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REVIEW ARTICLE

The branched-chain amino acid-related isoleucic acid: recent research advances

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ABSTRACT

Isoleucic acid (ILA) was identified in human patients with maple syrup urine disease (MSUD) half a century ago. MSUD patients, who are defective in the catabolism of branched-chain amino acids (BCAAs), that is, isoleucine, leucine, and valine, have urine with a unique maple syrup odour related to the accumulation of BCAA breakdown products, largely 2-keto acid derivatives and their reduced 2-hydroxy acids including ILA. A decade ago, ILA was identified in *Arabidopsis thaliana*. Subsequent studies in other plant species indicated that ILA is a ubiquitously present compound. Since its identification in plants, several efforts have been made to understand the biological significance and metabolic pathway of ILA. ILA plays a positive role in plant signalling for defence responses against bacterial pathogens by increasing the abundance of salicylic acid aglycone through competitive inhibition of SA deactivation by glucosylation. Here, we review recent progress in the characterization of ILA biosynthesis and function in plants and discuss current knowledge gaps and future directions in ILA research.

INTRODUCTION

Small molecules play diverse roles in plants, including defence against biotic and abiotic stresses, signalling, growth, and development. They exhibit great structural diversity and are based on a large variety of specific biosynthetic pathways, but also directly related to branches of primary metabolism (Palanivelu *et al.* 2003; Piasecka *et al.* 2015; Tian *et al.* 2024). The development of advanced analytical techniques has recently facilitated identification of several unknown and/or low-abundance regulatory metabolites, such as the 2-hydroxy-carboxylic acid isoleucic acid (ILA, 2-hydroxy-3-methylpentanoic acid). ILA was originally discovered in humans in the context of maple syrup urine disease (MSUD), which is caused by a genetic defect in the catabolic pathway of the branched-chain amino acids (BCAAs) leucine, isoleucine, and valine, leading to high levels of these amino acids and their intermediary degradation products in the urine of patients (Mamer & Reimer 1992; Podebrad *et al.* 1997). MSUD is a metabolic disorder caused by a mutation in one or more of the three subunits (E1, E2, and E3) of a multimeric enzyme complex called the branched-chain 2-ketoacid (2-KA) dehydrogenase complex (BCKDH) (Treacy *et al.* 1992). The different mutations in the BCKDH complex reduce the activity of the enzyme to different degrees. The residual activity of the mutant enzyme complex determines the severity of disease symptoms and the accumulation of BCAA-related metabolites. Metabolic characterization of MSUD patients with varying clinical symptoms revealed the presence of ILA (2-hydroxy-3-methylvaleric acid, HMVA) in urine samples (Lancaster *et al.* 1974; Jakobs *et al.* 1977). In addition, these patients accumulate a larger amount of BCAAs and related compounds, such as leucine (Leu), isoleucine (Ile), valine (Val), their corresponding α-ketoacids (BCKA), and 2-hydroxy acids (2-HAs) (Jakobs *et al.* 1977; Mamer & Reimer 1992; Frazier *et al.* 2014). However, the ratio of ketoacids to hydroxy acids detected in the blood samples of MSUD patients differs between the three BCAA catabolic pathways. Ketoacids derived from Ile and Leu metabolism, that is, KMVA (2-keto-3-methylvaleric acid) and KICA (2-ketoisocaproic acid), are more abundant than the corresponding hydroxy acids, ILA and leucic acid, respectively (Jakobs *et al.* 1977).

The presence of ILA in plants was first reported in a mutant of the model species *Arabidopsis thaliana* lacking the glucosyltransferase AtUGT76B1 gene. AtUGT76B1 modifies ILA, salicylic acid (SA), and N-hydroxypipecolic acid (NHP) by conjugating activated glucose molecules (von Saint Paul *et al.* 2011; Bauer *et al.* 2021). The glucosylated form of ILA (ILA-G) is reduced in the loss-of-function mutant *ugt76b1*, whereas ILA-G levels are significantly increased in lines constitutively overexpressing UGT76B1. ILA was indeed a substrate of UGT76B1 when tested *in vitro*, as suggested by these genetic and metabolic studies (von Saint Paul *et al.* 2011). However, the endogenous ILA aglycone was not determined *in planta* until several years later. Maksym *et al.* (2018) developed a sensitive method for the BCAA-related compounds, and they were finally able to detect unconjugated ILA in shoots and roots of *Arabidopsis* plants, as well as in leaves of different monocot and

dicot plant species. Interestingly, ILA was detected in all plant species studied, suggesting its widespread presence in plants (Maksym *et al.* 2018). In 2-week-old *Arabidopsis* shoots, approximately 750 ng g^{-1} DW (~0.6 μM estimated on a uniform distribution in fresh material) of ILA was measured, and this concentration almost halved as the age of the plant increased to 4 weeks (Maksym *et al.* 2018). In contrast, valic acid and leucic acid were detected in fewer species, but this may also be related to the sensitivity of the instrumentation to detect even less abundant molecules than ILA. In MSUD patients, valic acid is the most abundant of the three hydroxy acids derived from branched-chain amino acid metabolism, whereas in *Arabidopsis* and some other plant species, valic acid has not been detected (Jakobs *et al.* 1977; Maksym *et al.* 2018). This probably indicates a divergence in the metabolism and function of 2-HAs. In MSUD patients, 2-HAs may be mere catabolic products of branched-chain amino acids, but in plants, ILA appears to undergo regulated synthesis and shows specific biological activity. Despite these intriguing findings, the biosynthetic pathway of ILA in plants remains elusive. The elucidation of the ILA biosynthetic genes would significantly contribute to elucidation of the role of ILA in plant growth, development, and defence responses to biotic and abiotic factors.

