogenously applied caffeine enhances resistance to pathogens in tobacco plants by stimulating the expression of defense-related genes (Kim & Sano, 2008). Likewise, tobacco plants producing caffeine display constitutive expression of *PATHOGEN-RELATED PROTEIN GENE* (*PR1*) and *PROTEINASE INHIBITOR II*, resulting in higher resistance to pathogens, TMV, and *P. syringae* (Kim & Sano, 2008). Further study in rice showed that caffeine dramatically enhances resistance to a broad range of biotic stresses by activating Ca^{2+} signaling and salicylic acid biosynthesis (Park et al., 2022). Caffeine transport was first reported in coffee plants as early as 1972 (Baumann & Wanner, 1972). Application of labeled caffeine to leaves and grafting experiments between caffeine-containing and caffeine-free species showed that a small amount of caffeine can move to systemic leaves and a larger amount of caffeine can travel from the pericarp into the seed tissue (Baumann & Wanner, 1972). Experiments in cabbage, water spinach (*Ipomoea aquatica*), and cabbage (*Brassica campastris*) showed that caffeine can be taken up by the roots of these plants and accumulate in the lower leaves (Chen et al., 2021). Moreover, caffeine was detected in the xylem sap collected from coffee seedlings (Mazzafera & Gonçalves, 1999). Like other specialized metabolites, caffeine is stored in the vacuole (Ashihara & Suzuki, 2004). Functional assays in heterologous expression systems identified that a member of the purine permease (PUP) protein family from *Camellis sinensis* acts as a caffeine transporter in yeast, suggesting that plasma membrane-localized PUP may import external caffeine into plant cells (Zhang et al., 2022).

Melatonin plays a crucial role in both growth and development, as well as in responses to biotic and abiotic stresses (Zhang & Zhang, 2021). Moreover, melatonin has been proposed as a mobile metabolite. ^{3}H-melatonin was found in different tissues after being applied to the apex and cotyledons of young plants (Kolář et al., 2003). Moreover, the presence of melatonin in the xylem sap confirmed that melatonin can be transported to systemic leaves through the xylem (Li et al., 2017). Nevertheless, further research is required to understand the role of melatonin as a mobile metabolite.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Plants evolved sophisticated mechanisms to integrate environmental information with local responses and long-distance communication signals to maintain normal growth and activate stress responses. Metabolites play a major role in long-distance communication, and progress has been made in identifying novel mobile signals. However, compared to other types, e.g., mRNA, microRNAs, peptides, and proteins, substantially less is known regarding mechanisms mediated by long-distance mobile plant metabolites that do not belong to the renowned select group of hormones. Several major impediments in this field slow down the pace at which such novel metabolites are identified, let alone characterized, at the highest mechanistic depth. The biggest hurdle is unquestionably determining whether a particular metabolite is indeed the 'true' and 'ultimate' communication molecule that activates the systemic process or plays an intermediary role in it. Long-distance communication pathways, specifically signaling ones, are likely more intricate than we currently understand, as they might include more than one mobile and 'final' factor. These molecules could be involved as either intermediates or committed factors in multiple pathways and linked with renowned hormone-mediated processes acting in a long-distance manner. Particular assays, including reciprocal plant grafting and metabolite–protein binding assays, can be of great assistance. However, by themselves, these methods are not sufficient. In the past decade, several metabolite–protein interaction assays that could lead to the discovery of proteins taking part in long-distance communication mediated by metabolites have been reported. They include PROtein–Metabolite Interactions using Size separation (PROMIS) (Veyel et al., 2018), Limited proteolysis Small Molecule mapping (Lip-SMap) (Piazza et al., 2018), the cellular thermal shift assay (CETSA) (Jafari et al., 2014), and drug affinity-responsive target stability (DARTS) (Pai et al., 2015). Nonetheless,

most, if not all, methods have been established in non-plant model systems and they require significant modifications to be implemented successfully in plants.

An additional core obstacle impeding our gain of knowledge with respect to long-distance mobile metabolites is the highly limited knowledge on cell type-specific metabolite biosynthesis, i.e., the source where a mobile long-distance signaling molecule is produced. Close to most of our knowledge regarding metabolic enzyme activity is at the organ level, and very little effort has been made to date at the cell type level (Antoniadi et al., 2015; Moussaieff et al., 2013). Currently, the activity of metabolic enzymes in a specific cell type is predicted from mRNA expression profiles, data that are frequently far from forecasting actual activity of enzymes. Recent work with single-cell approaches coupled with transcriptome analysis might improve data quality but will not be providing better predictors for enzyme activity in particular metabolic pathways. Sampling metabolites at the single-cell level is still in its infancy, particularly in plant cells. As one (relatively rare) example, live single-cell mass spectrometry (LSC-MS) represents an efficient approach for profiling metabolites in single live plant cells in minutes by sucking out the cell content with nano-electrospray ionization and directly analyzing it by mass spectrometry (Masuda et al., 2018). This technique has been used successfully to monitor changes in plant hormone levels in response to stress (Shimizu et al., 2015). LSC-MS and other technologies, such as fluidic force microscopy (Guillaume-Gentil et al., 2017), are limited in use and will hopefully become more prevalent in the coming years.

The latter complications, including the capacity to trace long-distance mobile metabolites at the cell type and single-cell levels, are tightly related to our capability to characterize in mechanistic depth the plethora of thousands of putative predicted transporter proteins present in a given plant genome. The multiplicity of transport substrates, in many cases even structurally distant metabolites, makes the understanding of mobile metabolite signals even more complex. Apart from great promiscuity in substrates, transporter proteins associated with long-distance communication might at the same time be associated with subcellular transport of the metabolites and not only with loading and/or export to specific cell types following long-distance movement. They could also be moving symplastically or through the apoplast and even, as in some known cases, through both paths (Lim et al., 2016). Functional characterization of such transporters requires, in most cases, specific assays in heterologous host systems, e.g., *Saccharomyces cerevisiae* (Tao et al., 2022), *Xenopus laevis* oocytes (Nour-Eldin et al., 2012), mammalian cell lines (Dvorak et al., 2021), and plant-based systems such as *Nicotiana tabacum* BY-2 cells (Pawela et al., 2019) and *N. benthamiana* leaves (Zhao et al., 2021), as well as functional complementation studies. These are suitable for some transporter types and substrates, and not for others. Finally, movement of metabolites might be thorough additional mechanisms, such as encapsulation and vesicle trafficking.

Exploiting these long-distance communication metabolites could generate effective solutions for engineering crop plants with operative solutions against a variety of both abiotic and biotic stresses, as well as enhanced growth and increased yield. Mechanistic understanding of their mode of action and the genetic layers of associated molecules might allow 'turn-key', inducible, and surgical control of positive plant traits without significant side effects by activating molecular networks sustaining plant fitness.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

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