

# Survey of Genes Involved in Biosynthesis, Transport, and Signaling of Phytohormones with Focus on *Solanum lycopersicum*

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**ABSTRACT:** Phytohormones control the development and growth of plants, as well as their response to biotic and abiotic stress. The seven most well-studied phytohormone classes defined today are as follows: auxins, ethylene, cytokinin, abscisic acid, jasmonic acid, gibberellins, and brassinosteroids. The basic principle of hormone regulation is conserved in all plants, but recent results suggest adaptations of synthesis, transport, or signaling pathways to the architecture and growth environment of different plant species. Thus, we aimed to define the extent to which information from the model plant *Arabidopsis thaliana* is transferable to other plants such as *Solanum lycopersicum*. We extracted the co-orthologues of genes coding for major pathway enzymes in *A. thaliana* from the translated genomes of 12 species from the clade Viridiplantae. Based on predicted domain architecture and localization of the identified proteins from all 13 species, we inspected the conservation of phytohormone pathways. The comparison was complemented by expression analysis of (co-) orthologous genes in *S. lycopersicum*. Altogether, this information allowed the assignment of putative functional equivalents between *A. thaliana* and *S. lycopersicum* but also pointed to some variations between the pathways in eudicots, monocots, mosses, and green algae. These results provide first insights into the conservation of the various phytohormone pathways between the model system *A. thaliana* and crop plants such as tomato. We conclude that orthologue prediction in combination with analysis of functional domain architecture and intracellular localization and expression studies are sufficient tools to transfer information from model plants to other plant species. Our results support the notion that hormone synthesis, transport, and response for most part of the pathways are conserved, and species-specific variations can be found.

**KEYWORDS:** phytohormone biosynthesis, transport, signaling, orthologue search, domain analysis, expression profiling, pathway conservation

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## Introduction

A handful of small chemical compounds and their derivatives, the so-called phytohormones, coordinate the genetically determined growth and development of plants as well as the incessant integration of environmental signals.<sup>1–3</sup> Typically, phytohormones act at low concentrations. Further, at organismic level, the action of these compounds is separated frequently from their biosynthesis in a spatiotemporal manner.<sup>4–6</sup> Thereby, transport over short and long distances in the plant contributes to the distribution of hormones in different tissues, thus forming distinct morphogenetic gradients for each hormone.<sup>7,8</sup> Short- and long-term responses are observed at target cells after perceiving the signal by binding to specific hormone

receptors and integrating it into the cellular metabolism. This signal recognition finally leads to adaptation or differentiation of specific functions during growth and development and ensures maintenance of tissue and organ integrity.<sup>9,10</sup> It is widely accepted that the ratio among the levels of different hormones determine the outcome of their activity and function.<sup>2,11</sup> In part, this is attributed to the mutual effects on hormone-specific synthesis, transportation, signaling, and response pathways.

The role of phytohormones in plant physiology, growth, and development of numerous species has been explored since the late 19th century.<sup>12</sup> However, our current knowledge on the molecular mechanisms underlying the biosynthesis and



distribution of these compounds as well as signaling and cellular responses is still limited to only few model plants.<sup>1,13,14</sup> Most of the information exists from studies on the model system *A. thaliana*.<sup>15–21</sup> Although basic features of pathways involved in hormone biosynthesis and action are considered to be conserved, initial results suggest variations in different plant species. These variations are likely the consequences of optimizing these pathways with respect to differences in plant architecture and interaction with the environment, including both biotic and abiotic factors as well as breeding of highly productive cultivars for agricultural purposes.<sup>22,23</sup>

During the last decade, biochemical and cell biological analyses as well as the availability of information of the whole genome of *A. thaliana*<sup>24</sup> yielded vast information on the molecular pathways for hormone synthesis, transport, and signaling.<sup>25</sup> Generalization of data for the entire plant kingdom is still challenging, as information for other plant species is by far not as advanced as for *A. thaliana*. Thus, it is required to predict putative proteins involved in phytohormone pathways in different plant species by bioinformatics analysis, which can be validated subsequently in further experiments. In addition, it is of great importance to assess to which extent the information from the model plant *A. thaliana* can be transferred to other plants. This will be the base to establish species-specific variations. The identification of all genes contributing to the plant-specific regulatory phytohormone networks is a challenge of the current research. Such knowledge can be a valuable tool for improvement of plant productivity by more targeted species-specific breeding programs. Here, we focus on the pathways of seven phytohormone classes: auxin, ethylene, cytokinin, abscisic acid (ABA), jasmonic acid (JA), gibberellin (GA), and brassinosteroid (BR).

Auxin is a key regulator of many growth processes during plant life cycle and was the first phytohormone detected as a growth-promoting compound involved in the regulation of cell division and elongation, cell differentiation, photo- and gravitropism, apical dominance, flowering, and senescence.<sup>26–30</sup> Indole-3-acetic acid (IAA) was identified as the major naturally occurring auxin in plants.<sup>31</sup> IAA is mainly synthesized in shoot meristems and young tissues. Maintenance of auxin homeostasis requires the continuous transport of IAA conjugates through the entire plant.<sup>32</sup> This is achieved by long-distance transport in the phloem toward the root tip and by local cell-to-cell transport mechanisms over shorter distances forced by chemiosmotic gradients.

Ethylene, which is the simplest alkene (C<sub>2</sub>H<sub>4</sub>), was the first gaseous biological signaling molecule discovered. In 1901, Neljubow<sup>33</sup> reported that ethylene was the active compound in illuminating gas that caused altered growth of pea seedlings.<sup>34</sup> In addition, seed germination, seedling growth, organ development and senescence, leaf and petal abscission, fruit ripening, and stress and pathogen responses are among the many processes governed at least in part by ethylene.<sup>35</sup> The easy-to-score “triple response” phenotype of dark-grown *A. thaliana*

seedlings exposed to ethylene enabled the identification of ethylene-insensitive and constitutive-response mutants.<sup>36</sup> The analysis of these mutants led to the description of a primarily linear model for ethylene signal transduction, which starts with hormone perception and ends in transcriptional regulation.<sup>37,38</sup> Current models, however, suggest the existence of a more complex pathway with both positive and negative regulatory feedback loops by several phosphorylation cascades, feedback-regulated transcriptional networks, and protein and mRNA turnover regulatory modules.<sup>39,40</sup>

Searching for substances promoting cell division in plant tissue cultures led to the discovery of adenine derivatives. Kinetin (6-furfurylaminopurine) was the active compound contained in autoclaved herring sperm DNA,<sup>41</sup> and zeatin was identified as the naturally occurring cytokinin in maize endosperm.<sup>42,43</sup> Besides its proposed activity in cell division, cytokinins are involved in the control of most aspects of plant growth and development, eg, shoot initiation and growth, apical dominance, sink/source relationships, photomorphogenesis, gametophyte development, and leaf senescence.<sup>18,44</sup> Pathways deriving from purine and isopentenyl metabolism in meristems and differentiating young tissues are the major sources of cytokinin biosynthesis in plants.<sup>18,45,46</sup> Transport over short and long distances contribute to the spatial distribution of the hormone within the plant. The signal transduction pathway in cytokinin perception and signaling is reminiscent to bacterial two-component phosphorelays.<sup>47</sup>

ABA was discovered as a growth inhibitor accumulated in abscising cotton fruit, therefore originally named as “abscisin”, and during development of dormancy in sycamore trees, leading to the name “dormin”.<sup>48</sup> ABA has an inhibitory effect on growth processes including cell division and is a counterpart of growth-promoting hormones such as GA, auxin, or cytokinin. It is further involved in the regulation of seasonal dormancy in resting tissues and of drought, salt, and cold stress tolerance mechanisms, providing interesting agricultural aspects.<sup>49–52</sup> This led to the assignment of ABA as a global player in the regulation of growth and developmental processes including storage of proteins as well as biotic stress response.<sup>53,54</sup>

JA and its biosynthetic precursor compounds are lipid derivatives synthesized from  $\alpha$ -linolenic acid, first identified from the oil of *Jasminum grandiflorum*.<sup>55</sup> First described physiological responses to JAMe (JA methyl ester) were growth-inhibiting and senescence-promoting effects.<sup>56,57</sup> Later, altered gene expression and accumulation of jasmonate-induced proteins after external application of JA or its methyl ester have been reported.<sup>58,59</sup> This and the finding of the multiple actions of this compound as an intrinsic and volatile signal molecule in plants in biotic and abiotic stress response and in developmental programs stimulated its intensive exploration during the last decades.<sup>60,61</sup>

More than 130 different GAs have been identified so far.<sup>62</sup> The majority of these compounds are biologically



inactive and represent intermediates of GA biosynthesis or catabolism. Only few, eg, GA1, GA3, GA4, and GA7, are bioactive in plant growth and development.<sup>20,63</sup> Besides stem elongation, GAs are essential for fundamental developmental processes, such as seed germination, trichome development, pollen maturation, and induction of flowering.<sup>64</sup> GA deficiency causes dwarfism and late-flowering phenotypes. Unraveling the molecular basis of GA biosynthesis, action, and signaling pathways over the past decades<sup>64–68</sup> has a major impact on agriculture, with huge improvements in productivity, especially since the introduction of the semidwarf varieties during the “green revolution” in the 1960s.<sup>69</sup>

BRs are the least explored group of phytohormones and originally isolated from *Brassica napus* pollen.<sup>70</sup> Brassinolide (BL) and its immediate precursor castasterone (CS) are the biological active compounds among numerous related phytoosteroids.<sup>71</sup> The essential function of BRs in the control of growth and developmental processes became evident after the identification and characterization of mutants in BR biosynthesis and signaling, which exhibited severe dwarf-like growth phenotypes, altered leaf morphology, reduced male fertility, and de-etiolated phenotypes in dark-grown seedlings.<sup>72</sup> The biosynthetic pathway of BR was described mainly on the base of *A. thaliana* and cell cultures of *Catharanthus roseus*, while major key steps have been reported for other plants, such as rice, pea, and tomato.<sup>73–76</sup> More recent findings provided insights into the complexity of the regulation of BR biosynthesis and homeostasis in a spatiotemporal manner, crosstalk with other hormone signaling pathways and integration of environmental signals.<sup>77–80</sup>

We analyzed the proteins involved in the pathways of these seven phytohormone classes by gathering genome information of 13 different species (Fig. 1), representing green algae, moss, monocots, and eudicots in the clade of Viridiplantae. *Chlamydomonas reinhardtii* represents a unicellular system, and comparison of findings for the green algae and for the other organisms studied allowed the identification of the components required only for multicellular systems. At first, we extracted information from literature for the biosynthetic pathways, transport mechanisms, and sensing systems in *A. thaliana* for the individual hormone classes. Next, we employed bioinformatic tools to identify orthologous genes in the selected plant species based on the information available for *A. thaliana*. From this groundwork, co-orthologues of the proteins involved in the various processes leading to hormone response were assigned, and the conservation of the pathways with respect to the knowledge governed from *A. thaliana* was evaluated by domain comparison and prediction of the intracellular localization of the putative identified proteins. Finally, we described these pathways in tomato in more detail by evaluating the expression profiles of the (co-)orthologous genes in different tissues based on publically available datasets. The results are discussed concerning evolution and functional equivalence of phytohormone pathways in plants. Extending

our approach by analysis of expression patterns in different tissues of tomato gives insights in preferentially used pathways for the biosynthesis of phytohormones in this important crop plant.