ILA BIOSYNTHESIS THROUGH IMPAIRED ILE CATABOLISM

ILA biosynthesis is associated with defects in branched-chain amino acid catabolism. The first three enzymes, that is, branched-chain aminotransferases (BCATs), branched-chain ketoacid dehydrogenase (BCKDH) and isovaleryl-CoA dehydrogenase (IVD), are commonly involved in catabolism of the three BCAAs (Binder 2010; Schertl *et al.* 2017). A defect in BCKDH activity inhibits the catabolism of all three amino acids. As a result, the structurally related 2-HAs, which are formed by the reduction of the BCAA-related 2-KAs, accumulate in MSUD patients (Treacy *et al.* 1992). The catabolism of Ile in the mitochondria of plant cells begins with removal of the α amino group (Binder 2010; Schertl *et al.* 2017). This is catalysed by BCAT enzymes. The BCAT-catalysed transamination reaction produces KMVA, a potential precursor for ILA synthesis (Fig. 1). There are two isoforms of BCAT enzymes in mammals, BCAT1 and BCAT2. BCAT1 is cytosolic, whereas BCAT2 is mitochondrial. In plants, BCATs have been characterized in several species including tobacco, potato, barley, and spinach (Campbell *et al.* 2001; Malatrasi *et al.* 2006; Gao *et al.* 2009). Six isoforms of BCAT genes (*BCAT1*, *BCAT2*, *BCAT3*, *BCAT4*, *BCAT5* and *BCAT6*) have been identified in *A. thaliana*. These isoforms show organelle specificity. BCAT1 is localized in mitochondria, whereas BCAT2, BCAT3 and BCAT5 are localized in plastids. BCAT6 is localized in the cytosol (Binder 2010; Fig. 2). The cytosolic BCAT4 has no activity towards Ile or vice versa towards its corresponding KMVA in the opposite biosynthetic transamination (Schuster *et al.* 2006). In an intact Ile catabolic pathway, BCAT-generated KMVA undergoes an oxidative decarboxylation reaction catalysed by BCKDH (Binder 2010; Blackburn *et al.* 2017). BCKDH is a high molecular weight enzyme complex consisting of three subunits E1, E2, and E3. It is located

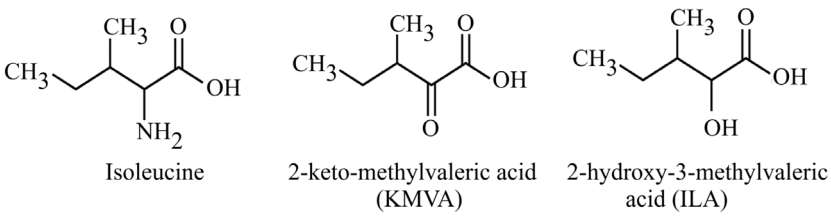

Fig. 1. Chemical structure of the branched-chain amino acid isoleucine and the related 2-oxo-methylvaleric acid (2-KMVA), being the first product of Ile catabolism and the immediate biosynthetic precursor of Ile. 2-hydroxy-3-methylvaleric acid (ILA) is the reduced form of 2-KMVA.

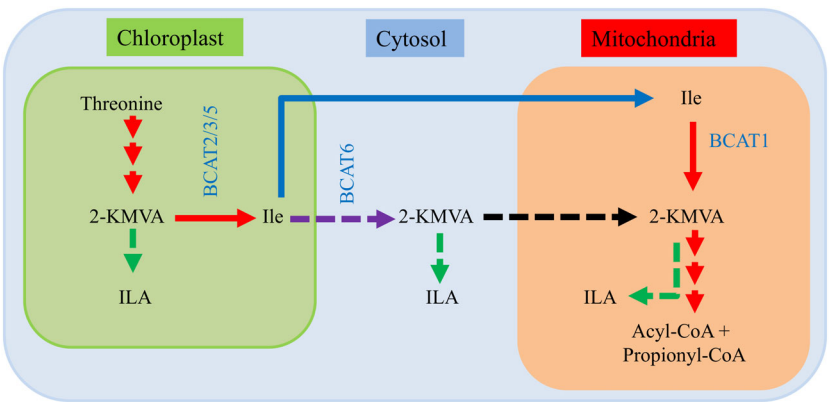

Fig. 2. Putative subcellular localization of ILA biosynthesis in plants. In the chloroplast, 2-KMVA is an immediate precursor for Ile biosynthesis, and in the presence of a reductase, 2-KMVA could be reduced to ILA (dashed green lines). In the cytosol and in mitochondria, 2-KMVA is the first product in the catabolism of Ile by transaminases. In the presence of appropriate reductases, 2-KMVA could be reduced to ILA (dashed green arrows). The dashed green lines are all hypothetical enzymatic steps.

on the inner membrane of mitochondria (Indo *et al.* 1987). The E1 subunit, itself composed of the E1α and E1β subunits, is a decarboxylase and catalyses the decarboxylation of 2-KMVA. The E2 subunit acts as an acyltransferase to transfer the acyl group from an intermediate lipoyl moiety to the reduced co-enzyme A; finally, the E3 subunit has a dehydrogenase activity and re-oxidizes the reduced lipoyl sulfur residues to produce NADH (Yeaman 1989). The catalytic activity of BCKDH on KMVA produces 2-methylbutyryl CoA, which undergoes a further catabolic reaction to the final product. Alternatively, KMVA could be reduced to ILA by the activity of an as yet unknown enzyme (Fig. 2).

