There and back again: an evolutionary perspective on long-distance coordination of plant growth and development

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Abstract

Vascular plants, unlike bryophytes, have a strong root-shoot dichotomy in which the tissue systems are mutually interdependent; roots are completely dependent on shoots for photosynthetic sugars, and shoots are completely dependent on roots for water and mineral nutrients. Long-distance communication between shoot and root is therefore critical for the growth, development and survival of vascular plants, especially with regard to variable environmental conditions. However, this long-distance signalling does not appear an ancestral feature of land plants, and has likely arisen in vascular plants to service the radical alterations in body-plan seen in this taxon. In this review, we examine the defined hormonal root-to-shoot and shoot-to-root signalling pathways that coordinate the growth of vascular plants, with a particular view to understanding how these pathways may have evolved. We highlight the completely divergent roles of isopentenyl-adenine and *trans*-zeatin cytokinin species in long-distance signalling, and ask whether cytokinin can really be considered as a single class of hormones in the light of recent research. We also discuss the puzzlingly sparse evidence for auxin as a shoot-to-root signal, the evolutionary re-purposing of strigolactones and gibberellins as hormonal signals, and speculate on the possible role of sugars as long-distance signals. We conclude by discussing the 'design principles' of long-distance signalling in vascular plants.

Introduction: a life of two halves

The conquest of land presented new opportunities for photosynthetic plant life, but also created many new problems, particularly relating to the restricted availability of water. The ancestral land plant solution to these new problems, still seen in the extant bryophyte plant lineages, involved small, prostrate growth habits, damp habitats, and exceptional drought tolerance. In typical bryophytes, the main gametophytic body is laterally flattened (as a thallus or a network of filaments), such that each part of the plant is in contact with the substrate, and can locally acquire water and nutrients [1]. Specialized rhizoids are generally present on the underside of these surfaces, to maximise acquisition of water and nutrients, and to provide anchorage. Furthermore, the laterally flattened body allows effective photosynthesis across the whole plant, meaning that each part of the plant is effectively self-sufficient in the acquisition of resources from the environment (Figure 1A).

The bryophyte lifestyle remains limited by the need for surface water, and constrained by the surface area available, since only a single layer of photosynthetic tissue can be developed. Thus, we fairly quickly see in the fossil record the evolution of upright shoot systems (in plants such as *Aglaophyton*) [2] to maximise light harvesting, and later the evolution of downward-growing root systems (e.g. *Asteroxylon*) [3]. This trend toward specialized organ systems culminated in the full 'division of labour' seen in extant vascular plants, which have completely separate root and shoot systems, respectively specialized for water and nutrient capture, and for gas exchange and light harvesting. Together with the evolution of a vascular system for efficient redistribution of resources between shoot and root, this division of labour allowed plants to conquer a huge new range of habitats, and in many cases to grow to vertiginous size.

However, the 'flip-side' of this division of labour is that the two vascular plant organ systems stopped being self-sufficient, and instead became completely mutually inter-dependent; roots absolutely require shoots for the supply of fixed carbon, while shoots absolutely require root systems for the supply of water and mineral nutrients (Figure 1B). A successful vascular plant must therefore produce of a suitable balance of roots and shoots, such that each system remains adequately supplied and can adequately supply the other system. From a developmental biology perspective, this raises intriguing questions as to how vascular plants achieve this balancing act, especially since it is not simply a case of producing equal amounts of shoot and root tissue. Rather, the optimal root-shoot balance at a given time depends on the external environment, and the relative abundance of resources above- and belowground. Furthermore, since resource availability is constantly changing, the optimal root-shoot balance is also constantly changing. How then are plants able to coordinate the relative growth of the root and shoot systems in a spatio-temporal and environmentally sensitive manner – especially when, in the largest plant species this may involve coordinating events over distances of 100 metres or more?