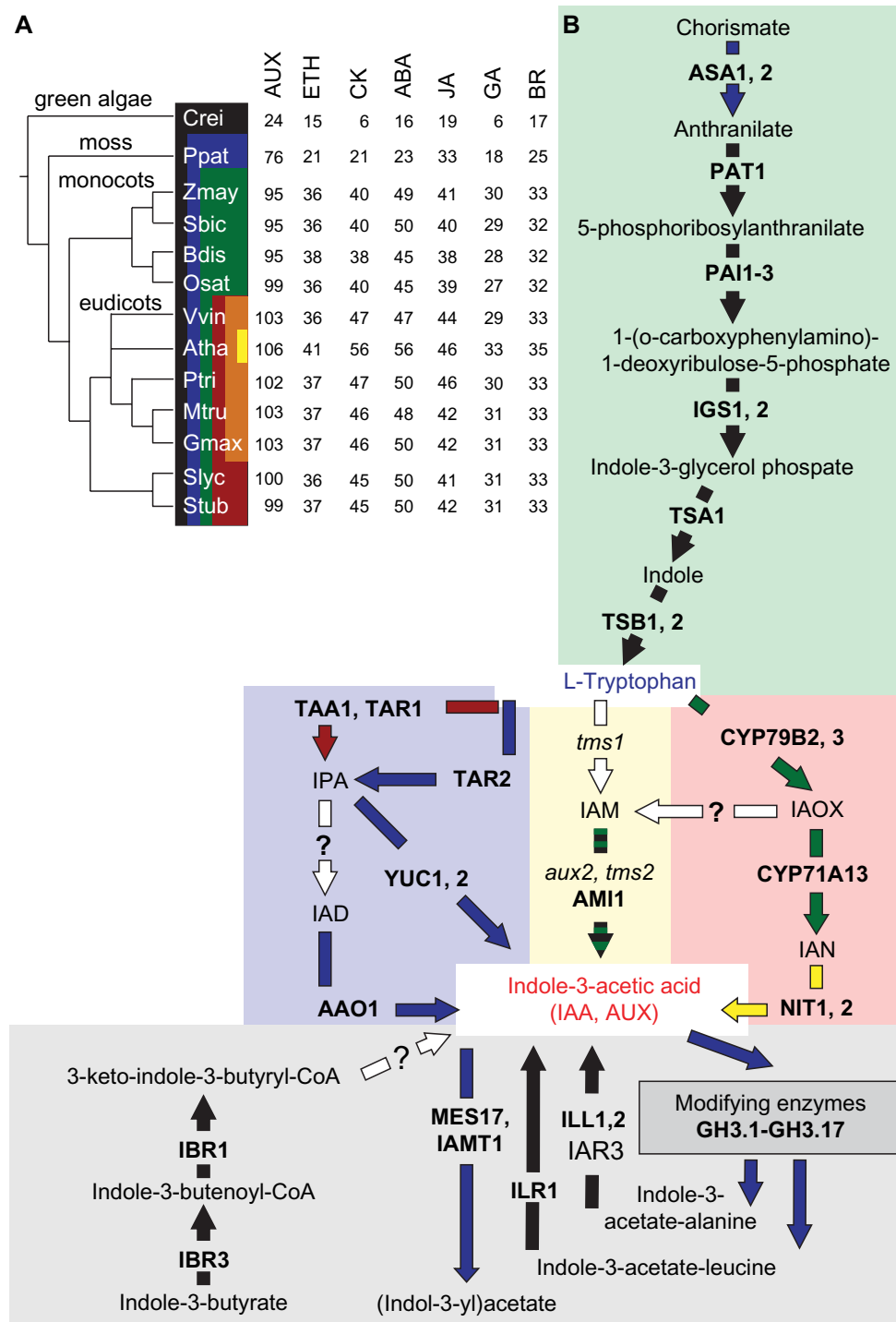
## Methods

### Curation of phytohormone pathway information.

Genes involved in biosynthesis, transport, and signaling pathways of seven phytohormone classes for the reference model organism *A. thaliana* were extracted from the Arabidopsis Hormone Database version 2.0 (AHD2.0; <http://ahd.cbi.pku.edu.cn/25>). Cross-evaluation from the AHD2.0 was performed to enzymes belonging to biosynthetic pathways extracted from the AraCyc (part of Plant Metabolic Pathways; <http://pathway.gamene.org/gamene/aracyc.shtml81>). Next, putative candidates and complex enzyme schematic diagrams of the AHD2.0 were verified by an extensive literature screening for the seven different phytohormone classes in book chapters and reviews from the last 10 years. For enzymes assigned to be putatively involved in chemical reactions of the phytohormone pathways in AraCyc and/or AHD2.0, we searched for experimental evidence as well. *A. thaliana* gene identifier (AGI) and associated description was curated based on Gene Ontology (GO) information deposited in The Arabidopsis Information Resource database (TAIR10; <http://www.arabidopsis.org/82>). The identified genes were assigned to the categories biosynthesis, transport, or signaling.

### Orthologue search and functional domain annotation.

The plant genomes of (i) *A. thaliana* (TAIR10; <https://www.arabidopsis.org/>), (ii) *Brachypodium distachyon* (bradi1.2 with GAEVAL; <http://www.plantgdb.org>), (iii) *C. reinhardtii* (JGI v4 with GAEVAL; <http://www.plantgdb.org>), (iv) *Glycine max* (Glyma1; <http://www.plantgdb.org>), (v) *Medicago truncatula* (Mt3.5v5 <http://jcv.org>), (vi) *Oryza sativa* (MSU Version 7.0 with GAEVAL; <http://www.plantgdb.org>), (vii) *Physcomitrella patens* (Phypa1.6; <http://phytozome.net>), (viii) *Populus trichocarpa* (Ptr v2.0 with GAEVAL; <http://www.plantgdb.org>), (ix) *Solanum lycopersicum* (ITAG2.4; [https://solgenomics.net/organism/Solanum\\_lycopersicum/genome](https://solgenomics.net/organism/Solanum_lycopersicum/genome)), (x) *Solanum tuberosum* (PGSC v3.4; <http://potatogenome.net>), (xi) *Sorghum bicolor* (JGI Sbi1; <http://www.plantgdb.org>), (xii) *Vitis vinifera* (Genoscope 12X; <http://genoscope.cns.fr>), and (xiii) *Zea mays* (B73 RefGen v2; <http://www.plantgdb.org>), which have been downloaded from PlantGDB,<sup>83</sup> have verified gene annotation in relation to alternative splicing and gene fusions/fissions by the gene annotation evaluation algorithm (GAEVAL 82).<sup>84</sup> For the orthologue search via OrthoMCL<sup>85</sup> and InParanoid,<sup>86</sup> we selected these plant species to sample the tree of the super kingdom Viridiplantae by at least one species of the phylum Chlorophyta (*C. reinhardtii*), one species of the class Bryopsida (*P. patens*), and four species of the branch of monocots (*S. bicolor*, *B. distachyon*, *O. sativa*, and *Z. mays*) of which *O. sativa* and *Z. mays* are of large agricultural relevance. Besides economic importance, the seven eudicot species (*A. thaliana*,



**Figure 1.** Biosynthesis pathway of auxin in selected species. **(A)** The phylogenetic relation of the selected 13 members of Viridiplantae. Species: *Atha* (*A. thaliana*), *Gmax* (*G. max*), *Mtru* (*M. truncatula*), *Ptri* (*P. trichocarpa*), *Stub* (*S. tuberosum*), *Slyc* (*S. lycopersicum*), *Vvin* (*V. vinifera*), *Bdis* (*B. distachyon*), *Osat* (*O. sativa*), *Sbic* (*S. bicolor*), *Zmay* (*Z. mays*), *Ppat* (*P. patens*), and *Crei* (*C. reinhardtii*). The color code indicates the distribution of the different clades demonstrating the distance from *A. thaliana*. The number of genes related to all analyzed phytohormone biosynthesis pathways in *A. thaliana* and of the identified co-orthologues in the other species are indicated. **(B)** Enzymes and intermediate products of the plastidial tryptophan biosynthesis pathway (green region) and distinct routes of Trp-dependent IAA biosynthesis in plants with IPA (blue region), IAM (yellow region), and TAM, IAOX and IAN (red region) as major biosynthesis intermediates. The route using IBA as precursor or storage intermediate and pathways of IAA conjugation are shown in the gray region. The arrows are colored according to the species in which the enzymes were found (taken from A) and multicolor dashed arrows indicate putative loss of co-orthologues genes in one clade during evolution.

**Abbreviations:** Proteins: AAO, *Arabidopsis aldehyde oxidase*; AMI, amidase; ASA, anthranilate synthase alpha subunit; CYP79B, cytochrome P450, family 79, subfamily B; GH, Gretchen Hagen; IBR, indole-3-butyric acid response; ILR1/ILL, IAA-leucine resistant; IAMT, IAA carboxylmethyltransferase; NIT, nitrilase; PAT, phosphoribosylanthranilate transferase; PAI, phosphoribosylanthranilate isomerase; IGS, indol-3-glycerol phosphate synthase 2; TSA, tryptophan synthase alpha chain; TSB, tryptophan synthase beta chain; TAA1, tryptophan aminotransferase of *Arabidopsis*; TAR, tryptophan aminotransferase related; YUC, YUCCA. Metabolites: IPA, indole-3-pyruvic acid; IAD, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAOX, indole-3-acetaldoxime; IAN, indole-3-acetonitrile.



*G. max*, *M. truncatula*, *P. trichocarpa*, *S. tuberosum*, *S. lycopersicum*, and *V. vinifera*) were chosen as representative model organisms of distinct plant families or with broad interest in specific plant research fields (Fig. 1A).<sup>87,88</sup> *G. max* was chosen due to agricultural relevance and *S. tuberosum* and *V. vinifera* were chosen due to their phylogenetic relation to *S. lycopersicum* and comparison with previous studies.<sup>89</sup>

The strategy to merge results of different orthologue algorithms has been justified in Paul et al.<sup>87</sup> OrthoMCL was used to determine cliques of likely orthologous genes (CLOGs) for more than two species, and results were verified using the pairwise approach of InParanoid version 8.<sup>86</sup> Genes were further analyzed when occurring in at least one of the two approaches. OrthoMCL filtered out poor-quality sequences based on the protein sequence length (<10 amino acids) and percentage of stop codons (marked by asterisks in the amino acid sequence; >20%). We searched in all CLOGs for all extracted genes of *A. thaliana* coding for enzymes involved in biosynthesis, transport, and signaling pathways of the seven phytohormone classes (373 genes based on our curation of phytohormone pathway information; Supplementary Tables 1–14).

**Prediction of domain architecture.** Domain prediction of proteins of all 13 plant species was performed using Pfam database (version 26.0),<sup>90</sup> WebMGA,<sup>91</sup> and HMMsearch.<sup>92</sup> Customized Python scripts (www.python.org) were used to analyze domain architecture of the protein and conservation of domains of the co-orthologues in a particular orthologous group. Within one orthologous group, the domain architecture of the *A. thaliana* reference protein sequence was compared to the other co-orthologues concerning the order of the domains, the absence of domains, or the occurrence of new ones. The name of the PFAM domain is indicated when discussed, and the detailed description of the individual domains is available in the PFAM database (<http://pfam.sanger.ac.uk/>).

**Prediction of protein localization.** The prediction of the localization of identified proteins was performed by MultiLoc2, TargetP, and Predotar, which enable automated prediction by submitting  $\geq 2$  sequences at once or using local versions of the software. The predictor MultiLoc2<sup>93</sup> distinguishes between 11 different cellular compartments (extracellular, nucleus, Golgi, endoplasmic reticulum (ER), mitochondrion, plastid, plasma membrane, peroxisome, vacuole, cytosol, and cytoskeleton). The localization results of MultiLoc2 for vacuole, ER, Golgi, and plasma membrane are merged and represented as endomembranes. TargetP<sup>94</sup> and Predotar<sup>95</sup> are widely used programs and can distinguish between chloroplast, mitochondria, and secretory pathway localization and are used for a more accurate prediction of the localization of proteins in these plant compartments.

**Transcriptome profiling.** Existing *S. lycopersicum* transcriptome expression data (RNA-seq libraries; GSE33507)<sup>89</sup> were obtained from the gene expression omnibus (GEO).<sup>96</sup> The RNA-seq data contain *S. lycopersicum* (cv. MoneyMaker) libraries from different organs: root, stem, leaf,

flower, and fruits at three developmental stages (mature green, breaker stage, and 10 days after turning red).

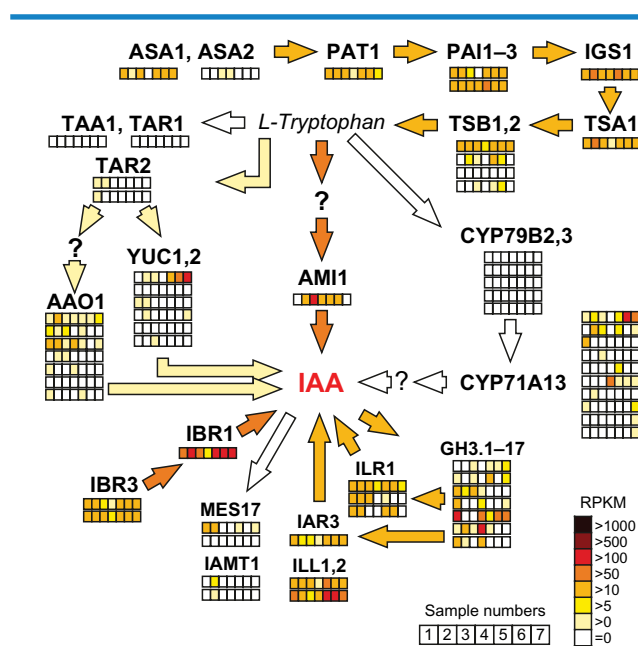
## Results and Discussion

**General procedure for information extraction.** We extracted the information for the phytohormone pathways established for *A. thaliana* (“Methods” section) and assigned co-orthologues to the proteins involved in these pathways in 13 photosynthetic eukaryotes including Arabidopsis (Fig. 1A, Supplementary Tables 1–7). In contrast to other orthologue studies in plants such as the study by Wu et al.<sup>97</sup>, our approach was not based on a single plant clade like Euasterid but attempt coverage of the whole Viridiplantae super kingdom by using model organisms from algae, mosses, monocots, and eudicots. The orthologue search was based on proteomes, and by this, the referenced tools OrthoMCL and InParanoid allowed detection of co-orthologues and putative paralogs without being restricted to single-copy orthologous genes.<sup>97</sup> The approach in our study allowed the detection of CLOGs for phytohormone biosynthesis, transport, and signaling. Identified co-orthologues and co-orthologues (multiple co-orthologues of the same species)<sup>98</sup> in one CLOG were analyzed for the presence of conserved functional domains found in the *A. thaliana* bait as it was performed in earlier studies.<sup>88,99</sup> Co-orthologues containing all functional domains were assumed to be functionally related; however, we also considered proteins with additional or missing domains as relevant co-orthologues due to domain “stealing, swapping, or swiveling”.<sup>100</sup> Additionally, putative targeting signals for intracellular localization were predicted to verify that the proteins within a CLOG share enzyme functionality and similar intracellular localization of the *A. thaliana* bait. This limited the number of co-orthologues with putative function in phytohormone pathways, because most pathways or parts of them are defined to specific organelles (Supplementary Tables 8–14). Focusing on tomato, the expression patterns of co-orthologues in the phytohormone pathways in tissues and fruit developmental stages gave hints for rate-limiting steps and tissue-specific expression. Publically available expression values in different tissues and fruit developmental stages based on “reads per kilobase per million mapped reads” (RPKM) normalized datasets were extracted from the GEO<sup>96</sup> and Sol Genomics Network (SGN; Supplementary Tables 15–21).<sup>101</sup> The expression data of co-orthologues in CLOGs were categorized as low expression (RPKM < 5), moderate expression (RPKM < 100), and high expression (RPKM  $\geq 100$ ). All information are deposited in a database,<sup>102</sup> focusing on the assignment of the recently published tomato genome.<sup>89</sup>

**The indole-3-pyruvic acid pathway is a major, but not the only, route of auxin biosynthesis in tomato.** Two major pathways of IAA biosynthesis have been proposed so far.<sup>26</sup> The *tryptophan (Trp)-independent pathway* derives from the plastidial Trp-biosynthesis route, which is initiated with the conversion of chorismate to anthranilate (Fig. 1B, green

region). This pathway consumes immediate precursors of Trp, ie, indole-3-glycerol phosphate or indole for the production of IAA. However, the molecular identity of the required enzyme remains unknown up to now. The *Trp*-dependent *IAA* synthesis unifies several routes of IAA biosynthesis deriving from Trp. These routes vary in the formation of distinct intermediates leading to the names of the particular pathways, ie, the indole-3-pyruvic acid (IPA) pathway (Fig. 1B, blue area), the indoleacetamine (IAM) pathway (Fig. 1B, yellow area), and the indole-3-acetaldoxime (IAOX) pathway (Fig. 1B, red area). The high number of intermediates and identified genes proposed to be involved in their enzymatic processing (Supplementary Tables 1 and 8) provides a high complexity to auxin biosynthesis concerning different possible biosynthetic pathways, which is still under debate.<sup>26</sup>

The IPA pathway includes two enzymatic reactions to convert Trp to IAA.<sup>21,103,104</sup> Either TAA1/TAR1 or TAR2 catalyzes the first reaction. While we could observe TAR2 co-orthologues in all plants except for green algae, TAA1/TAR1 was specific for eudicots (Fig. 1B). Remarkably, tomato co-orthologues to TAA1/TAR1 were not expressed in any of the analyzed tissues (Fig. 2). The second is catalyzed by



**Figure 2.** Expression of tomato genes encoding Trp and IAA biosynthesis enzymes. Major routes and enzyme activities involved in auxin biosynthesis are represented as in Figure 1B with focus on the expression of corresponding co-orthologues, genes identified in the tomato genome. Relative transcript levels in different tissues and developmental stages are shown in the color code as indicated in the right lower corner.