ILA BIOSYNTHESIS IN PLANTS

ILA biosynthesis in plants is thought to originate from isoleucine catabolism or from Ile biosynthesis, since both pathways involve KMVA as a product or educt of the transamination reaction (Fig. 2). A significant increase in Ile abundance in *Arabidopsis* shoots following exogenous application of ILA supports the hypothesis that ILA is metabolically related to Ile (Maksym *et al.* 2018). The degradation of Ile takes place in mitochondria and therefore ILA might be produced in the same organelle. Isoleucine biosynthesis is a unique feature of photosynthetic organisms, such as plants, and takes place in chloroplasts (Ellerstrom *et al.* 1992; Diebold *et al.* 2002). ILA biosynthesis could therefore also take place in the chloroplast. Alternatively, any other compartment could be involved in ILA biosynthesis as long as KMVA is translocated to it. In humans, *de novo* biosynthesis of isoleucine is not possible and therefore isoleucine is derived from the diet (Blackburn *et al.* 2017). A putative ILA-synthesizing plant enzyme probably competes with BCKDH in mitochondria and BCAT in chloroplasts for the common substrate KMVA. Under normal conditions, the catabolic reaction of KMVA by BCKDH in mitochondria and the biosynthetic reaction by BCAT in chloroplasts appear to be the favoured pathways. MSUD patients, who have severely reduced BCKDH activity, accumulate higher levels of ILA. Nevertheless, the accumulated ILA is still nine- to tenfold lower than its precursor KMVA (Jakobs *et al.* 1977; Shigematsu *et al.* 1983; Blackburn *et al.* 2017), suggesting that ILA synthesis is a less preferred route of Ile catabolism in humans. Mamer & Reimer (1992) proposed that a member of the L-lactate dehydrogenase enzyme family catalyses the reduction of KMVA to synthesize ILA in humans. Although LDHs from various sources can reduce other 2-KAs (Kim & Whitesides 1988), the involvement of L-lactate dehydrogenase in ILA biosynthesis in humans has not been demonstrated experimentally. In plants, the *Arabidopsis* L-lactate dehydrogenase mutant (*l-ldh*; At4g17260) accumulates ~166 ng g^{-1} DW ILA, which is similar to the amount measured in shoots of wild-type plants (~137 ng g^{-1} DW) (Fig. 3a). The D-lactate dehydrogenase mutant (*d-ldh*, AT5G06580) also accumulates a similar amount of ILA (~159 ng g^{-1} DW) (Fig. 3a). The similar levels of ILA in the *l-ldh* and *d-ldh* mutants compared to the wild type suggests that these *Arabidopsis* L-lactate and D-lactate dehydrogenases are unlikely to be involved in ILA biosynthesis. L-lactate and D-lactate dehydrogenases are involved in the redox

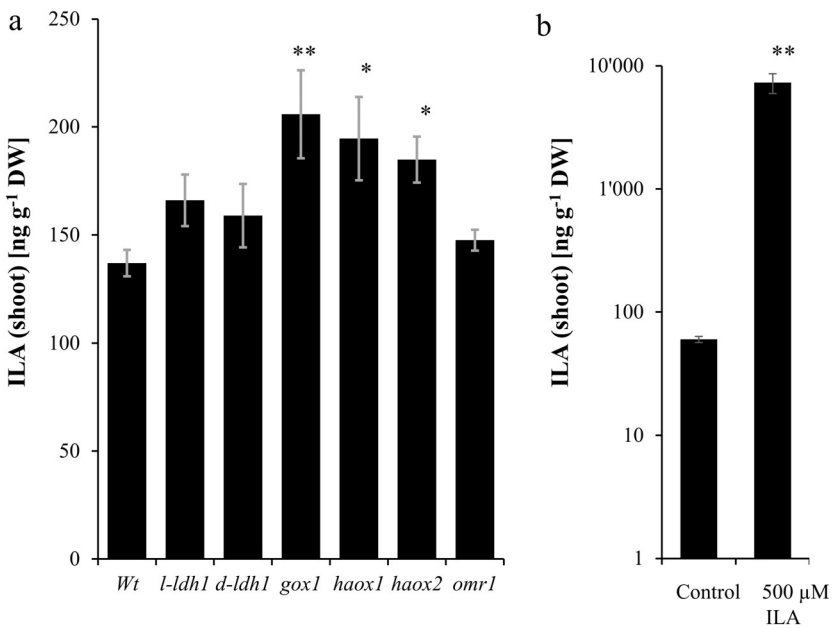

Fig. 3. (a) ILA content in 3-week-old shoots of various *Arabidopsis* mutant lines compared to the wild type. Leaf samples were collected from soil-grown plants for ILA quantification. ILA content was measured in leaf extracts after anion-exchange solid-phase extraction (SPE) followed by silylation and GC-MS analysis (Maksym *et al.* 2018). Values represent the average of three biological replicates; error bars indicate the standard error of means. Statistical difference compared to wild type (Benjamini-Hochberg adj. *P*-values **, <0.01; *, <0.05; n.s., non-significant; ANOVA, Holm-Sidak post-hoc method). The experiment was repeated independently and with comparable statistical results. Abbreviations: WT, wild type; *l-ldh*, L-lactate dehydrogenase mutant (Alonso *et al.* 2003); *d-ldh*, D-lactate dehydrogenase mutant; *gox1*, glyoxylate oxidase 1; *haox1*, hydroxy acid oxidase 1; *haox2*, hydroxy acid oxidase 2; *omr1*, L-O-methythreonine resistance 1, threonine deaminase gain of function mutant (Mourad & King 1995). (b) ILA content in shoots of 4-week-old soil-grown *Arabidopsis* plants irrigated once with 500 µM ILA. ILA was analysed 3 days after its application. Values are the average of four biological replicates. Error bars are standard error of means (**$P < 0.01$, ANOVA).

reaction of pyruvate interconversion to lactate and vice versa (Engqvist *et al.* 2009; Maurino & Engqvist 2015). Thus, ILA production in plants may be catalysed by another oxidoreductase as a side reaction involved in other processes, or by an enzyme dedicated to ILA synthesis.