Clearly, long-distance signalling must be deployed in order to achieve this coordination, with signals moving between the shoot and root and back again. And since this need for long-distance coordination of growth is peculiar to vascular plants, we might expect that long-distance communication itself has only evolved within vascular plants. Thus, the central thesis of this review is that major innovations in long-distance hormonal signalling must have occurred in vascular plants, and the overall aim of this review is therefore to examine long-distance coordination of root and shoot growth signalling in vascular plants, with respect to understanding how this coordination may have evolved.

A plethora of long distance, root-to-shoot (R-S) or shoot-to-root (S-R) signals have now been identified in the regulation of a wide range of plant processes, including plant physiology, plant defence responses and plant symbioses. For instance, the stress hormone abscisic acid (ABA) has been studied extensively as a possible R-S signal. ABA accumulates in the roots of drought-stressed plants, and there is evidence for its shootward transport via the xylem to signal water limitation, leading to shoot responses such as stomatal closure [4,5]. However, ABA is also synthesised in leaves in response to drought, and the relevance of root-shoot ABA transport is questionable [6–9]. Jasmonates (JA) are produced in response to tissue damage by pathogens and insect herbivores, which leads to activation of systemic-induced immunity in both above and belowground tissues [10]. Various microRNAs have been identified to move in the vasculature in response to nutrient deficiencies in the soil [11]. Shoot derived *miR399,* for example, triggers both the uptake and the R-S transport of phosphate under low phosphate conditions [12]. Small signalling peptides have also been identified to travel long distances across plants. Members of the CEP peptides family have been identified as R-S signals communicating physiological responses to nitrogen deficiency in the rhizosphere [13,14], while CLE-ROOT SIGNAL 1 and 2 (CLE-RS1 and CLE-RS2) are small peptides which are transported R-S in the xylem in response to root nodulation in leguminous plants [15]. As a broader phenomenon, these long-distance signals have been recently reviewed elsewhere [11,16], and this review will focus instead solely on hormonal signals that coordinate growth and development between the shoot and root systems. Of course, there are likely to be more, as-yet-unidentified signals that also coordinate root and shoot development than we cover here; given recent progress in identifying new signals in plants [17,18], we are likely to discover these sooner rather than later.

Trans-Zeatin type cytokinins are key root-to-shoot signals

Cytokinins (CKs) are adenine derivatives which stimulate a wide spectrum of developmental processes including cell differentiation and division, shoot and root meristematic activity, and vasculature development to name a few [11,19]. CKs are key hormones in shoot development, but have been more recently shown to regulate root development [20]. CK signalling is relatively well studied, however less is known about the molecular basis of CK control of development [20] and its role as a long-distance information carrier. Many cytokinin species have been identified, but the main

types are isopentenyl-adenine (iP), *trans*-Zeatin (*tZ*), aromatic CK and *cis*-Zeatin *(cZ)* [21] (Figure 2). Generally speaking, the 'free base' cytokinins are active species whereas the ribotide or riboside versions of the same molecule are inactive [22]. *Trans*-Zeatin type cytokinins are present and active in all higher plants whereas the *cis* type are active in fewer plant species, despite being ubiquitously present [22,23]. The biosynthesis of iP and *tZ* forms of CKs is well understood, however that of cZ type is less clearly defined [22].

In Arabidopsis, CK synthesis begins with the generation of iP-ribotides by isopentenyl-transferase enzymes (IPTs) [22], which are expressed in many tissue types in the roots, including root phloem companion cells [24]. Following this, trans-hydroxylation of iP ribotides is the dominant pathway for forming *tZ*-type cytokinins and is catalysed by cytochrome P450 enzymes of the CYP735A class [25]. CYP735A1 and CYP735A2 are highly expressed in the root vascular bundle in Arabidopsis [26], indicating *tZ* production is predominantly in the roots (Figure 2). Both *tZ*- and iP-ribotides must undergo a conversion to free base active forms by cytokinin nucleoside 5′-monophosphate phosphoribohydrolases of the LONELY GUY (LOG) enzyme family [27,28] (CK biosynthesis is discussed in more detail in [22]). LOG genes are expressed across the plant, indicating CK activation can occur throughout the plant [27]. This could suggest that active forms are not required to be transported long distances, since ribotides could be converted to active forms at their site of action.