**Notes:** Analyzed samples in each row from left to right are: 1, root; 2, stem; 3, leaf; 4, flower; 5, fruit – mature green; 6, fruit – breaker stage; 7, fruit – ripening 10 days after turning red. Number and order of the rows at indicated co-orthologues corresponds to the presentation of expression data in Supplementary Table 15. Genes coding for enzyme activities not yet identified in tomato are indicated by question mark. Color intensities of the arrows from light to dark orange indicate the general pathway expression at low, moderate, and high levels, respectively.

YUC1 and YUC2, two proteins encoded by the YUCCA gene family of flavin monooxygenases. Co-orthologues of all these genes were found in all species with the exception of *C. reinhardtii* (Fig. 1B, Supplementary Tables 1, 8, and 15). Again, the expression of tomato YUC1, 2 co-orthologues, was low in most of the tissues (RPKM < 5) compared to other genes of the synthesis pathway (Fig. 2). This might point to conversion of IPA to indole-3-acetaldehyde (IAD) by an indole-3-carboxylase, an enzymatic activity described for IAA synthesis in plant growth-promoting rhizobacteria species, which has not been identified in plants yet. Co-orthologues of AAO1, the proposed aldehyde oxidase activity required for the subsequent conversion of IAD to IAA, were detected by our analysis in all plants, and their moderate expression in tomato exceeded that of YUC co-orthologues (RPKM > 5; Fig. 2). Nevertheless, it needs to be mentioned that broad substrate specificity was observed for the AAO1 multigene family that might link its activity to ABA synthesis as well, which is still discussed.<sup>105,106</sup>

The IAM pathway also predicts two steps for the conversion of Trp to IAA with IAM as an intermediate product (Fig. 1B). The pathway resembles the conversion of Trp to IAA found in *Agrobacterium* strains.<sup>107</sup> In our study, only co-orthologues of AMI1, the enzyme that catalyzes the second step,<sup>108,109</sup> were identified in all plants except for *P. patens*. AMI co-orthologues were highly expressed in tomato leaves compared to other organs (Fig. 2). In contrast, proteins similar to the bacterial proteins encoded by *aux1/iaaM/tms1* genes were not identified. Recently, the conversion of IAOX to IAM was suggested as an alternative route to generate IAM.<sup>110</sup>

The activity of YUCCA enzymes is assigned to the IAOX pathway for converting tryptamine (TAM) into IAOX (Fig. 1B). However, we detected neither tomato co-orthologues to *A. thaliana* NIT1, 2 enzymes converting tryptophan to TAM nor to enzymes converting indole-3-acetonitril (IAN) to IAA (Fig. 1B). This observation stands in line with discussion that the IAOX pathway is present in Brassicaceae only.<sup>111</sup> Furthermore, the identified co-orthologues of the cytochrome P450 oxidases CYP79B2/B3 involved in IAOX production in *A. thaliana*<sup>110</sup> were also not expressed in the examined tissues in tomato (Fig. 2, Supplementary Table 15). This supports the current model that the IPA pathway is the major route of auxin biosynthesis in tomato. Nevertheless, we cannot exclude that several Trp-dependent auxin biosynthesis pathways may coexist and operate in different tissues.<sup>103</sup>

**IAA conjugation, storage, and degradation is conserved among species.** The mechanism of stimulation of adventitious root formation by indol-3-butyric acid (IBA) is well established. Further, IBA is a naturally occurring IAA precursor in many plant species, which requires a peroxisomal  $\beta$ -oxidation activity and the IBA synthase<sup>112</sup> for its conversion to the biologically active IAA (Fig. 1B, gray region). The compound IBA appears as reversible auxin storage form, which is transported independent of IAA.<sup>113</sup> In tomato, the orthologue to IBR1 and



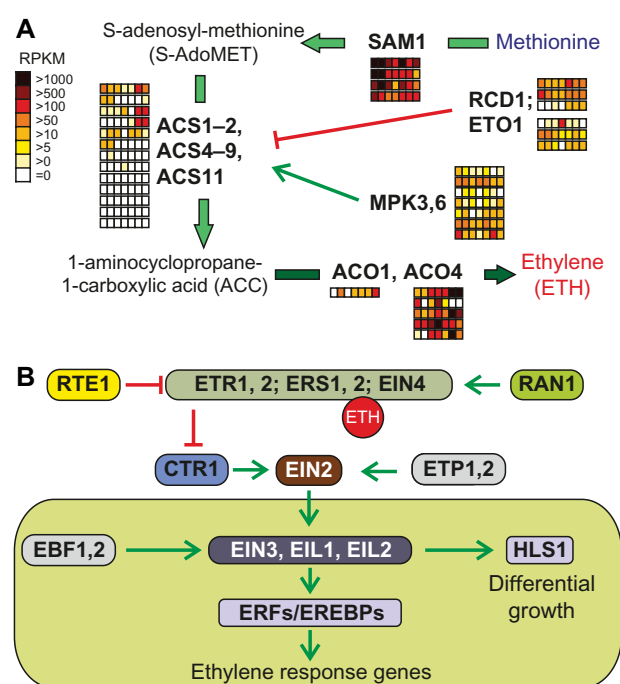
consisting of P-glycoproteins of the ABCB transporter family (ABCB/PGP). While most PIN proteins are plasma membrane proteins, PIN5, PIN8, and PIN-LIKE proteins are localized at the ER membrane and regulate the intracellular distribution of IAA.<sup>116</sup> Consequently, in our analysis, PIN5 and PIN8 were grouped into two distinct CLOGs containing none of the other PIN genes (PIN1–4, PIN6–7). Further, co-orthologues of PIN5 and PIN8 were found only in monocots and eudicots and tended to occur as single-copy genes (Fig. 3A, Supplementary Tables 1 and 8). With respect to their function in intracellular transport, co-orthologues to all other PINs and NRT1.1 existed in all plants, but not in *C. reinhardtii*, and the number of co-orthologues varied between 3 and 14 (Fig. 3B).

**Auxin perception is tightly linked to the regulation of auxin-responsive gene.** Two classes of interacting transcription factors are involved in the control of auxin-regulated gene expression (Fig. 3C<sup>115–117</sup>). AUX/IAA transcriptional repressors were found to be present in all monocots and eudicots and were represented by a single CLOG (Supplementary Tables 1 and 8) with varying numbers of co-orthologues ranging from 5 in tomato to 15 in *A. thaliana*. Remarkably, one tomato orthologue was found to be highly expressed only in fruits (Solyc09g065850), while all others were not expressed in this tissue (Supplementary Table 15). AUX/IAAs generally consist of four functional domains. The “N-terminal domain I” harbors an ethylene response factor (ERF)-associated amphiphilic repressor (EAR) motif required for recruitment of TOPLESS (TPL), which is acting as a transcriptional corepressor in the absence of auxin. Interestingly, co-orthologues to TPL were identified in all analyzed plant genomes except in *C. reinhardtii*. For *P. patens*, we could identify two TPL co-orthologues but no co-orthologues to AUX/IAA (Supplementary Table 1). Domain II of AUX/IAA proteins is required for the control of their auxin-stimulated degradation, and domains III and IV participate in the formation of homo- and hetero-oligomeric complexes including interactions with members of the second class of transcription regulators, the auxin-responsive factors (ARFs). ARFs recognize and bind through their B3 DNA-binding domain-specific auxin-response elements and act as either transcriptional repressors or activators.<sup>118</sup> In *A. thaliana*, the ARF subfamily consists of 15 members while we identified only six ARF co-orthologues in the genome of tomato (Supplementary Table 15).

In the presence of auxin, the activation of ARFs is controlled by degradation of AUX/IAAs and release of TPL-mediated repression of DNA-binding and transcriptional activation (Fig. 3C). Degradation of AUX/IAAs is triggered by binding of auxin to one of the F-box proteins TIR1/AFB1–5, the substrate recognition subunit in the SCF<sup>TIR1/AFB</sup> E3 ubiquitin ligase complex, which per se is acting as an intracellular auxin receptor in plants. The stability of the E3 complex is regulated by modification of the scaffold subunit Cullin1 (CUL1) through covalent binding of the ubiquitin-related small protein

RUB1. Our results suggest that the regulatory regime of auxin signaling can be transferred to all phototrophic eukaryotes containing co-orthologues with the exception for *C. reinhardtii* (Supplementary Tables 1 and 8) in which, if present, auxin signaling must be differently perceived. Although all components of the basic SCF complex were also present in the green algae co-orthologues of the TIR/AFB, F-Box proteins were not present.

**ACC synthases form a diverse gene family within the ethylene pathway.** The biosynthesis pathway of ethylene consists of only three enzymes (Fig. 4). *S*-adenosylmethionine is synthesized from methionine by *S*-adenosyl-L-methionine synthetase (SAM1) and is converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). Our orthologue search shows that each selected plant species contains at least one orthologue for SAM1. More interestingly, the *A. thaliana* genome encodes 12 ACS-like genes, which could



**Figure 4.** Pathways of ethylene synthesis and signaling. (A) Enzymes and intermediate products of the ethylene production unify to methionine (blue) as precursor for ethylene (red) synthesis. The enzymatic step catalyzed by ACS is inhibited by the action of RCD1 and ETO1 or activated by MPK3 and MPK6. Further details are presented in the text. The arrows are colored according to the species in which the enzymes were found (Fig. 1A). Expression of the identified genes in tomato is shown as explained in Figure 2. (B) The components involved in ethylene signaling are represented as interaction scheme, at which activation of downstream components is indicated by green arrows and inhibition by red bar-headed lines.

**Abbreviations:** Proteins: SAM, *S*-adenosylmethionine synthetase; ACS, ACC synthase; RCD, radical-induced cell death; ETO, ethylene overproducer; MPK, mitogen-activated protein kinase; ACO, ACC oxidase; ETR/ERS, ethylene response; EIN, ethylene-insensitive; RAN, RAS-related nuclear protein; RTE, reversion-to-ethylene sensitivity; CTR, constitutive triple response; EDR, enhanced disease resistance; EIN, ethylene-insensitive; ETP, EIN2 targeting protein; EIL, ethylene-insensitive 3-like; EBF, EIN3-binding F-box protein; HLS, HOOKLESS.



be divided into three CLOGs. *ACS3* is an *A. thaliana*-specific pseudogene with a short protein sequence, whereas *ACS10* and *ACS12* belonged to the same CLOG (Supplementary Table 2), and it has been shown that these aminotransferases can complement the *Escherichia coli* aminotransferase mutant *DL39*.<sup>119</sup> Thus, there are nine authentic ACS genes in the *A. thaliana* genome,<sup>120</sup> which were grouped in one CLOG containing co-orthologues from green algae, monocots, and eudicots but without co-orthologues from the moss *P. patens*. In tomato, there are at least eight characterized ACS genes,<sup>121</sup> but we could assign 13 co-orthologues to the nine *A. thaliana* ACS genes involved in ethylene synthesis (Supplementary Table 16). In the last step of ethylene biosynthesis, ACC oxidase (ACO, four genes in *A. thaliana*) converts ACC to ethylene. Remarkably, ACO2 and ACO3 build their own CLOG without other plant co-orthologues and ACO1 and ACO4 further fell in different CLOGs. Overall, our analysis revealed six genes in the tomato genome in the three CLOGs of ACOs, which has been shown in earlier results.<sup>121</sup> Coherent with the importance of ethylene for fruit development, the high expression of two genes (RPKM > 1000; Solyc01g095080, Solyc05g050010) was observed during ripening (Fig. 4<sup>121</sup>). The current model considers the formation of ACC as the rate-limiting step. SAM1 and EFE/ACO co-orthologues were highly expressed in all tissues in tomato, while the ACS co-orthologues showed only moderate expression (Fig. 4; Supplementary Table 16). However, the activity of ACS is regulated by phosphorylation at protein level as it was documented for SIACS2 (Solyc01g095080).<sup>122</sup>

The ethylene signaling cascade starts with binding of ethylene to ER-localized receptors with protein kinase activities.<sup>50,123,124</sup> In all monocots, eudicots, and mosses, ethylene receptors represent a gene family that is composed of ETR1, ERS1, ETR2, ERS2, and EIN4 in *A. thaliana* (Supplementary Tables 2 and 9). Our analysis revealed the presence of seven co-orthologues in *S. lycopersicum*, from which five have been previously described (SIETR1, -2, -4, -5, and Never ripe), while two have not been yet tested in tomato (SIETR6 and -7<sup>125</sup>; Supplementary Table 16).