ILA DEGRADATION

ILA can be degraded or transformed into other products. Exogenous application of ILA increased endogenous levels of Ile, suggesting its degradation via oxidation to KMVA and subsequent transamination (Maksym *et al.* 2018). Oxidases that use molecular oxygen as an electron acceptor produce a low ratio of 2-HA to 2-KA when the reaction is at equilibrium (Maurino & Engqvist 2015). The *Arabidopsis* recombinant hydroxy acid oxidase proteins, HAOX1, HAOX2, and GOX1, showed slight activity towards ILA in *in vitro* experiments (Engqvist *et al.* 2015). However, this activity was observed at 5 mM ILA, which is a non-physiological concentration. Shoot extracts from 3-week-old *Arabidopsis haox1* (At3g14130) and *haox2* (At3g14150) single T-DNA insertion mutants contain about 190 ng g^{-1} DW ILA, which is significantly higher than the wild type (Fig. 3a). This observation suggests that both HAOX1 and HAOX2 may play an oxidative role in ILA degradation *in vivo*. HAOX1 and HAOX2 have different substrate specificities; HAOX1 shows the highest preference for 2-hydroxydodecanoic acid followed by L-lactate, whereas HAOX2 shows the highest preference for leucic acid (Esser *et al.* 2014). Similarly, the *Arabidopsis gox1* single mutant contains significantly higher ILA than the wild type, suggesting the presence of another possible oxidation of ILA *in vivo* (Fig. 3a). GOX1 mainly catalyses the oxidation of the 2-HA compound glycolate to glyoxylate in peroxisomes (Maurino & Engqvist 2015). Since GOX1 is localized in the peroxisome, the oxidative reaction of GOX1 on ILA requires the transport of ILA into the peroxisome or its synthesis in the peroxisome. The reduction of glyoxylate back to glycolate is catalysed by glyoxylate reductases in the cytosol and plastid after glyoxylate export from the peroxisomes (Hoover *et al.* 2007; Simpson *et al.* 2008; Maurino & Engqvist 2015). Similar to the glycolate–glyoxylate interconversions, the interconversion between KMVA and ILA may also involve two or more distinct enzymes and different organelles. Significant change in the ILA content of *haox1*, *haox2*, and *gox1* single mutants confirms the results of the *in vitro* assay with the recombinant proteins. The involvement of HAOX and GOX proteins in the oxidation of ILA needs to be validated through the generation of higher order mutants.

In addition to degradation, ILA can also be glucosylated by a UGT (UDP-glucosyltransferase). In *Arabidopsis*, this reaction is catalysed by UGT76B1, which inactivates ILA as part of pathogen defence signalling (von Saint Paul *et al.* 2011; Bauer *et al.* 2021).

ROLE OF ILA *IN PLANTA*

The 2-HAs play a central role in various plant metabolic pathways, such as the glyoxylate cycle and the TCA cycle (Maurino & Engqvist 2015). Although detailed characterization is required, the 2-HA ILA appears to have regulated production and function in plants, rather than being a mere byproduct of Ile biosynthesis or degradation. First, the abundance of ILA is negatively correlated with plant age, that is, its level in shoots is reduced in older *Arabidopsis* plants, whereas the level of Ile remains unchanged (Maksym *et al.* 2018). Second, ILA levels are poorly correlated with the increased endogenous Ile levels of the *omr1* mutant. The EMS-derived *Arabidopsis omr1* mutant accumulates 20-fold more Ile in shoots than the wild type (Mourad & King 1995). However, ILA of 3- and 4-week-old *omr1* mutant plants only increased by 10%–30% compared to the wild type (Fig. 3a). The fact that ILA abundance is less affected by endogenous Ile levels suggests that ILA abundance is independently regulated.

Current knowledge of the role of ILA in plants comes from studies involving its exogenous application to *Arabidopsis* plantlets. Incubation with 500 μM ILA increased the endogenous ILA level up to 7300 ng g^{-1} DW (~5.6 μM) (Fig. 3b). As the genes involved in ILA biosynthesis have not yet been identified, it has not been possible to manipulate ILA biosynthesis and study its effect *in planta*. ILA treatment inhibited root growth of *Arabidopsis* (Bauer *et al.* 2020). The ILA-induced inhibition of root growth is not related to its putative precursor Ile, since the same concentration of Ile caused less inhibition. Thus, ILA itself or possibly other compounds derived from ILA are responsible for the inhibition (von Saint Paul *et al.* 2011; Bauer *et al.* 2021). One possible product derived from exogenous ILA is oxidized KMVA. The effect of KMVA on plant growth has not yet been characterized; however, this compound inhibited growth of *Escherichia coli* strains, such as DH5α (Lorenz *et al.* 2013). The ILA-induced inhibition of root growth is also not mediated by the major phytohormones, such as abscisic acid (ABA), jasmonic acid (JA), and ethylene, since single mutant lines defective in the biosynthesis of these hormones behave similarly to the wild type after ILA treatment (Bauer *et al.* 2020). The ILA-induced inhibition of root growth does not require an intact SA signalling pathway. The root growth of an SA-depleted *Arabidopsis* NahG *sid2* plant was inhibited to the same extent as the wild type after exogenous ILA application. This suggests that ILA has a unique signalling pathway, independent of the major hormonal pathways, to regulate plant growth.

ILA-induced inhibition of root growth involves a defect in cell elongation at the root meristem and in the differentiation zone. Cells in the elongation zone of ILA-treated plants are deformed compared to control plants. In addition, the development of root cap columella cells is either delayed or inhibited, which may be related to the observed loss of root gravitropic response (Bauer *et al.* 2020). There is an induction of reactive oxygen species (ROS) in roots following ILA treatment. However, genetic and pharmacological studies excluded the involvement of O_2^- and H_2O_2 in the growth inhibition by ILA (Bauer *et al.* 2020). Nevertheless, other forms of reactive species cannot be excluded as mediators of ILA-induced growth inhibition. The effect of ILA is not limited to root growth. Shoot growth of ILA-treated seedlings was also reduced, although this was not characterized in detail (von Saint Paul *et al.* 2011). The decrease in shoot ILA content with increasing age of *Arabidopsis* plants (Maksym *et al.* 2018) may contribute to alleviation of the inhibitory effect of ILA on plant growth.