CKs have been identified to move over both short and long distances [29]. Early research into CK transport involving exogenous application of radioactive CKs to leaves suggested that although most CKs are retained at the application site, some are translocated to other areas of the plant [30]. CKs have been identified in both phloem and xylem sap, indicating CK transportation via these routes [31,32]. It was originally suggested that CKs are solely produced in the roots and transported shootward [33], however more recent evidence has uncovered that CKs are synthesised in both roots and shoots, in many different tissue types [22] (Figure 2). *tZ*-type CKs are transported via the xylem with the major form being *tZ*-ribotides [29,33,34], though free base *tZ* is also present [35], in addition to other free base and conjugates at much lower quantities [29,34,36]. Conversely, iP-type CKs are primarily transported rootward in the phloem [37], with the main types including iP-ribosides and iP-ribotides [29]. However, understanding of rootward iP transport is limited compared to that of *tZ* transport. Grafting studies in Arabidopsis with the CK synthesis mutant *ipt1357* supports the idea that there are differing transport routes for *tZ* and iP CKs [21]. When WT rootstocks were grafted to *ipt1357* mutant scions, *tZ* CKs were recovered in shoots but iP types were absent. However, in the opposite grafting arrangement, iP CKs were recovered in the mutant root stocks but *tZ* type was not fully recovered [21], giving an indication on the directional nature of *tZ* and iP transport. Since iP and *tZ* have been shown to be transported in different directions in different tissues this indicates that they might have distinct roles in plant development (Figure 2).

Long distance shootward *tZ* transport in Arabidopsis requires *ABCG14*, a G-type ABC transporter. *ABCG14* is expressed in root stele cells, localising to the plasma membrane, and loads *tZ*-type cytokinins into the xylem [24,38]. *abcg14* knockouts show phenotypic similarities to CK biosynthesis mutants, with altered vasculature in the shoot, thin and short stems and smaller rosettes and inflorescences compared to WT [24,38]. They also have altered *tZ* distribution, with a 90% reduction in xylem CK content. Furthermore, this defective transport results in reduced *tZ* content in the shoot and over-accumulation in root tissues [38]. The defect in shootward transport of *tZ* was confirmed by shoot growth rescue of *abcg14* knockouts with exogenous application of *tZ* type CK. However, the *abcg14* mutants could not be recovered by iP-type cytokinins, further suggesting iP has a different role to *tZ*. When *abcg14* scions are grafted to WT root stocks, their shoot growth is restored to WT. Together, these findings show that *ABCG14* is essential for *tZ* transport and normal shoot development [24,38] (Figure 2).

The importance of root-derived *tZ* for shoot growth is further supported by the role of CYP735A enzymes [26]. Arabidopsis *cyp735a1 cyp735a2* double mutants have shoot growth defects such as a reduction in flower and flower bud numbers, short primary inflorescence stem, smaller rosette leaves and a smaller shoot meristem. Since these are similar phenotypes to *ipt357* (reduced CK levels) and *ahk2 ahk3* (reduced signalling) mutants, this suggests diminished CK activity in the shoots of the *cyp735a1cyp735a2* double mutant [26]. These shoot phenotypes can be rescued by *tZ* application whereas application of iP was unable to rescue the double mutant phenotype even at high concentrations [26]. Furthermore, when the *cyp735a1 cyp735a2* double mutant scions were grafted to WT rootstocks, their phenotype was rescued, and their shoot *tZ* concentration was the same as in WT plants. Interestingly, when WT scions were grafted to double mutant root stocks, there was little change to shoot phenotypes and CK concentration suggesting that *tZ* production in the shoot was sufficient to overcome the lack of *tZ* from the roots. Similarly, *log1234578* septuple mutants have severe shoot growth retardation associated with a lack of active CKs, which can be rescued by grafting to WT rootstocks, increasing the tZ content of the shoots back to WT levels [35]. Collectively, these data show that *tZ* has specific effects on shoot development that iP type cytokinins do not have, and that – since the primary site of *tZ* production is in the roots – *tZ* is a key R-S signal controlling shoot growth.