In contrast to the orthologue search, previous sequence analysis divided ethylene receptors into subfamily I and II. Subfamily I members tend to have higher similarity to histidine kinases, whereas subfamily II members have acquired serine kinase activities.<sup>126</sup> The ethylene signaling in *A. thaliana* is more dependent on subfamily I members, which cannot be functionally replaced by subfamily II members.<sup>127</sup> In turn, reduced expression of the subfamily II receptor genes, *SIETR4* or *SIETR6*, in tomato results in substantially increased ethylene sensitivity.<sup>124</sup> This phenotype cannot be restored to that of wild-type by overexpression of the subfamily I receptor. Thus, the subfamily II receptors might have a more important function in tomato than in *A. thaliana*.

In the absence of ethylene, the receptors activate the negative regulator CTR1 (constitutive triple response1), thus

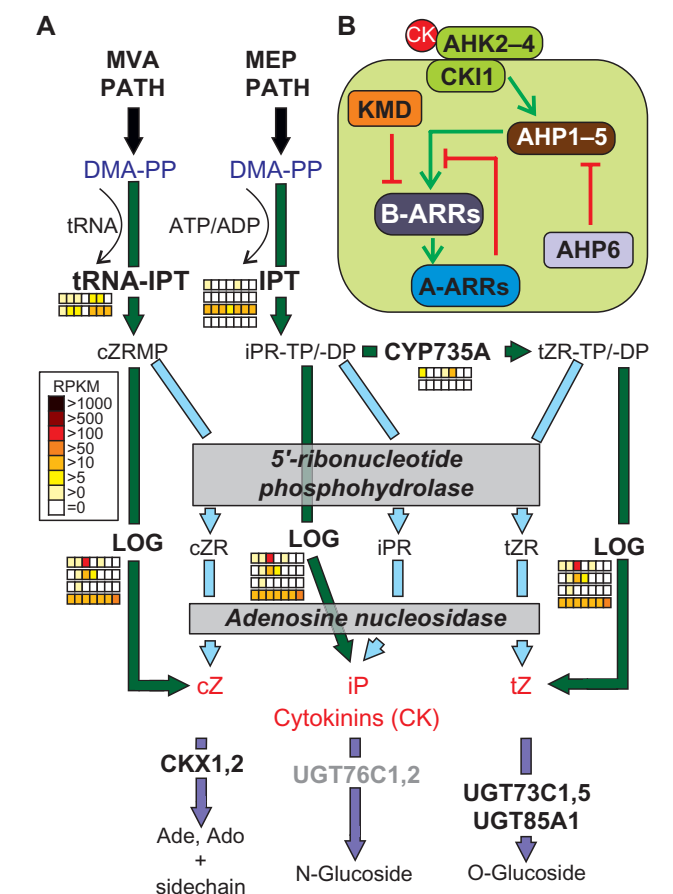
repressing the activity of downstream ethylene signaling components.<sup>39</sup> In *A. thaliana*, the Ser/Thr protein kinase CTR1 is localized at the ER membrane due to its physical interaction with the receptors<sup>128</sup> and directly phosphorylates the C-terminal domain of EIN2 in the absence of the ethylene signal. Our orthologue search detected co-orthologues of the two *A. thaliana* CTR1 genes only in monocots and eudicots, while in line with former studies, four co-orthologues were found in tomato (Supplementary Table 16<sup>124</sup>).

The physical movement of EIN2 C-terminal region from the ER membrane to the nucleus allows the ethylene signal to activate the downstream transcription factors EIN3 and EIN3-like (EILs) in the nucleus. Phosphorylation at specific sites in EIN2 region regulates translocation.<sup>39</sup> In tomato, we identified six co-orthologues to these transcription factors (EIN4/EIL1, four co-orthologues; EIL3, two co-orthologues; Supplementary Tables 2 and 9). The ethylene signal stabilizes EIN3 and EIL transcription factors, which are short-lived proteins in the absence of ethylene, and consequently activates ERFs, inducing various physiological responses.<sup>129</sup>

Recent advances propose a more complex route that includes feedback-regulated transcriptional networks as well as protein and mRNA turnover regulatory modules. According to this regime, ETP1 and ETP2 control EIN2 levels, whereas EBF1 and EBF2 (EIN3-binding factor) regulate the levels of EIN3 in response to the ethylene signal.<sup>39</sup> Interestingly, the orthologous group including ETP1 and ETP2 contains 134 additional co-orthologues of *A. thaliana*, most of them containing both or at least one F-Box and F-Box associated (FBA\_1) domains (Supplementary Table 9). Remarkably, only one representative of *B. distachyon* and *S. tuberosum* was detected in the same CLOG, which could be due to the fact that these F-Box proteins evolve very fast or they are species-specific in their primary sequence. Four orthologues were identified for EBF1 and EBF2 in tomato and at least three of them showed moderate or high expression in all tissues (Supplementary Tables 2 and 16). However, *A. thaliana* ETP1 and ETP2 were grouped in one CLOG containing 134 additional putative co-orthologues but only one orthologue of *B. distachyon* and *S. tuberosum* each. With respect to the high number of co-orthologues only in Arabidopsis and the low conservation within the super kingdom of Viridiplantae, the functional relation of all the 136 co-orthologues in the ethylene signaling pathway is unlikely.

**Cytokinin biosynthesis seems to be specific for monocots and eudicots.** Enzymes encoded by isopentenyltransferase (IPT) genes catalyze the initial step of cytokinin biosynthesis. Earlier work based on sequence alignments by ClustalW<sup>130</sup> proposed that IPTs likely evolved in plants, although similar sequences exist in some cyanobacteria.<sup>45</sup> In contrast, our orthologue search approach (see “Methods” section) confirmed the occurrence of IPT genes in all monocots and eudicots, but not in mosses or algae. The only exception was tRNA-IPT (IPT9), which was detected in all selected

plant species. Nevertheless, functional equivalent enzymes for IPT in species like green algae are expected, because cytokinin has been detected in the green alga *Chara globularis* and putative cytokinin receptors have been found in different green algae.<sup>131,132</sup> We identified six co-orthologues in tomato for the nine *A. thaliana* IPTs, from which only one showed high expression in all tissues (Supplementary Table 17).



**Figure 5.** Pathways of cytokinin synthesis and signaling. **(A)** The precursor of cytokinin synthesis is DMA-PP, which is derived from the MVA or MEP pathways. The intermediate products cZRMP, iPRTP/-DP, or tZRTP/-DP are converted to the final products such as cZ, iP, or tZ (red) by the action of LOG or by yet not clearly defined 5'-ribonucleotide phosphohydrolases and adenosine nucleosidases. For further details, see text. The arrows are colored according to the species in which the enzymes were found (Fig. 1A). Expression of the identified co-orthologues in tomato is shown as explained in Figure 2. Genes coding for enzyme activities not expressed by any orthologue are colored in gray. **(B)** The components involved in cytokinin signaling are presented as interaction scheme.

**Abbreviations:** Proteins: IPT, isopentenyltransferase; CYP735A, cytochrome P450, family 735, subfamily A; LOG, LONELY GUY; UGT73C, UDP-glucosyl transferase 73C; UGT85A, UDP-glucosyl transferase 85A; CKX, cytokinin oxidase/dehydrogenase; AHK, Arabidopsis histidine kinase; CKI, cytokinin-independent; AHP, histidine-containing phosphotransmitter; ARR2, response regulator; KMD, KISS ME DEADLY. Metabolites: ATP, adenosine triphosphate; ADP, adenosine diphosphate; DMA-PP, dimethylallyl pyrophosphate; cZRMP, cZR 5' monophosphate; iPRTP, iPR 5' triphosphate; tZRTP, tZR 5' triphosphate; cZR, cis-zeatin riboside; iPR, isopentenylribose; tZR, trans-zeatin riboside; cZ, cis-zeatin; iP, isopentenyladenine; tZ, trans-zeatin.

IPT utilizes adenosine tri- or diphosphate (ATP/ADP) and dimethylallyl-diphosphate (DAMPP), the reactive isomer of isopentenyl diphosphate, to synthesize N<sup>6</sup>-( $\Delta^2$ -isopentenyl) adenosine-5'-triphosphate and -diphosphate ribonucleotides iPRTP and iPRDP, respectively (Fig. 5A<sup>133</sup>). Both compounds are converted subsequently into the corresponding trans-zeatin ribonucleotides (tZRTP/tZRDP) by cytokinin trans-hydroxylases, such as the cytochrome P450-dependent monooxygenases CYP735A1 and CYP735A2. Both enzymes were identified in *A. thaliana* and found to be conserved in monocots and eudicots. In tomato, two co-orthologues were detected in one CLOG, which showed different expression patterns in tissues and developmental stages (Supplementary Table 17). Only CYP735A1 showed moderate expression in root, flower, and mature fruit, whereas CYP735A2 was not expressed in any of these tissues. The formation of cytokinin ribonucleosides and the free bases iP and tZ has been originally assumed to be catalyzed by 5' ribonucleotide phosphohydrolases and adenosine nucleosidases.<sup>134,135</sup> However, the identification of the LONELY GUY (LOG) family of enzymes in rice and *A. thaliana* suggests the direct conversion into the bioactive forms of the two cytokinins. Interestingly, one CLOG containing LOG1, LOG3, and LOG4 was found to be conserved in all plant species but the remaining six *A. thaliana* LOG genes were clustered in different CLOGs and could be divided into Arabidopsis specific (LOG2, LOG6, and LOG9), eudicot specific (LOG7), or monocot/eudicot specific (LOG5 and LOG8). In summary, we identified four co-orthologues of the LOG family in tomato (Supplementary Table 10). Remarkably, almost all co-orthologues of IPT, CYP735A, and LOG showed low expression in fruit ripening stages (Supplementary Table 17), which on the one hand suggests that cytokinins are not produced in fruits at high levels, but on the other hand, studies on the metabolite cytokinin showed that it has an unclear influence on fruit development.<sup>136</sup>

Although found in substantial amounts, cis-zeatin (cZ) has no biological hormone activity and cannot be converted sufficiently into trans-zeatin (tZ).<sup>137</sup> Moreover, the synthesis of cZ seems to be completely independent of the tZ synthesis pathway because two of the nine IPT genes in *A. thaliana* (IPT2 and IPT9) are specifically involved in the formation of cZRMP by utilizing AMP and DMA-PP.<sup>45</sup> We were able to assign one specific tomato orthologue for both enzymes (IPT2: Solyc11g066960; IPT9: Solyc12g014190), suggesting that this pathway is also present in tomato. cZRMP is mainly derived from the cytosolic mevalonate (MVA) pathway of IPP synthesis, while the tZ pathway preferentially consumes IPP derived from the plastidial methylerythritol phosphate (MEP) pathway.

Conjugation to glucose and irreversible cleavage are the two major reactions efficiently reducing the levels of bioactive cytokinins (Fig. 5A<sup>18</sup>). N-glycosyltransferases catalyze the glycosylation of nitrogen residues in the purine ring, while O-glycosylation occurs on the oxygen in the side

chain of tZ or dihydrozeatin (dhZ). The latter reaction likely produces storage forms that can be rapidly reactivated by  $\beta$ -glucosidases.<sup>138</sup> Removal of the unsaturated side chains of tZ and iP by the activity of cytokinin oxidases (CKX) leads to their irreversible inactivation and is important for the regulation of active cytokinin levels and limitation of cytokinin effects. The members of both IPT and CKX gene families show remarkably distinct expression patterns in *A. thaliana* and expression of several CKX genes can be stimulated upon cytokinin application.<sup>139</sup> For tomato, only one orthologue of IPT5 and another of IPT9 were found to be expressed in all tissues, while transcripts of the other IPT co-orthologues were absent in most of the tissues. None of the co-orthologues of the tomato CKX gene family was expressed in all tissues, and a high variance in the expression patterns was observed as it was shown for *A. thaliana* in previous studies (Supplementary Table 17).<sup>139</sup> Interestingly, with the exception of the CKX family, we could show that always one gene of each enzyme family of the cytokinin biosynthesis path in tomato was expressed in every tissue. In summary, for the green algae *C. reinhardtii* and the moss *P. patens*, no co-orthologues were identified for cytokinin trans-hydroxylases and glucosyltransferases, while *C. reinhardtii* also lacked the CKX family. Furthermore, only the tRNA-IPT (IPT9) and a small set of LOG family genes were found to be present in the unicellular algae.