The best-studied effect of ILA is its defence-stimulating action. ILA promotes plant resistance to pathogens by enhancing the SA-mediated defence response. This beneficial effect was demonstrated using the SA-depleted NahG *sid2* mutant,

which is unable to induce a defence response following exogenous ILA application. The enhancement of the ILA-triggered defence response is facilitated by competitive inhibition of SA glucosylation, which inactivates the bioactive phytohormone SA. NHP is another substrate of UGT76B1 that competes with ILA and SA for glucosylation and is critical for defence activation (Bauer *et al.* 2021; Holmes *et al.* 2021). NHP is a lysine-derived compound that is required to induce systemic acquired resistance (SAR) of plants in response to a primary pathogen infection (Hartmann *et al.* 2018). Indeed, *Arabidopsis* seedlings grown in liquid culture and treated with ILA accumulated more SA and NHP and exhibited increased expression of their biosynthetic genes compared to untreated seedlings (von Saint Paul *et al.* 2011; Bauer *et al.* 2021). SA and NHP trigger local and SAR defence signalling cascades against biotrophic pathogens. In the absence of pathogen infection and to attenuate defence responses, SA is inactivated to SA-*O*-glucoside by UGT76B1. In parallel, NHP is glucosylated and inactivated to its *O*-glucoside by UGT76B1 after its initial rise in response to pathogen infection (Bauer *et al.* 2021; Cai *et al.* 2021; Holmes *et al.* 2021; Mohnike *et al.* 2021). Finally, ILA is another substrate that competes with NHP and SA conjugation and therefore enhances the defence response when applied exogenously (Bauer *et al.* 2020). *In vitro* studies suggest that UGT76B1 has a higher preference for NHP than for ILA and SA (Holmes *et al.* 2021). When comparing SA and ILA, UGT76B1 has a lower K_m for SA than ILA, but also a lower k_{cat}. Thus, the enzymatic efficiency is almost the same for both substrates, SA (k_{cat}/K_m = 2.2) and ILA (k_{cat}/K_m = 1.9) (Maksym *et al.* 2018; Holmes *et al.* 2021). Endogenous SA levels in *Arabidopsis* shoots range from 250 to 1000 ng g^{-1} FW (Vicente & Plasencia 2011). A slight local increase in ILA concentration may be sufficient to compete effectively with SA glucosylation. Unexpectedly, endogenous ILA aglycone levels were also reduced in *ugt76b1* mutants after infection, suggesting an additional regulation of endogenous ILA levels independent of its UGT76B1-dependent glucosylation. The ILA-enhanced defence response of *Arabidopsis* plants requires the functioning of UGT76B1. In the *ugt76b1* mutant, the stimulatory effect of ILA on the SA-mediated defence response is abolished, confirming the importance of UGT76B1 (Bauer *et al.* 2020). In summary, ILA promotes plant defence indirectly by competitively inhibiting the attenuating effect of UGT76B1 on SA and NHP. So far, this could only be studied by exogenous application of ILA, so the real *in planta* scenario is still not fully understood.

All the above discussed studies have been performed using the model plant *A. thaliana*. However, it is likely that similar metabolic and regulatory scenarios are conserved in other plant species. Apart from the fact that ILA itself has been detected in a wide variety of plants (see above; Maksym *et al.* 2018), the detection of putative ILA glucoside (*m/z* 293.1242) by ultra-high resolution mass spectrometry in extracts of leaves from Brassicaceae, Solanaceae, wheat, barley, and poplar may be more instructive (Fig. 4). The responsible glucosyltransferases may be homologues of AtUGT76B1, at least in the closely related Brassicaceae, e.g., the *Brassica napus* genome harbours two putative orthologs, encoded by *BnaA05g27700D* and *BnaC05g41870D*, with 94% similar amino acids compared to AtUGT76B1. Less sequence homology may not be a stringent criterion to identify ILA (and SA and NHP) conjugating UGT enzymes (Vogt & Jones 2000; Bowles *et al.* 2006). For example, wheat harbours numerous homologues of AtUGT76B1;

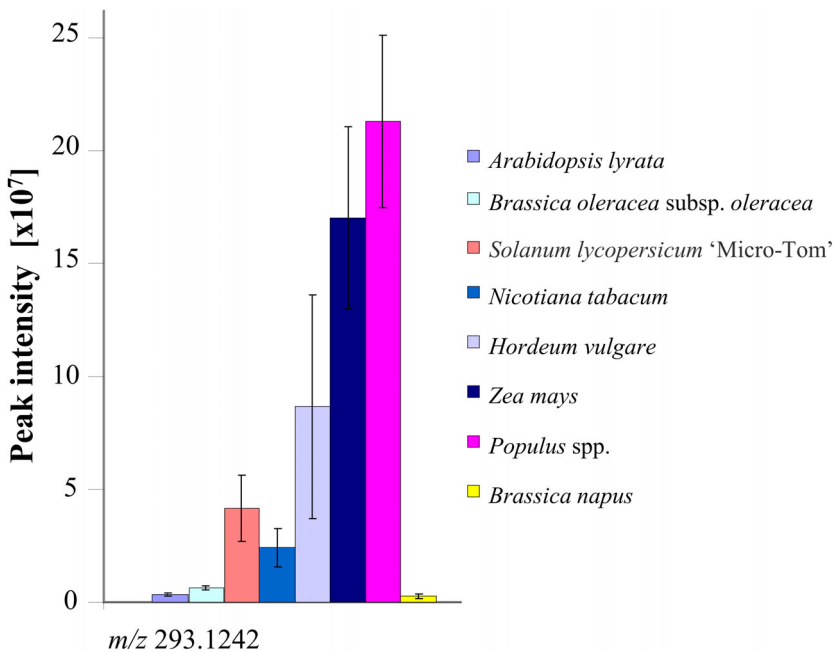

Fig. 4. Qualitative assessment of putative ILA glucoside (*m/z* 293.1242, $C_{12}H_{21}O_8$, negative mode) by ultrahigh-resolution Fourier transform ion cyclotron resonance mass spectrometry. *Arabidopsis lyrata*, *Brassica oleracea* subsp. *oleracea*, *Brassica napus*, *Solanum lycopersicum* cv. 'Micro-Tom', *Nicotiana tabacum*, *Hordeum vulgare* L. cv. Barke, *Zea mays*, and *Populus* spp. were grown in a greenhouse with 10 h light at 24°C and at 18°C during the dark period with 60% relative humidity. Leaf samples (n = 5; mean ± SD) were collected at young seedling stage, extracted and analysed as described previously (von Saint Paul *et al.* 2011).