But what is the purpose of this R-S *tZ* pathway? What is being communicated, and why? For certain, the *tZ* pathway is involved in communicating the availability of mineral nutrients in the soil to the shoot system. The induction of cytokinin synthesis in roots by high nitrate levels is well established [34,39–41]; high nitrate stimulates the expression of CYP735A2 and IPT3 in roots, which increases *tZ* translocation from R-S and in turn activates shoot CK signalling [29,42–44]. IPT3 expression is also induced when additional iron, phosphate and sulphate are supplied to plant roots [29,42,45]. The ability to respond to mineral nutrient availability by increasing *tZ* production allows plants to

modify their shoot growth to match resource availability, for instance by increasing shoot branching and the rate of shoot meristematic activity [46,47]. Thus, the *tZ* pathway acts as 'integrated information', providing information to the shoot on the overall nutrient status of the soil, and promoting shoot growth in proportion to this nutrient status. Thus, although some nutrients – particularly nitrate and phosphate – have signalling functions in their own right, the value of the *tZ* pathway is that it provides a simplified, non-specific source of information. And since the signal is non-specific, it is reasonable to suppose that other soil stimuli could also influence *tZ* production. Thus, inhibitory factors such as limited soil volume or the presence of neighbouring plants could reduce *tZ* production even when nutrient levels are high, to prevent excessive shoot growth. The value of 'integrated information' signals such as *tZ* is therefore that the shoot does not have to integrate multiple conflicting signals, because this has already been performed at the point of signal generation – forming a highly parsimonious and effective system.

Cytokinins – two hormones for the price of one?

As discussed above, in contrast to *tZ*, iP-type CKs seem to be predominantly transported from S-R, and their presence in the shoot cannot compensate for loss of *tZ*. Similarly, *tZ* in the root cannot compensate for the loss of shoot-derived iP. iP-type CKs thus appear to act as a S-R signal in a way that is independent of the role of *tZ* as a R-S. Thus, by any meaningful functional definition, *tZ* and iP can hardly be considered to be the same hormone. In turn, this rather suggests that the whole concept of 'cytokinins' is erroneous, a 'bracketing' accident arising from the history of their discovery. Among the historically well-known effects of cytokinins, many of those effects are actually specific to *tZ*, while others are specific to iP. It would therefore not be completely unreasonable to suggest that plant science should abandon the monolithic concept of cytokinins entirely.

And yet, the exceptionally close structural relationship between iP and *tZ* can hardly be denied, nor their shared synthesis pathway, nor their interconvertibility, nor their common signalling pathway. A plausible hypothesis is therefore that early on in land plant evolution, cytokinins <u>*were*</u> a monolithic signal, with a single purpose. Indeed, this might still be the case in bryophytes, but although studies of cytokinin signalling in *Marchantia polymorpha* and *Physcomitrella patens* are starting to provide insights, it is probably too early to conclude whether there are separate *tZ* and iP effects [48–50]. So how did cytokinins become long-distance signals? And how did the dual-hormone system present in flowering plants evolve; how have two such closely related molecules ended up acting as such different signals? Fully resolving these questions will require, in particular, better understanding of the role of iP as a S-R signal, including the mechanism S-R transport, and their exact developmental role. In terms of *tZ* long-distance signalling, the evolution of ABCG14-type transporters to load *tZ* into the xylem must have been an important innovation in converting cytokinin from a local signal to a long distance one. And in terms of the evolution of the dual hormone system, it is clear that a major part of the answer to this question lies in the evolution of the cytokinin receptor family.