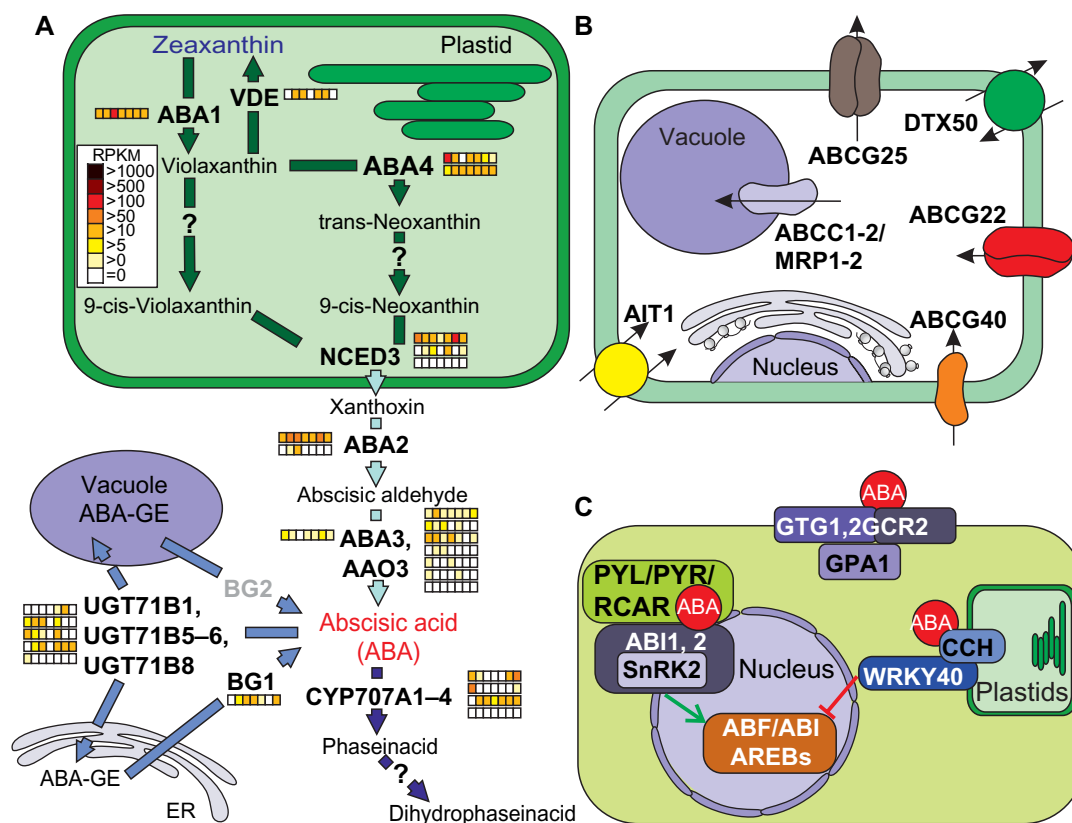
**Cytokinin transport, perception, and signaling.** tZ-ribonucleoside (tZR) is preferentially transported toward the shoot through the xylem, while the rootward transport of cytokinins occurs through the phloem. Initially, it was discussed that cytokinin receptors (AHK2, 3, and 4 in *A. thaliana*), with typical domain structure of membrane-bound histidine sensor kinases, represent the extracellular recognition site for cytokinin binding. Recent studies identified most of the receptor proteins in the ER membrane, which opened the discussion whether perception of cytokinins occurs in the ER lumen rather than on the cell surface.<sup>140–142</sup> AHK2, 3, and 4 are proposed to form heterodimeric receptor complexes upon cytokinin perception, while AHK1 is located in a distinct orthologous group,<sup>143</sup> which was confirmed by our orthologue search. Cytokinin response1 (CRE1) and Woodenleg (WOL1) are allelic to AHK4 and were identified in mutant screens for cytokinin phenotypes.<sup>144</sup> Cytokinin insensitive1 (CKI1) is one additional, more distantly related histidine kinase in *A. thaliana*<sup>145</sup>; however, its involvement in cytokinin signaling remains to be shown. In tomato, three co-orthologues were also present in the CLOG of AHK2–4 (Supplementary Table 17), which were expressed at high levels in all analyzed tissues. In contrast, for the tomato orthologue of CKI1, no expression was observed (Supplementary Table 17).

Binding of cytokinins triggers the activation of the histidine kinase domain of AHK on the cytosolic site, leading to its autophosphorylation. Components acting downstream in the phosphorelay signaling pathway include histidine-containing phosphotransfer proteins, named AHP in *A. thaliana*. The AHP

family consists of five members (AHP1–5) and one pseudo AHP considered as a negative regulator (AHP6). We detected four co-orthologues of AHP in tomato falling in four different CLOGs. One of the four co-orthologues was expressed in all tissues (Supplementary Table 17). For the AHP4 and AHP6 co-orthologues, no expression was detected. AHP has been shown to function in the transfer of the signal to the nucleus to regulate the activity of response regulators (ARRs) acting as transcription factors. ARR form a large family with 15 proteins in *A. thaliana*, which can be divided according to their domain structure into type-A AARs acting as repressors (ARR3–9, 15–17) and type-B ARR acting as activators (ARR1–2, 10–12).<sup>140,146,147</sup> In tomato, six type-A AAR and five type-B AAR co-orthologues exist (Fig. 5B; Supplementary Tables 3 and 17). For ARR16 and ARR17, only a weak response to cytokinin was reported.<sup>148</sup> These two AARs were found only in eudicots in our analysis, which could indicate redundant or newly developed functions in cytokinin response. The stability of some of the type-B AARs is controlled by their recruitment to specific SCF E3 ubiquitin ligase complexes containing one of the four F-box proteins KISS ME DEADLY (KMD1–KMD4). Two co-orthologues of KMD were detected in tomato (Fig. 5B, Supplementary Table 3).

Type-A ARR do not possess a defined output domain, and most of them are transcriptionally induced in response to cytokinins (Supplementary Table 10). Interestingly, for the six co-orthologues in tomato, we observed that each orthologue corresponded to a pair of Arabidopsis type-A ARR except for the group containing ARR8 and ARR9. Furthermore, in tomato type-A ARR were expressed in all analyzed tissues with two exceptions. The orthologue of ARR5/6 was not expressed in tomato fruit, while the orthologue of ARR16/17 was only expressed in flowers and fruits (Supplementary Table 17). Altogether, the function of type-A ARR as negative regulators of cytokinin response by interfering with the function of positive regulators in the signal transduction pathway is widely assumed. One exception so far is ARR4, which has been shown in Arabidopsis to interact positively with phytochrome B,<sup>149</sup> and it will be interesting to see whether this particular function is conserved in tomato and other plant species as well.

**Putative rate limitations in biosynthesis of ABA in tomato.** ABA synthesis is initiated in plastids and derives from the metabolism of carotenoids.<sup>17,150</sup> Moreover, ABA-specific defects are connected to mutations in genes acting downstream of zeaxanthin synthesis. Zeatin epoxidase (ZEP), also called ABA1, catalyzes the formation of violaxanthin, and this step is reversed by the action of violaxanthin de-epoxidase (VDE; Fig. 6A). The process known as xanthophyll cycle is assumed to provide sufficient amounts of zeaxanthin for an increased photo-oxidative protection under high-light conditions. Each of the two enzymes is encoded by a single gene in Arabidopsis and has one orthologue in tomato, whereas VDE1 was not found in *C. reinhardtii* (Supplementary Tables 4 and 11). In



**Figure 6.** Pathways of abscisic acid synthesis, transport, and signaling.

**Notes:** (A) Enzymes and intermediate products of the plastidal, endoplasmatic, and cytosolic pathways for abscisic acid production unify to the plastidal production of zeaxanthin (blue) as precursor for abscisic acid (red) synthesis in the cytosol. The storage pathway is catalyzed by BG1 and BG2 as well as by members of the UGT71B gene family. For further details, see text. The arrows are colored according to the species in which the enzymes were found (Fig. 1A). The expression of the identified genes in tomato is shown as explained in Figure 2. Genes coding for enzyme activities not expressed by any orthologue are indicated in gray. (B) Survey and localization of main transporters involved in uptake and intracellular distribution of abscisic acid. (C) The components involved in abscisic acid signaling are represented as interaction scheme.

**Abbreviations:** Proteins: ABA, ABA deficient; VDE1, violaxanthin de-epoxidase; NCED, nine-cis-epoxycarotenoid dioxygenase; CYP707A, cytochrome P450, family 707, subfamily A; UGT71B, UDP-glucosyl transferase 71B; BG, beta-1,3-glucanase; ABCG, ATP-binding cassette G25; DTX, detoxification efflux carrier; ABCG, ATP-binding cassette G; AIT, ABA-importing transporter; ABCC1, ATP-binding cassette C; GTG, GPCR-type G protein; GCR, G protein coupled receptor; GPA, G PROTEIN alpha subunit; CCH, conditional chlorina; WRKY, WRKY DNA-binding protein; PYL1, PYR1-like 1, PYR, pyrabactin resistance; RCAR, regulatory component of ABA receptor; SNRK, sucrose nonfermenting 1(SNF1)-related protein kinase; ABI, ABA insensitive; ABF, abscisic acid responsive elements-binding factor.

contrast to ZEP1, which was at least moderately expressed in all tissues (Supplementary Table 18), VDE1 was not expressed in roots, flowers, and ripe fruits.

Formation of 9-cis-violaxanthin and 9-cis-neoxanthin via its intermediate trans-neoxanthin are the last steps occurring in plastids before cleavage by 9-cis-epoxycarotenoid dioxygenase (NCED; five genes in *A. thaliana*<sup>151</sup>) and releasing of xanthoxin from the C40 precursor into the cytosol. While the enzyme-encoding genes are known for the ABA4-dependent formation (ABA4, AT1G67080) of trans-neoxanthin, the activities required for the isomerization to the 9-cis derivatives remain to be identified. Two co-orthologues of ABA4 were identified in tomato, which are globally expressed (Fig. 6A; Supplementary Table 18). The subsequent cleavage of xanthophyll by NCED is the rate-limiting step in ABA synthesis,<sup>17</sup> and expression of NCED is tightly regulated in response to stress and developmental signals. In contrast to previous results,<sup>151</sup> we observed a localization signal for only three (NCED 2, 3, 5) NCEDs in

*A. thaliana*. The number of co-orthologues in the 13 plant species ranges from none in *C. reinhardtii* to nine in *Z. mays*, all containing the carotenoid oxygenase-specific domain (retinal pigment epithelial membrane (RPE)<sup>66</sup>, Supplementary Tables 4 and 11). In tomato, three NCED co-orthologues were present and one appeared to be globally expressed (Fig. 6A; Supplementary Table 18).

The final two steps of ABA synthesis occur in the cytoplasm and include the conversion of xanthoxin to ABA aldehyde catalyzed by SDR1/ABA2 and the formation of the carboxyl group mainly by abscisic acid oxidase 3 (AAO3), which is one of the three AAOs in *A. thaliana*.<sup>152</sup> All three enzymes depend on a molybdenum cofactor.<sup>153</sup> Consequently, a mutant of the sulfurase in *A. thaliana* that produces the functional cofactor was named *aba3*, because strong ABA deficiency phenotypes were caused due to abolishing all AAO activities.<sup>154</sup> In tomato, we identified two co-orthologues of ABA2, six of AAO3, and one of ABA3 (Fig. 6A; Supplementary Tables 4

and 18). However, not all co-orthologues of AAO3 might be involved in ABA synthesis. On the one hand, three of them showed only moderate expression (Fig. 6A), while on the other hand, it has been shown that *Arabidopsis* AAO3 is the major AAO in ABA biosynthesis.<sup>152,153</sup>

ABA catabolism plays an important role in the regulation of ABA homeostasis, and currently, two pathways have been described.<sup>17</sup> The first is initiated by 8'-hydroxylation of ABA, which leads to the isomerization of the intermediate 8'-OH-ABA to phaseic acid (PA) and the subsequent degradation via conversion to dihydrophaseic acid (Fig. 6A<sup>17</sup>). Four P450-type monooxygenases CYP707A were identified to catalyze the initial step in *A. thaliana*.<sup>155</sup> All of them were present in the same CLOG together with four co-orthologues of tomato (Supplementary Tables 4 and 11; Solyc08g005610, Solyc08g075320, Solyc04g078900, and Solyc01g108210). The expression patterns showed spatial and temporal differences, indicating that individual members might have different importance during growth and development (Fig. 6A; Supplementary Table 18). The second pathway leads to the formation of glycoside ester conjugates (ABA-GE) by the activity of several glycosyltransferases (UGT71Bs<sup>17</sup>). ABA-GE is considered as storage or transport form of ABA, which accumulates in vacuoles and the apoplast. Translocation to the ER and rapid reactivation of ABA by cleaving the glycoside ester conjugates by  $\beta$ -glucosidases (BG1 and BG2) is assumed to occur in response to dehydration. The five tomato co-orthologues to UGT71B and the one to BG1 were found to be expressed in most tissues. BG2, which was assumed to catalyze the conversion of vacuolar stored ABA conjugates, was expressed at very low level (Fig. 6A; Supplementary Table 18).

**Transport of ABA needs functional assignment within orthologous groups.** Several transporters encoded by distinct transporter gene families are involved in the transport of ABA or ABA conjugates across the various membranes (Fig. 6B).<sup>156</sup> The members of the subfamily G of the large ATP-binding cassette (ABC) protein family are one group of these transporters (ABCG).<sup>157</sup> In *A. thaliana*, ABCG25 was the first ABA efflux carrier identified in the plasma membrane. It is expressed mainly in vascular tissues.<sup>158,159</sup> ABCG22 and ABCG40 reside in guard cells and contribute to enhanced import of ABA into stomatal cells.<sup>160</sup> The CLOGs of ABCG22 and ABCG40 contained five additional co-orthologues to *Arabidopsis* ABCG22/25 and 14 co-orthologues to ABCG40, for which the functional assignment remains to be elaborated. In total 31 ABCG co-orthologues have been detected in tomato (Supplementary Table 18).

The ABA-importing transporter 1 (AIT1), originally characterized as nitrate transporter (NRT1.2), is involved in cellular import of ABA in the vascular tissues of inflorescence stems, leaves, and roots.<sup>7</sup> In the genome of tomato, four co-orthologues to AIT1 were identified (Supplementary Table 18). DTX50 is an ABC transporter of the detoxification efflux carriers/multidrug and toxic compound extrusion

(DTX/MATE) family shown to function in efficient ABA export after drought stress.<sup>161</sup> DTX50 plays an important role in the removal of excess ABA levels to prevent hyperaccumulation of the hormone.<sup>157</sup> Again, four co-orthologues were detected in the genome of tomato (Supplementary Table 18). Finally, two members of the ABCC subfamily in *A. thaliana*, AtABCC1/MRT1 and AtABCC2/MRT2 (multidrug resistance-associated protein), have been shown to transport ABA-GE across the tonoplast into the vacuole. Both belong to the same CLOG in which 14 tomato genes were also clustered.