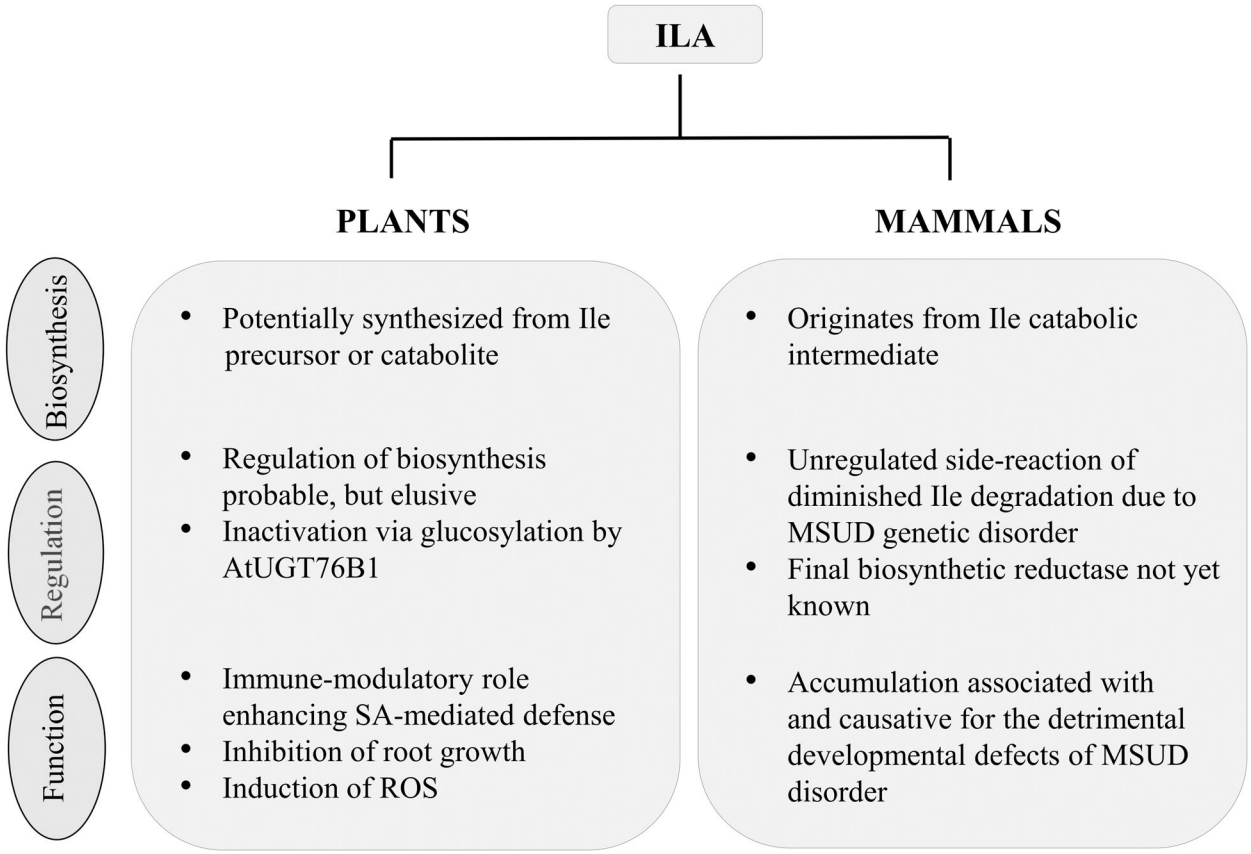

Fig. 5. Summary of the differences in ILA biosynthesis, regulation, and functional roles in plants and mammals.

however, all are more distant than other family 76 UGTs found in *A. thaliana*. Thus, these genes evolved and diversified after the evolutionary split of monocots and dicots, excluding a simple homology-based relationship.

CONCLUSIONS

The 2-HA ILA has divergent impacts affecting development, stress responses, and plant defence of *A. thaliana*. In particular, metabolic data suggest that ILA and its interplay with ILA glucoside also play a role in other plant species. Although the biosynthetic steps in plants (and in mammals) are not yet (fully) elucidated, the analogy with the human genetic disorder MSUD strongly implies that ILA originates from Ile biosynthetic or catabolic reactions (Fig. 5). Identification of the biosynthetic components in plants would be decisive and instructive to initiate genetic approaches with mutants manipulating endogenous ILA levels rather than relying on exogenous application of ILA; yet the putatively close link to the amino acid metabolism may jeopardize such a strategy. Genome-wide association studies (GWAS) based on ILA analytics using collections of *Arabidopsis* accessions may serve as an alternative strategy to identify loci that control ILA levels in plant cells. In contrast to MSUD, ILA levels in plants (*A. thaliana*) appear to be regulated by yet unknown mechanisms, since ILA concentrations do not correlate with altered Ile levels. Furthermore, *A. thaliana* has evolved a means of inactivating ILA by glucosylation, a reaction which is intrinsically linked with the inactivation of the immune-modulatory key players SA and NHP, since a single glucosyltransferase, AtUGT76B1, accepts all three substrates in a competitive manner. Thus, ILA itself impacts immune responses. The molecular actions of ILA related to its other independent roles in stress response and development remain elusive (Fig. 5).

AUTHOR CONTRIBUTIONS

D. W. Mekonnen, A. Ghirardo, and W. Zhang analysed the metabolite data. D. W. Mekonnen wrote the manuscript with contributions from A. Ghirardo and A. R. Schäffner.

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The corresponding author will provide the data included in this paper upon request.