Cytokinins, both *tZ* and iP, are perceived by transmembrane receptors of the HISTIDINE KINASE family, which trigger a complex cytoplasm-to-nucleus phosphorelay transduction pathway upon activation [51–53]. In Arabidopsis, there are three defined CK receptors, ARABIDOPSIS HISTIDINE KINASE 2 (AHK2), AHK3 and AHK4 (also called CYTOKININ RESPONSE1/CRE1 and WOODEN LEG/WOL) [54–57]. Mutations in any individual AHK lead only to subtle phenotypes, but double and triple mutants have more severe phenotypes, particularly *ahk2 ahk3* [53,57–60]. Coupled with their ability to at least partially complement each other, it would be easy to treat them as a redundant gene family [61,62]. However, detailed investigation of their functions has revealed major differences in both their expression pattern across the plant body and their ligand binding affinities [55,56,62]. For example, using live-cell binding of transgenic CK receptor-expressing bacteria, both AHK3 and AHK4 were found to have high affinity towards *tZ*, whereas AHK4 had a 10-fold higher affinity for iP than AHK3 [63]. Similarly, the maize ZmHK2 (orthologous to Arabidopsis AHK3) is most sensitive to *tZ*, while ZmHK1, the maize orthologue of AHK4, has highest affinity for iP-type CK [64,65]. AHK2 also has a somewhat higher affinity for tZ than for iP [62], while the maize ortholog of AHK2, ZmHK3a, showed a similar preference to *tZ*, iP and *cZ* [65]. Loosely speaking, AHK3/ZmHK2 therefore seem to be *tZ* receptorsand AHK4/ZmHK1 seem to be iP receptors, while AHK2/ZmHK3 seem to act as multi-functional CK receptors. Consistent with the specific function of iP in roots, both AHK4 and ZmHK1 are highly expressed in roots, and much less so in shoots [55,56,65]. Thus, the inability of iP to rescue lack of tZ in shoots occurs because shoots are effectively insensitive to iP. Conversely, AHK2 and AHK3 are strongly expressed in shoots, consistent with the sensitivity of shoots to *tZ* [55,58]. Overall, the distinct R-S and S-R activities of *tZ* and iP can be seen to arise as a consequence of the expression of their receptors in the plant body (Figure 2). It should be noted that AHK3 is also expressed in the root meristem [66], where it mediates meristem size; this may reflect a local developmental effect of *tZ*, after its synthesis but before its translocation to the shoot.

Cytokinins thus represent a fascinating class of long-distance signals in plants, in which two completely distinct activities have likely arisen from a single origin. Their ‘split personality’ raises clear implications for experimental design; when we perform an exogenous cytokinin treatment, are we really stimulating the right pathway, or activating two distinct signalling systems together? Typically, studies use 6-benzylaminopurine (BAP; also known as 6-benzyladenine, BA) as an exogenous cytokinin treatment; where tested, BAP seems to bind to cytokinin receptors with rather weak and aspecific affinity [67,68]. As their complexity continues to become apparent, a whole raft of new questions regarding their function arise, and much more work is therefore needed to understand the evolution of this remarkable system.

Strigolactones: root-to-shoot signals, but how?

Strigolactones (SLs) were first identified as soil chemicals that stimulate the germination of the parasitic plant *Striga lutea* [69] and have since been identified as being involved in the stimulation of mychorrizal associations with plant roots [70] and as endogenous regulators of plant development (i.e. hormones) [71]. The first identified strigolactones were strigol and the more commonly found 5-deoxystrigol (5DS). Canonical strigolactones such as 5DS consist of a so-called 'ABC' tricyclic lactone moiety, attached to a butenolide 'D' ring by an enol-ether bridge [72]. A stereocentre in the ABC ring divides strigolactones into two stereoisomeric classes, the 4-deoxyorobanchol (4DO) and 5DS (described in detail in [72]) (Figure 3A). However, a large number of biologically active strigolactone molecules do not fit this canonical SL structure, although their activity is comparable to that of canonical SLs. These 'non-canonical' strigolactones lack the characteristic ABC structure but still have a clear D ring [72]. Therefore this suggests strigolactone activity depends primarily on the D ring [73]. To date, a large number of different canonical and non-canonical strigolactones identified.