Three ABA-specific receptor types associated with distinct cellular localizations have been described (Fig. 6C).<sup>17,48,162</sup> One type is localized to the ER, Golgi, and peripheral plasma membranes and consists of the G protein coupled receptor proteins GTG1, GTG2, and GCR2. Interaction with the G-alpha subunit GPA1 regulates the binding of ABA. Signal transduction from these receptors mainly targets the regulation of membrane channels involved in Ca<sup>2+</sup> transport and control of water permeability, eg, in guard cells. We identified three tomato co-orthologues of GCR2, and one for GTG1 and GTG2. Interestingly, the latter was only expressed in flower and fruit tissues (Supplementary Table 18).

The subunit H of the Mg<sup>2+</sup> chelatase complex (CCH) localized in plastids was proposed as an additional ABA receptor.<sup>163</sup> The tomato orthologue of CCH was found to be highly expressed in all tissues (Fig. 6C; Supplementary Table 18). Binding of ABA enhances the interaction of subunit H on the cytosolic side with a group of transcription factors from the WRKY family, thereby preventing their translocation to the nucleus. In the absence of ABA, the transcription factors act as negative regulators of ABA response genes.

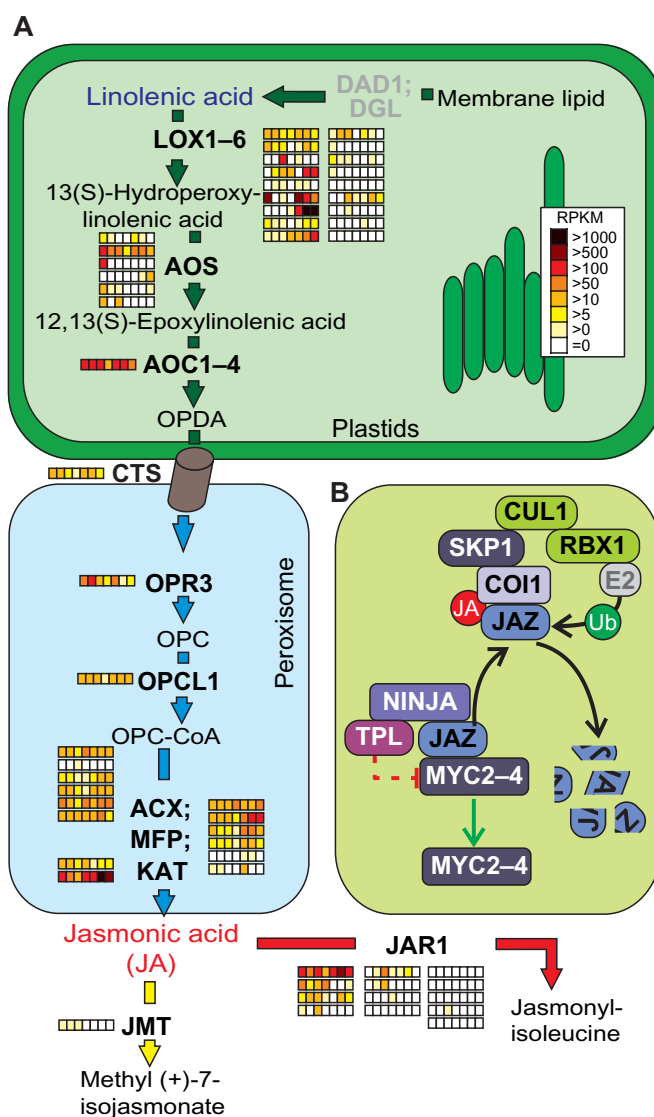
The third class of ABA receptors consists of soluble proteins seen as entrance components of the "core ABA signaling pathway" (Fig. 6C<sup>48</sup>). These receptors belong to a gene family (14 genes in *A. thaliana*) known as pyrobactin resistant (PYR), PYR-like (PYL), or regulatory component of ABA receptor (RCAR) which was represented in our study by eight co-orthologues in *S. lycopersicum* (Supplementary Table 4). The proteins control the activity of downstream components of the signaling pathway by the interaction with protein phosphatase 2C (PP2C), which acts as a negative regulator of ABA response. This inhibition is maintained by the interaction with the active ABI1, 2 enzymes containing the PP2C domain (conserved in all 13 species, Supplementary Table 11). Further, SNF1-related kinases (SnRKs; seven co-orthologues in tomato) regulate the ABA signaling in the absence of ABA or at low concentrations of ABA. This interaction prevents the phosphorylation and activation of numerous transcription factors, ion channels, and other mediators of ABA responses. Alternating binding of PYR/PYL/RCAR to ABI1, 2 controls release and activity of the SnRKs and thereby the activation of a transcription factor cascade cooperating in the regulation of numerous ABA response genes.<sup>164</sup>

### Differences in the localization of biosynthesis enzymes from JA in tomato.

Jasmonates are lipid-derived compounds synthesized from  $\alpha$ -linolenic acid released from plastid membranes by one of the seven different branches of the lipoxygenase (LOX) pathway.<sup>165</sup> Subsequently,  $\beta$ -oxidation occurs in peroxisomes and the final step of JA synthesis takes place in the cytoplasm.<sup>166</sup> Defective in anther dehiscence 1 (DAD1), phospholipase 1 (PLA1), and phospholipase 2 (PLA2) are involved in  $\alpha$ -linolenic acid production.<sup>61</sup> In *A. thaliana*, *dad1* mutant results in a male sterile phenotype,<sup>167</sup> indicating that DAD1 is responsible for JA biosynthesis in flowers, while in leaves JA is synthesized by the DGL (Dongle) gene.<sup>168,169</sup> Both enzyme CLOGs contained no co-orthologues from *C. reinhardtii* and *P. patens*; thus, it is likely that they evolved in the ancestor of monocots and eudicots (Supplementary Table 5). We identified two co-orthologues for DGL and one orthologue for DAD1 in tomato, but none of them were expressed in the tissues and developmental stages of fruits analyzed (Fig. 7A; Supplementary Table 19). This suggests that another pathway for linolenic acid in tomato may exist or that the pathway is only activated under stress conditions.

$\alpha$ -linolenic acid (18:3) acts as a fatty acid substrate for synthesis of 13(*S*)-hydroperoxylinolenic acid by LOXs.<sup>170</sup> Except in *C. reinhardtii*, all plant species contained more than six co-orthologues, with a maximum of 43 co-orthologues in *G. max*. In total, 18 co-orthologues were identified in tomato; however, a chloroplast targeting signal was only predicted for three of them (Solyc05g014790, Solyc03g122340, and Solyc01g006560), which most likely indicated their involvement in jasmonate synthesis (Fig. 7A; Supplementary Table 12). At least one of them was expressed in each tissue and Solyc01g006560 was highly expressed in leaves. Allene oxide synthase (AOS; six co-orthologues in tomato)<sup>171</sup> catalyzes the dehydration of 13(*S*)-hydroperoxylinolenic acid to 12,13(*S*)-epoxylinolenic acid, which is converted to *cis*(+)-12-oxophytodienoic acid (OPDA) likely by allene oxide cyclase (AOC). Interestingly, we detected only two co-orthologues of AOS containing a chloroplast targeting signal (Solyc04g079730 and Solyc11g069800) in tomato. At least one of them was expressed in all tissues, similar to the unique tomato AOC orthologue (Fig. 7A). In *A. thaliana*, transcription of AOC is induced within two hours after wounding and occurs in anthers and pollen grains,<sup>172</sup> while we observed moderate expression of the tomato orthologue in all tissues (Supplementary Table 19<sup>173,174</sup>).

OPDA is transported from plastids to peroxisomes by the action of CTS,<sup>175</sup> and the expression of the tomato orthologue was at least low or moderate in all tissues (Fig. 7A; Supplementary Table 19). The OPDA reductase (OPR3) catalyzes the reduction of OPDA to 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC<sup>176</sup>) and the orthologue in tomato showed high variance in expression from low to high levels in the tissues analyzed (Fig. 7A). OPC-CoA intermediate is formed in peroxisomes by 4-coumarate:CoA ligase like (4-CL-like) enzymes such as OPCL1, whose orthologue in tomato is globally expressed (Fig. 7A).<sup>177</sup> The subsequent



**Figure 7.** Pathways of jasmonic acid synthesis and signaling.

**Notes:** (A) Enzymes and intermediate products of the plastidial and peroxisomal pathways for jasmonic acid production starting from linolenic acid as precursor and ending with delivering of jasmonic acid from the peroxisome into the cytosol. The last steps catalyzed by JMT and JAR1 mark the conversion of jasmonic acid to its methyl ester as volatile transport form and jasmonyl-isoleucine as biological active form, respectively. For further details, see text. The arrows are colored according to the species in which the enzymes were found (Fig. 1A). Expression of the identified co-orthologues in tomato is shown as explained in Figure 2. Genes coding for enzyme activities not expressed by any orthologue are indicated in gray. (B) The components involved in jasmonic acid signaling are represented as interaction scheme. The bar-headed dashed line in red indicates indirect suppression mechanisms.

**Abbreviations:** Proteins: DAD, defective anther dehiscence; DGL, DONGLE; LOX, lipoxygenase 2; AOS, allene oxide synthase; AOC, allene oxide cyclase; CTC, COMATOSE; OPR, oxophytodienoate-reductase; OPCL, OPC-8:0 CoA ligase; ACX, acyl-CoA oxidase; MFP2, multifunctional protein; AIM, abnormal inflorescence meristem; KAT, 3-keto-acyl-CoA thiolase; JMT, jasmonic acid carboxyl methyltransferase; JAR, jasmonate resistant; ST2a, sulfotransferase 2A; JAZ, jasmonate-ZIM-domain protein; COI, coronatine-insensitive; NINJA, novel interactor of JAZ; TPL, TOPLESS; SKP1, S-phase kinase-associated protein 1; CUL, Cullin; RBX, RING-box. Metabolites: OPC, 3-oxo-2-(20(Z)-pentenyl)-cyclopentane; OPC-CoA, 3-oxo-2-(20(Z)-pentenyl)-cyclopentane-coenzyme A.

$\beta$ -oxidation to synthesize JA occurs in three cycles<sup>178</sup> by the action of an acyl-CoA oxidase (ACX1), a multifunctional protein (MFP), and an L-3-ketoacyl CoA thiolase (KAT).<sup>60</sup> While two peroxisome targeted co-orthologues of ACX1 and six of MFP were identified in tomato, we could not predict a peroxisomal targeting signal in one of the two co-orthologues of KAT (Fig. 7A; Supplementary Table 12). Interestingly, MFP2 and AIM1 were located in one orthologous group in *A. thaliana* while six co-orthologues were found in tomato of which the peroxisomal-located enzymes Solyc12g007170, Solyc12g099440, Solyc07g019670, and Solyc01g066620 were at least low expressed in all tissues except Solyc01g066620 (Supplementary Table 19).

JA is subsequently secreted into the cytoplasm, where it can be further utilized either by *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) to form the volatile JA methyl ester (methyl (+)-7-isojasmonate) or conjugated to isoleucine by the activity of JAR1, which converts JA into the biologically active jasmonyl-isoleucine.<sup>4,179,180</sup> JMT was only present in eudicots and represented by a single orthologue in tomato, which exhibited a low expression in root, stem, and leaf. In tomato, (+)-7-iso-JA-Ile is exclusively synthesized upon wounding, and in *SILJAR1*-RNAi lines, JA-Ile is downregulated by 50%–75%, confirming the importance of JAR1 for the conversion of JA to JA-Ile.<sup>181</sup> Remarkably, we observed that the CLOG of JAR1 contained 13 tomato co-orthologues, and one of them (Solyc01g095580) was highly expressed in all tissues except of flowers and stems. Interestingly, the co-orthologue Solyc10g011660 showed a high expression in flowers and stems, and by this, we could compensate Solyc01g095580 in both tissues (Fig. 7A; Supplementary Tables 5, 12, and 19). Thus, it needs to be experimentally proven whether Solyc01g095580 is the essential JAR1 orthologue in jasmonate signaling and whether rapid induction of the gene is caused by stress induction during the harvesting procedure.