REFERENCES

Alonso J.M., Stepanova A., Leisse T.J., Kim C.J., Chen H., Shinn P., Stevenson D.K., Zimmerman J., Barajas P., Cheuk R., Gadrinab C., Heller C., Jeske A., Koesema E., Meyers C.C., Parker H., Prednis L., Ansari Y., Choy N., Deen H., Geralt M., Hazari N., Hom E., Karnes M., Mulholland C., Ndubaku R., Schmidt I., Guzman P., Aguilar-Henonin L., Schmid M., Weigel D., Carter D.E., Marchand T., Risseeuw E., Brogden D., Zeko A., Crosby W.L., Berry C.C., Ecker J.R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Bauer S., Mekonnen D.W., Geist B., Lange B., Ghirardo A., Zhang W., Schäffner A.R. (2020) The isoleucic acid triad: distinct impacts on plant defense, root growth, and formation of reactive oxygen species. *Journal of Experimental Botany*, **71**, 4258–4270.

Bauer S., Mekonnen D.W., Hartmann M., Janowski R., Zhang W., Lange B., Geist B., Zeier J., Schäffner A.R. (2021) UGT76B1, a promiscuous hub of small molecule-based immune signaling, glucosylates N-hydroxypipecolic acid and controls basal pathogen defense. *The Plant Cell*, **33**, 714–734.

Binder S. (2010) *Branched-chain amino acid metabolism in Arabidopsis thaliana*. The Arabidopsis Book, e0137. American Society of Plant Biologists. https://doi.org/10.1199/tab.0137

Blackburn P.R., Gass J.M., Veiro F.P., Farnham K.M., Arwal H.K., Macklin S., Klee E.W., Atwal P.S. (2017) Maple syrup urine disease: mechanisms and management. *The Application of Clinical Genetics*, **10**, 57–66.

Bowles D., Lim E.K., Poppenberger B., Vaistij F.E. (2006) Glycosyltransferases of lipohilic small molecules. *Annual Reviews in Plant Biology*, **57**, 567–597.

Cai J., Jozwiak A., Holoidovsky L., Meijler M.M., Meir S., Rogachev I., Aharoni A. (2021) Glycosylation of N-hydroxy-pipecolic acid equilibrates between systemic acquired resistance response and plant growth. *Molecular Plant*, **14**, 440–455.

Campbell M.A., Patel J.K., Meyers J.L., Myrick L.C., Gustin J.L. (2001) Genes encoding for branched-chain amino acid aminotransferase are differently expressed in plants. *Plant Physiology and Biochemistry*, **39**, 855–860.

Diebold R., Schuster J., Däschner K., Binder S. (2002) The branched-chain amino acid transaminase gene family in *Arabidopsis* encodes plastid and mitochondrial proteins. *Plant Physiology*, **129**, 540–550.

Ellerstrom M., Josefsson L.G., Rask L., Ronne H. (1992) Cloning of a cDNA for rape chloroplast 3-isopropylmalate dehydrogenase by genetic complementation in yeast. *Plant Molecular Biology*, **18**, 557–566.

Engqvist M., Drincovich M.F., Flügge U.I., Maurino V.G. (2009) Two D-2-hydroxy-acid dihydrogenases in *Arabidopsis thaliana* with catalytic capacities to participate in the last reactions of the methylglyoxal and beta-oxidation pathways. *Journal of Biological Chemistry*, **284**, 25026–25037.

Engqvist M.K.M., Schmitz J., Kuhn A., Florian A., Jaspert N., Arif M., Balazadeh S., Mueller-Roeber B., Fernie A.R., Maurino V.G. (2015) GOX3, a glycolate oxidase homologue of yeast *L*-lactate cytochrome c oxidoreductase, supports *L*-lactate oxidation in roots of *Arabidopsis thaliana*. *Plant Physiology*, **169**, 1042–1061.

Esser C., Kuhn A., Groth G., Lercher M.J., Maurino V.G. (2014) Plant and animal glycolate oxidases have a common eukaryotic ancestor and convergently duplicated to evolve long- chain 2-hydroxy acid oxidases. *Molecular Biology and Evolution*, **31**, 1089–1101.

Frazier D.M., Allgeier C., Homer C., Marriage B.J., Ogata B., Rohr F., Splett P.L., Stembridge A., Singh R.H. (2014) Nutrition management guideline for maple syrup urine disease: an evidence- and consensus-based approach. *Molecular Genetics and Metabolism*, **112**, 210–217.

Gao F., Wang C., Wei C., Li Y. (2009) A branched-chain aminotransferase may regulate hormone levels by affecting *KNOX* genes in plants. *Planta*, **230**, 611–623.

Hartmann M., Zeier T., Bernsdorff F., Reichel-Deland V., Kim D., Hohmann M., Scholten N., Schuck S., Bräutigam A., Hölzel T. (2018) Flavin monooxygenase-generated N- hydroxyl-pipecolic acid is a critical element of plant systemic immunity. *Cell*, **173**, 456–469.

Holmes E.C., Chen Y.C., Mudgett M.B., Sattely E.S. (2021) *Arabidopsis* UGT76B1 glycosylates *N*-hydroxypipecolic acid and inactivates systemic acquired resistance in tomato. *The Plant Cell*, **33**, 750–765.

Hoover G.J., van Cauwenberghe O.R., Breitkreuz K.E., Clark S.M., Merrill A.R., Shelp B.J. (2007) Characteristics of an Arabidopsis glyoxylate reductase: general biochemical properties and substrate specificity for the recombinant protein, and developmental expression and implications for glyoxylate and succinic semialdehyde metabolism in planta. *Canadian Journal of Botany*, **85**, 883–895.

Indo I., Kitano A., Endo F., Akaboshi I., Matsuda I. (1987) Altered kinetic properties of the branched-chain alpha-keto acid dehydrogenase complex due to mutation of the beta-subunit of the branched-chain alpha-keto acid decarboxylase (E_1) component in lymphoblastoid cells derived from patients with maple syrup urine disease. *The Journal of Clinical Investigation*, **80**, 63–70.

Jakobs C., Solem E., Ek J., Halvorsen K., Jellum E. (1977) Investigation of the metabolic pattern in maple syrup urine disease by means of glass capillary gas chromatography and mass spectrometry. *Journal of Chromatography*, **143**, 31–38.

Kim M.J., Whitesides G.M. (1988) *L*-lactate dehydrogenase: substrate specificity and use as a catalyst in the synthesis of homochiral 2-hydroxy acids. *Journal of the American Chemical Society*, **110**, 2959–2964.

Lancaster G., Mamer O.A., Scriver C.R. (1974) Branched-chain alpha keto acids isolated as oxime derivatives: relationship to the corresponding hydroxy acids and amino acids in maple syrup urine disease. *Metabolism*, **23**, 257–265.

Lorenz E., Klatte S., Wendisch V.F. (2013) Reductive amination by recombinant *Escherichia coli*: whole cell biotransformation of 2-keto-3-methylvalerate to l-isoleucine. *Journal of Biotechnology*, **168**, 289–294.

Maksym R.P., Ghirardo A., Zhang W., von Saint Paul V., Lange B., Geist B., Hajirezaei M.R., Schnitzler J.P., Schäffner A.R. (2018) The defense-related isoleucic acid differentially accumulates in Arabidopsis among branched-chain amino acid-related 2-hydroxy carboxylic acids. *Frontiers in Plant Science*, **9**, 766.

Malatrasi M., Corradi M., Svensson J.T., Close T.J., Gulli M., Marmiroli N. (2006) A branched-chain amino acid aminotransferase gene isolated from *Hordeum vulgare* is differentially regulated by drought stress. *Theoretical and Applied Genetics*, **113**, 965–976.

Mamer O.A., Reimer M.L.J. (1992) On the mechanisms of the formation of L alloisoleucine and the 2-hydroxy-3-methylvaleric acid stereoisomers from L-isoleucine in maple syrup urine disease patients and in normal humans. *The Journal of Biological Chemistry*, **267**, 22141–22147.

Maurino V.G., Engqvist M.K.M. (2015) *2-hydroxy acids in plant metabolism*. The Arabidopsis Book, e0182. American Society of Plant Biologists. https://doi.org/10.1199/tab.0182

Mohnike L., Rekhter D., Huang W., Feussner K., Tian H., Herrfurth C., Zhang Y., Feussner I. (2021) The glycosyltransferase UGT76B1 modulates *N*-hydroxypipecolic acid homeostasis and plant immunity. *The Plant Cell*, **33**, 735–749.

Mourad G., King J. (1995) L-O-methylthreonine-resistant mutant of Arabidopsis defective in isoleucine feedback regulation. *Plant Physiology*, **107**, 43–52.

Palanivelu R., Brass L., Edlund A.F., Preuss D. (2003) Pollen tube growth and guidance is regulated by POP2, an Arabidopsis gene that controls GABA levels. *Cell*, **114**, 47–59.

Piasecka A., Jedrzejczak-Rey N., Bednarek P. (2015) Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. *New Phytologist*, **206**, 948–964. https://doi.org/10.1111/nph.13325

Podebrad F., Heil M., Leib S., Geier B., Beck T., Mosandl A., Sewell A.C., Böhles H. (1997) Analytical approach in diagnosis of inherited metabolic diseases: maple syrup urine disease (MSUD) – Simultaneous analysis of metabolites in urine by enantioselective multidimensional capillary gas chromatography-mass spectrometry (enantio-MDGC-MS). *Journal of High Resolution Chromatography*, **20**, 355–362. https://doi.org/10.1002/jhrc.1240200703

Schertl P., Danne L., Braun H.P. (2017) 3-Hydroxyisobutyrate dehydrogenase is involved in both valine and isoleucine degradation in *Arabidopsis thaliana*. *Plant Physiology*, **175**, 51–61.

Schuster J., Knill T., Reichelt H., Gershenzon J., Binder S. (2006) Branched-chain aminotransferase 4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in Arabidopsis. *The Plant Cell*, **18**, 1–16.

Shigematsu Y., Kikuci K., Momoi T. (1983) Organic acids and branched-chain amino acids in body fluids before and after multiple exchange transfusions in maple syrup urine disease. *Journal of Inherited Metabolic Disease*, **6**, 183–189.

Simpson J.P., Di Leo R., Dhanoa P.K., Allan W.L., Makhmoudova A., Clark S.M., Hoover G.J., Mullen

R.T., Shelp B.J. (2008) Identification and characterization of a plastid-localized Arabidopsis glyoxylate reductase isoform: comparison with a cytosolic isoform and implications for cellular redox homeostasis and aldehyde detoxification. *Journal of Experimental Botany*, **59**, 2545–2554.

Tian L., Hossbach B.M., Feussner I. (2024) Small size, big impact: small molecules in plant systemic immune signalling. *Current Opinion in Plant Biology*, **81**, 102618.

Treacy E., Clow C.L., Reade T.R., Chitayat D., Mamer O.A., Scriver C.R. (1992) Maple syrup urine disease: interrelations between branched-chain amino-, oxo- and hydroxy acids; implications for treatment; associations with CNS dysmyelination. *Journal of Inherited Metabolic Disease*, **15**, 121–135.

Vicente M.R.S., Plasencia J. (2011) Salicylic acid beyond defence: its role in plant growth and development. *Journal of Experimental Botany*, **62**, 3321–3338.

Vogt T., Jones P. (2000) Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. *Trends in Plant Science*, **5**, 380–386.

von Saint Paul V., Zhang W., Kanawati B., Geist B., Faus-Keßler T., Schmitt-Kopplin P., Schäffner A.R. (2011) The Arabidopsis glucosyltransferase UGT76B1 conjugates isoleucic acid and modulates plant defense and senescence. *The Plant Cell*, **23**, 4124–4145.

Yeaman S.J. (1989) The 2-oxo acid dehydrogenase complexes: recent advances. *Biochemical Journal*, **257**, 625–632.