**Jasmonate response co-orthologues show differences between tomato and Arabidopsis.** The main components of JA response are transcription factors of the JA-ZIM-domain (JAZ) repressor family, calcium-related signaling molecules, and JA-related transcription activators. Ca<sup>2+</sup>-dependent phosphorylation and MAPK cascades are also involved in the regulation of JA biosynthesis.<sup>61</sup> Among the targets of JA signaling are JA biosynthesis genes that form a positive feedback system.<sup>182</sup>

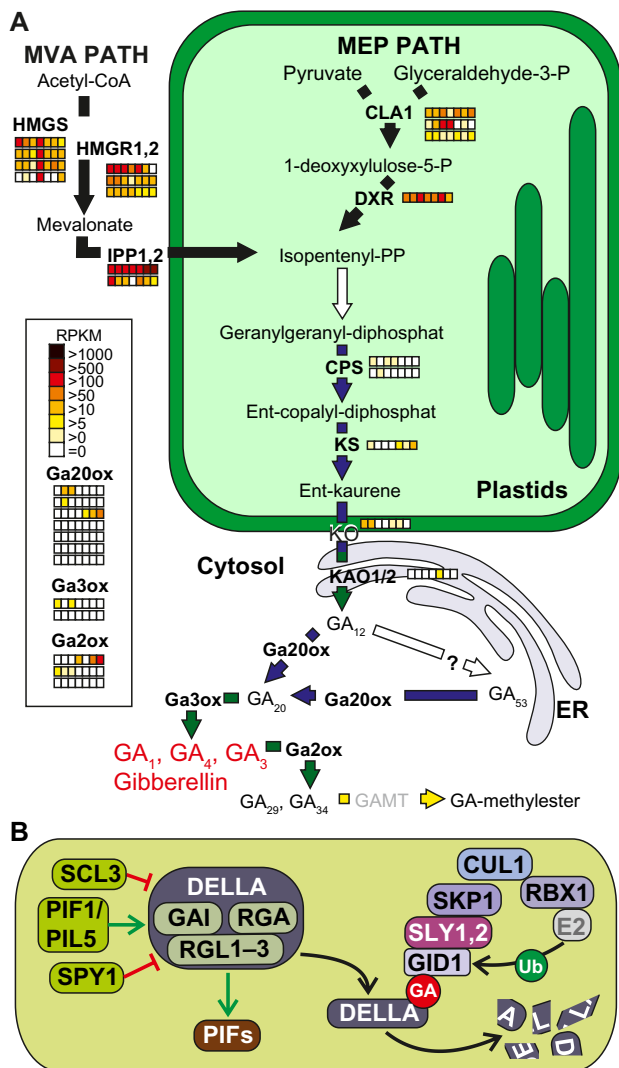
We observed that the regulation of transcriptional changes triggered by JA synthesis was conserved in eudicots and monocots (Fig. 7B; Supplementary Tables 5 and 12).<sup>183–185</sup> The primary regulators are MYC transcription factors and JAZ repressors are associated in corepressor complexes with NINJA and TPL (Topless; Fig. 7B). None of the eight JAZ repressors in *A. thaliana* were represented in green algae and moss, and JAZ5, 6, and 9 occurred only in eudicots or were found specifically in *A. thaliana* (Supplementary Table 5). Degradation of JAZ repressors is mediated by binding of JA-Ile to the F-box

protein coronatine-insensitive 1 (COI1) bound to the SCF<sup>COI1</sup> E3 ubiquitin ligase complex (Fig. 7B), which in turn allows the expression of early JA response genes.<sup>186–188</sup> JAZ proteins also bind to MYB21 and MYB24, two transcription factors encoded by genes strongly induced by JA in flower tissues that control stamen development and pollen maturation in *A. thaliana*.<sup>189,190</sup> For tomato, only three JAZ co-orthologues were identified in two distinct CLOGs, showing the same domain architecture containing a TIFY (transcription factor domain) and a CCT\_2 (short plant protein motif) domain (Supplementary Table 12). For two co-orthologues (Solyc07g042170 and Solyc03g118540), high expression levels were found in roots and Solyc07g042170 was at least moderately expressed in all other tissues (Supplementary Table 19).

Impairment of JA signaling leads to severe phenotypes like male sterility in *A. thaliana*, while in the tomato mutant, *jai1-1* female sterility was observed.<sup>191</sup> In line with the essential role of JAI1, the tomato orthologue was expressed in all tissues. Interestingly, expression of the COI1 orthologue was not observed in tomato flowers (Supplementary Table 19),<sup>165</sup> although it is interacting with JAI1.

**Low-expressed enzymes in tomato influence GA biosynthetic pathway.** GAs derive from the diterpene geranylgeranyl diphosphate (GGDP) synthesized in plastids from the isoprenoid precursor isopentenyl pyrophosphate, which is provided mainly by the plastid-resident MEP pathway, but also by the cytosolic MVA pathway (Fig. 8A).<sup>192</sup> Our analysis showed that both pathways were conserved in mosses, monocots, and eudicots, but the MVA pathway was missing in the green algae *C. reinhardtii*.<sup>193,194</sup> Remarkably, the co-orthologues from the MEP and MVA pathway showed moderate or high expression in all tomato tissues, whereas the expression of co-orthologues catalyzing the subsequent steps of GA biosynthesis was low in tomato or even not present in some tissues (Fig. 8A, Supplementary Table 20). The chloroplast-localized *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), which are involved in formation of *ent*-kaurene, were detected in all plants except in the green algae (Fig. 8A, Supplementary Table 6). Interestingly, expression of tomato CPS and KS co-orthologues was detected if at all at low levels only (Fig. 8A, Supplementary Table 20). GA1 (CPS) and GA2 (KS) in *A. thaliana* were discriminated on the basis of their domain architecture, which could be confirmed by our orthologue search due to the fact that they fall into two different CLOGs. Inspection of the domain architecture of co-orthologues within the two CLOGs indicated that orthologue search and functional domain architecture might differ, but it could also exemplify an interesting case of domain stealing.<sup>100</sup> For instance, some of the monocot co-orthologues in the CPS group contain a Prenyltrans\_1 domain. This domain is absent in the CPS of *A. thaliana*, but present in the KS orthologue (Supplementary Table 13).

Conversion of *ent*-kaurene to GA<sub>12</sub> requires the transport of *ent*-kaurene into the cytoplasm and several oxidation



**Figure 8.** Pathways of gibberellin synthesis and signaling. **(A)** Enzymes and intermediate products of the plastidial and cytosolic pathways for isopentenyl pyrophosphate production unify to the plastidial production of *ent*-kaurene as precursor for gibberellin synthesis ( $GA_1$ ,  $GA_4$ , and  $GA_3$ ) in the cytosol.  $GA_{20ox}$  and  $GAMT$  mark the biological inactivation of gibberellin. For further details, see text. The arrows are colored according to the species in which the enzymes were found (Fig. 1A). Expression of the identified genes in tomato is shown as explained in Figure 2. **(B)** The components involved in gibberellin signaling are presented as interaction scheme. Enzyme activities not expressed by any orthologue gene in tomato are indicated in gray.

**Abbreviations:** Proteins: CLA, chloroplastos alterados; DXR, 1-deoxy-xylulose 5-phosphate reductoisomerase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl diphosphate isomerase; CPS, ent-copalyl diphosphate synthetase; KS, ent-kaurene synthase; KO, ent-kaurene oxidase; KAO1, ent-kaurenoic acid oxydase;  $GA_{20ox}$ , gibberellin 20-oxidase;  $GA_{3ox1}$ , gibberellin 3-oxidase;  $GA_{2ox}$ , gibberellin 2-oxidase;  $GAMT$ , gibberellin methyltransferase;  $GID$ , GA insensitive dwarf;  $GAI$ , gibberellic acid insensitive;  $RGA$ , repressor of  $GA_{1-3}$ ;  $RGL$ , RGA-like;  $SLY$ , SLEEPY;  $PIF$ , PHY-interacting factor;  $SPY$ , SPINDLY;  $SCL$ , scarecrow-like.

steps. The first step is catalyzed by the *ent*-kaurene oxidase (KO), localized in the outer envelope of plastids, which is involved in the simultaneous transfer of *ent*-kaurenoic acid to the ER.<sup>195</sup> Subsequent oxidization requires the ER-associated

*ent*-kaurenoic acid oxidase (KAO) for conversion to  $GA_{12}$ . However, the nature of the enzyme activity involved in the formation of  $GA_{53}$  by hydroxylation on C-13 is still not clear. The subsequent hydroxylation on C-20 of  $GA_{12}$  or  $GA_{53}$  is catalyzed by cytoplasmic  $GA_{20ox}$  oxoglutarate-dependent dioxygenases ( $GA_{20ox}$ ). The activity of other oxoglutarate-dependent dioxygenases like the  $GA_{3ox}$  further leads to the production of bioactive GA compounds such as  $GA_1$ ,  $GA_4$ , or  $GA_3$ . Deactivation of GAs by C-2 hydroxylation is catalyzed by  $GA_{2ox}$ . As in the case of KS, KAO1 and KAO2, co-orthologues of  $GA_{3ox}$  and  $GA_{2ox}$  were not found in *P. patens* and *C. reinhardtii* (Supplementary Table 6), while their expression was low in most of the analyzed tissues (Supplementary Table 20).

Oxidization and conversion of GAs into their epoxides by cytochrome P450 monooxygenases as well as formation of GA methyl esters by the activity of GA methyl transferases ( $GAMTs$ ) are discussed as other mechanisms of GA deactivation.<sup>66</sup> However, these mechanisms appear to be species-specific as co-orthologues to the *A. thaliana*  $GAMT$  were not identified by our approach in any of the selected species except for *V. vinifera* (Supplementary Table 6).

**GA perception and signaling is absent in green algae and mosses.** Current models for GA perception and signaling consider three key components.<sup>196</sup> The first is the soluble, nuclear-localized GA receptor  $GID1$ ,<sup>197</sup> which we identified in monocots and eudicots (Supplementary Table 6). The second are the growth repressing DELLA proteins  $GAI$ ,  $RGA$ , and  $RGL1$ , 2, and 3 identified in *A. thaliana*<sup>198</sup> as well as  $SLR1$  found in rice,<sup>199</sup> which form a subfamily of the plant-specific GRAS transcription regulators. All of these proteins were grouped in one CLOG also containing co-orthologues from the moss in addition to monocots and eudicots. However, in contrast to *A. thaliana* that has five GRAS enzymes, many eudicots and monocots contained only one orthologue. The third are the F-box proteins  $SLY1$  and  $SLY2$ ,<sup>200</sup> which are called  $GID2$  in rice (Fig. 8B<sup>201</sup>). No co-orthologues of these factors were detected in the moss and the green algae. By this, the GA signaling pathway seems to have emerged after mosses. However, co-orthologues of  $SLY$  were not found in *V. vinifera* (Supplementary Table 6). Remarkably,  $SLY2$  co-orthologues were not expressed in the analyzed tomato tissues under normal conditions (Supplementary Table 20).

In the absence of GA, DELLA proteins act as central repressors of GA response by suppressing the activity of  $PIF$  transcription factors ( $PIF3$ , 4), which are involved in the regulation of growth-promoting GA-responsive genes.<sup>202,203</sup> Binding of GA to the receptor  $GID1$  triggers its interaction with DELLA proteins and causes their release from repression complexes.<sup>204</sup> In turn, simultaneous binding of  $SLY1/GID2$  recruits DELLA proteins for ubiquitination by the  $SLY1/GID2$ -dependent E3-ligase complex and subsequent degradation by the proteasome.<sup>204</sup> Furthermore, the level of DELLA proteins is also controlled by the activity of the

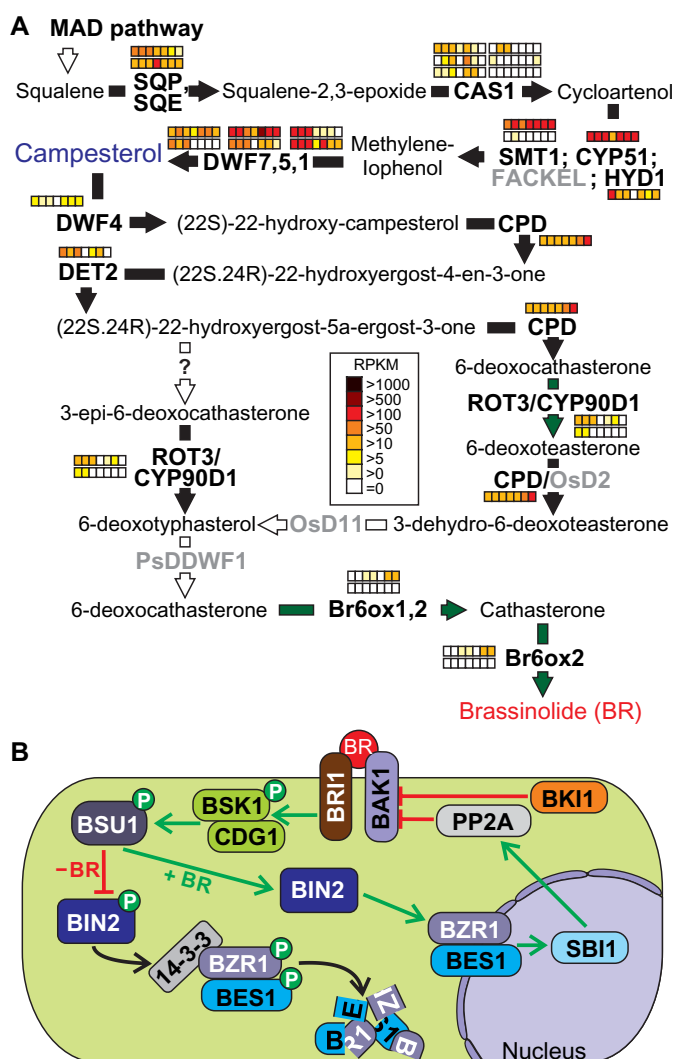


phytochrome-interacting factor PIF1/PIL5, and the regulation of DELLA activity is done by proteins SCL3 and SPY1.<sup>67,205</sup> In general, PIFs have been evolved specifically in different species, as PIF6 was present only in *A. thaliana* and we observed PIF1 co-orthologues only in some eudicots (*S. tuberosum*, *S. lycopersicum*, *M. truncatula*, and *G. max*; Supplementary Tables 6 and 13). Further, our analysis for expression in tomato revealed that most of the enzymes were expressed at low levels under normal conditions (Supplementary Table 20).

**BR synthesis is conserved in Viridiplantae until synthesis of campesterol.** As for all other phytoosteroids, the biosynthesis of BR derives from isopentenyl pyrophosphate (isopentenyl PP; Fig. 9A), which is conjugated to the triterpene squalene (Fig. 9A).<sup>16,206,207</sup> By the action of CAS1, SMT1, CYP51, FACKEL, HYD1, DWF7, DWF5, and DWF1, campesterol is synthesized,<sup>73</sup> which is the direct C28 precursor of the BR specific pathway. In line with the fact that campesterol is not exclusively a precursor for BR, the pathway was conserved in all analyzed plants with the exception of FACKEL and DWF5 (Fig. 9A, Supplementary Tables 7 and 14). The enzyme FACKEL was only present in *Z. mays*, *S. tuberosum*, and *A. thaliana*, whereas DWF5 co-orthologues were only missing in *C. reinhardtii*. In tomato, CAS1 co-orthologues showed a moderate expression at maximum and could potentially limit the rate of campesterol synthesis. In contrast, SMT1, CYP51, and DWF5 showed the tendency to be highly expressed in most of the tissues (Supplementary Table 21). Furthermore, HYD1 orthologue was not expressed in flower tissue.

The subsequent processing of BR synthesis starting from campesterol as a basic precursor is not yet fully described and the exact order of enzymatic reactions in this part of the BR pathway is still under debate. For our analysis, we depicted the two pathways described by Zhao and Li,<sup>76</sup> which are branching during the action of DET2, DWF4 (annotated as CYP724B2 and CYP90B3 in tomato, respectively), and CPD (annotated as CYP90A1 in *A. thaliana*). Remarkably, only DET2 co-orthologues were found in the green algae and the moss included in our analysis (Supplementary Table 14). For eudicots, the reaction catalyzed by the P450 monooxygenase DWF4 has been proposed as a rate-limiting step in BR synthesis,<sup>208</sup> and transcriptome profiling of tomato co-orthologues revealed only low expression in all tissues for DWF4. In contrast, CPD showed moderate or high expression in all tissues (Supplementary Table 21). For co-orthologues in both routes, mostly no or only low expression was observed in flower tissue. The two routes lead to the production of 6-deoxytyphasterol and the only two additional enzymes identified in *A. thaliana* are ROT3 (CYP90C1) and CYP90D1, but it is unknown yet whether both enzymes indeed participate in both pathways.

The conversion of 6-deoxytyphasterol to 6-deoxocathasterone is catalyzed by DDWF1, which is only described in pea,<sup>209</sup> while the corresponding gene in *A. thaliana* has not been identified yet. Finally, oxidation on the C-6 by Br6ox leads to CS, the



**Figure 9.** Pathways of brassinosteroide synthesis and signaling. **Notes:** (A) Brassinosteroide synthesis uses the precursor compound campesterol, which is derived from the MAD pathway. Two major routes are shown leading to the formation of the bioactive brassinosteroids castasterone and brassinolide. For further details, see text. The arrows are colored according to the species in which the enzymes were found (Fig. 1A). Expression of the identified genes in tomato is shown as explained in Figure 2. (B) The components involved in brassinosteroid signaling are presented as interaction scheme. Enzyme activities not expressed by any orthologue gene in Arabidopsis or tomato are indicated in gray. D2 and D11 were only detected in *O. sativa* and DDWF1 was identified in *Pisum sativum*.

**Abbreviations:** Proteins: SQP, squalene monooxygenase; SQE, squalene epoxidase; CAS, cycloartenol synthase; SMT, sterol methyltransferase; CYP, cytochrome P450; HYD, Hydra; DWF, dwarf; CPD, constitutive photomorphogenic dwarf; DET2, de-etiolated; ROT, rotundifolia; Br6ox, brassinosteroid-6-oxidase; BRI1, brassinosteroid-insensitive; BAK1, BRI1-associated receptor kinase; BK1, BRI1 kinase inhibitor; BSK, brassinosteroid-signaling kinase; CDG, constitutive differential growth; CDL, CDG1-LIKE; BSU, BRI1 suppressor; BIN, brassinosteroid-insensitive; BZR, brassinazole-resistant; BES, BRI1-EMS-suppressor 1; SBI, suppressor of BRI; PP2A, serine/threonine protein phosphatase 2A.

immediate precursor of BL. Br6ox (CYP85A1 and CYP85A2 in *A. thaliana*) has been originally identified as an *extreme dwarf* ( $d^x$ ) allele of the tomato *DWARF* locus (D). CYP85A2 (Br6ox2),



but not CYP85A1 (Br6ox1), drives the conversion of CS to BL. Expression analysis of tomato co-orthologues in different tissues showed that only one co-orthologue was expressed in both cases. For the co-orthologues of Br6ox, only moderate expression was detected in fruit ripening stages, whereas for ROT3/CYP90D1, moderate expression was observed in root, stem, and leaf (Supplementary Table 21).

**Alternative modes of BR signaling in monocots and moss.** Because long-distance transport is not shown for BR or derivatives so far, it is widely assumed that they act in close vicinity to the places of synthesis.<sup>210</sup> Binding of BRs to the extracellular leucine-rich repeat domain of the serine/threonine receptor kinase BRI1 initiates the release of the inhibitor protein BKI1 and formation of heterodimers with BAK1 (Fig. 9B<sup>211,212</sup>). While we detected co-orthologues of BAK1 and BRI1 in all plants except the green algae *C. reinhardtii*, the inhibitor BKI1 was identified only in eudicots, which suggests an alternative mode of regulation in monocots (Supplementary Table 7). Interestingly, the CLOG of BAK1 contained seven to 30 co-orthologues per species in eudicots and monocots. However, the identified co-orthologues showed a large heterogeneity in their domain architecture (Supplementary Table 14).

Autophosphorylation and sequential transphosphorylation of the cytoplasmic kinase domains fully activate the receptor complex and triggers the downstream signal transduction by activating BSK1, which in turn phosphorylates BSU1. Interestingly, BSK1 was low expressed during fruit ripening and in the mature fruit (Supplementary Table 21). BSU1 acts as a protein phosphatase and inactivates the serine/threonine kinase BIN2 by dephosphorylation. In the absence of BR, BIN2 is constitutively active and controls the phosphorylation of BZR1 and BES1/BZR2, two transcription factors involved in the regulation of BR-responsive genes. We observed that this regulatory network was mainly conserved in all plants except for green algae. Both transcription factors are kept inactive by either rapid proteasomal degradation or cytoplasmic retention through interaction with 14-3-3.<sup>74</sup> Only BZR1 and BES1/BZR2 were not identified in *P. patens*, which suggests that species-specific transcription factors might exist in moss (Supplementary Table 7).

Besides controlling the expression of growth-promoting genes, BZR1 and BZR2 also feedback the expression of upstream signaling components and genes involved in BR biosynthesis. Among these, SBI1 mediates the methylation of PP2A, which in turn is translocated to the plasma membrane and promotes the inactivation of the internalized BRI1 receptor by dephosphorylation (Fig. 9B). In contrast to PP2A, SBI1 was not identified in the eudicot *S. tuberosum*, which could be related to the incomplete genome draft or an analogous redundant function.

## Conclusion

We applied an integrated bioinformatics approach combining orthologue search, domain analysis, prediction of protein localization based on signal sequence detection, and expression

analysis to transfer the accumulated knowledge on hormone pathways from the model plant *A. thaliana* to other plant systems. In general, the orthologue search revealed that most of the analyzed pathways, especially the signaling pathways, were only partially covered by co-orthologues from the green algae *C. reinhardtii*. Further, the biosynthetic pathways in the moss *P. patens* were more complete than in the green algae compared to the model plant *A. thaliana*. However, for example, the biosynthetic pathway of GA seemed to be conserved in *P. patens* only within the first part (until production of GGDP), while downstream, some acting enzymes such as KS, KAO1, GAMT1, 2, GA3ox1, and GA2ox1 could not be detected. Interestingly, concerning the signaling of GA, we observed enzymes of the DELLA complex as well as proteins activated by DELLA (PIF3) and proteins regulating DELLA (SCL3 and SPY). Thus, alterations of the pathways in moss could be expected as well. Remarkably, the analysis of the conservation of phytohormone pathways in 13 different species (Fig. 1) leads to the proposal of evolutionary routes and distinctions of pathways between, eg, eudicots and monocots or between multicellular and unicellular photosynthetic eukaryotes. For example, GH3 enzymes and PIN transporter involved in auxin pathways were not present in *C. reinhardtii* (Figs. 1 and 3), as these enzymes mark features of multicellular systems. In turn, TAA1/TAR1 proteins involved in auxin synthesis were only present in eudicots and thus have evolved very late in plant diversification. A similar situation was found for BKI1, involved in BR signaling (Fig. 9). This suggests that eudicots have an increased complexity of signal perception. Moreover, the complexity of central components in jasmonic acid (JAZ5, JAZ5, and JAZ9; Fig. 7) or GA signaling (PIF1; Fig. 8) appears to be larger in eudicots than in monocots. In contrast, we could show that the two auxin transporters PIN5 and PIN8 (Fig. 3), IPTs involved in cytokinin biosynthesis (Fig. 5), and DAD1 and DGL1 involved in JA biosynthesis (Fig. 7) are proteins that can likely be traced back to the common ancestor of monocots and eudicots. Further, the existence of Brassicaceae-specific routes, like the IAOX pathway for auxin, could be confirmed based on the results presented here.

Remarkably, the application of the combination of orthologue search and functional domain prediction of the CLOGs led to the identification of putative domain-stealing events during the evolution of the GA synthesis pathway (Fig. 8). We observed a domain exchange in some species between the two subsequently acting enzymes CPS and KS involved in GA biosynthesis, which suggests that these enzymes operate in a larger complex. Both CLOGs (CPS and KS) contained proteins with at least one terpene synthase domain, but the CPS orthologue in *A. thaliana* is missing the functional domain to transfer allylic prenyl groups, which is present in some CPS co-orthologues of monocots (*S. bicolor*, *Z. mays*), whereas the KS orthologue of *A. thaliana* contains the prenyltransferase domain.

The localization prediction in combination with the orthologue search had the advantage to allow the dissection of large

CLOGs and categorized these enzymes according to their localization to be putatively involved in hormone synthesis. For example, we detected 18 and 6 co-orthologues of the LOX and AOS gene families, respectively, involved in JA synthesis. Prediction of the protein localization limited the number of enzymes with likely equivalent function in JA synthesis to three LOX and two AOS co-orthologues (Fig. 7). Therefore, this approach provides an advantage for the assignment of protein members in equivalent pathways. Additionally, inspection of the expression profile provides information on putative active pathways in the case that multiple pathways exist. For example, three alternative routes for conversion of tryptophan to auxin exist, but only genes coding for enzymes of two routes were expressed in the analyzed tissues of tomato (Fig. 2).

Expression analysis of the orthologous enzymes involved in phytohormone biosynthesis, transport, and signaling in different tissues of tomato gave insights in the expression patterns of orthologue groups containing more than one enzyme of the same species and allowed conclusions on functions depending on the developmental stage or tissue. Interestingly, the co-orthologues of DGL and DAD1 in the JA biosynthesis pathway (Fig. 7) as well as the co-orthologues of BG2 in the ABA biosynthetic pathway (Fig. 6) were not expressed in tomato. Moreover, the expression at specific developmental stages could help to assign the function to specific co-orthologues. For example, Br6ox involved in BR synthesis had a high expression during fruit ripening, while ROT3 showed the highest expression in root, stem, and leaf tissues (Fig. 9). Further, the ABA receptor GTG2 was only expressed in flower and fruit tissues of tomato (Fig. 6), and a similar observation was presented for ACS and ACO involved in ethylene synthesis (Fig. 4), which was in line with the importance of the two phytohormones ABA and ethylene during reproduction and fruit ripening.

In general, we can conclude that the information derived from the model plant *A. thaliana* can largely be transferred to eudicots, while some distinctions, particularly in the signaling mechanisms, became obvious in comparison between monocots and *A. thaliana*. In turn, the pathways in the moss *P. patens* and green algae *C. reinhardtii* are in parts distinct or even missing and the transfer of information from *A. thaliana* to these species is mostly difficult. Our results provide the platform for future work on experimental confirmation of the phytohormone pathways in a broader variety of plant species and for the identification of species-specific components.

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## Author Contributions

Conceived and designed the bioinformatics approaches: ES, SS. Searched the databases and analyzed the data: SS. Contributed to writing of the manuscript: SS, KDS, NF, SJ,

MLC, ES. All the authors reviewed and approved the final manuscript.

## Supplementary Material

**Supplementary Tables 1–7.** Survey of genes involved in phytohormone biosynthesis, transport, and signaling in *A. thaliana* and their co-orthologues in selected Viridiplantae. Analyzed phytohormones are auxin (S1), ethylene (S2), cytokinin (S3), abscisic acid (S4), jasmonic acid (S5), gibberellin (S6), and brassinosteroid (S7). The corresponding AGI number, gene, and mutant names, description in database, number of species containing co-orthologues, number of co-orthologues in the indicated plant species and the overall distribution in the groups of eudicots, monocots, mosses, and algae are indicated. The selected plant species are: Atha, *A. thaliana*; Bdis, *B. distachyon*; Crei, *C. reinhardtii*; Gmax, *G. max*; Mtru, *M. truncatula*; Osat, *O. sativa*; Ppat, *P. patens*; Ptri, *P. trichocarpa*; Sbic, *S. bicolor*; Slyc, *S. lycopersicum*; Stub, *S. tuberosum*; Vvin, *V. vinifera*; Zmay, *Z. mays*.

**Supplementary Tables 8–14.** Survey of co-orthologues involved in phytohormone biosynthesis, transport, and signaling in the analyzed plant genomes with prediction of intracellular localization and domain structure. Analyzed phytohormones are auxin (S8), ethylene (S9), cytokinin (S10), abscisic acid (S11), jasmonic acid (S12), gibberellin (S13), and brassinosteroid (S14). The selected plant species are: Atha, *A. thaliana*; Bdis, *B. distachyon*; Crei, *C. reinhardtii*; Gmax, *G. max*; Mtru, *M. truncatula*; Osat, *O. sativa*; Ppat, *P. patens*; Ptri, *P. trichocarpa*; Sbic, *S. bicolor*; Slyc, *S. lycopersicum*; Stub, *S. tuberosum*; Vvin, *V. vinifera*; Zmay, *Z. mays*.

**Supplementary Tables 15–21.** Expression of tomato co-orthologues involved in phytohormone biosynthesis, transport, and signaling in different tissues: root, stem, leaf, flower, and fruits at the three developmental stages, namely, mature green (fruit\_mature), breaker stage (berry\_breaker), and 10 days after turning red (berry\_10 days). Analyzed phytohormones are auxin (S15), ethylene (S16), cytokinin (S17), abscisic acid (S18), jasmonic acid (S19), gibberellin (S20), and brassinosteroid (S21).

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