The SL synthesis pathway begins with the conversion of β-carotene to the SL precursor carlactone (CL) by the enzymes CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), CCD8 and the isomerase DWARF27 (D27) [74]. Carlactone is then converted to active strigolactone by the MORE AXILLARY GROWTH1 (MAX1) cytochrome P450 enzyme, along with other biosynthesis steps that are not fully understood to date [75]. SL biosynthesis genes are highly expressed in the root, hence this is usually viewed as the key site of SL biosynthesis. In Arabidopsis, CCD7 and CCD8 are expressed in the root cap columella and MAX1 is expressed mainly in root vascular parenchyma cells [76]. Strigolactone is known to be exuded from roots into the soil to stimulate mycorrhizal symbioses [70], further supporting that idea SL biosynthesis occurs primarily in the roots. Consistent with this, root SL concentrations are 30 to 1000 times greater than in the shoot [77–79].

However, the effects of SLs on root development are rather weak and inconsistent between species [73,80,81]. Conversely, SLs control many aspects of shoot development including branch number and angle, internode elongation and leaf elongation [71,73,82,83]. Since mutations in any aspect of SL biosynthesis and signalling results in severe shoot phenotypes, it is clear that SLs are needed to regulate shoot growth, in turn suggesting that SLs are mobile R-S signals, an idea provided with strong support by grafting studies. Grafting of wild-type (WT) rootstocks to SL synthesis mutant scions in Arabidopsis, pea and petunia results in complementation of the shoot phenotypes by the rootstock, indicating R-S transmission of SLs across the graft junction [82,84–86]. However, the opposite graft arrangement (synthesis mutant rootstock grafted to WT scion), also results in a WT shoot phenotype, showing that SL synthesis can also occur in the shoot [76,82,87,88]. Synthesis intermediates can also be transported R-S, since grafting of *max1* roots to *ccd7* or *ccd8* mutant shoots produces a WT phenotype [76]. The carlactone synthesised by D27, CCD7 and CCD8 can

be transported to the shoot and acted upon by MAX1 there [75]. Furthermore, exogenous application of carlactone or other SLs to roots also rescued SL synthesis mutant shoot phenotypes [89–91]. Collectively, these data suggest a clear SL transport route from R-S.

It was originally proposed that SLs are transported R-S via the xylem [92,93]. In Arabidopsis and tomato, SL was found in the xylem sap following phosphate starvation when analysed by mass spectrometry. The xylem from phosphate-starved WT Arabidopsis was used in a germination assay, and induced 4-fold higher germination of parasitic plants than xylem sap from phosphate-sufficient plants [92]. Furthermore, root applied GR24 (an SL analogue) (Figure 3A) in hydroponically grown Arabidopsis was found to be transported to the stem and hypocotyl [92], further suggesting SL is transported R-S in the xylem. However, despite these rather convincing studies, the presence of SL in the xylem had not been confirmed in other studies [72]. Xylem sap contents were examined in rice, cucumber, sorghum, Arabidopsis, tomato and tobacco, but no SL was recorded in the xylem sap of these species [79]. Furthermore, application of strigol to rice roots resulted in a strong inhibition of shoot branching despite the lack of apparent strigol transport to the shoots [71]. Similarly, when GR5 (another SL analogue) (Figure 3A), was applied to Arabidopsis roots, branch production was strongly inhibited, but when GR5 was applied to buds this had a much smaller response [94]. These data suggest that some SLs might not be transported R-S, but might instead activate the transport of other signals to the shoot (which might be other SL species) [72].

Some species, such as sorghum, produce SLs of the 5DS-type, while others produce the 4DO-type (rice), or both types (tobacco) [95]. R-S transport of SLs was explored in hydroponically grown rice, tobacco and sorghum by applying four stereoisomers of 5DS- and of 4DO- type strigolactones to the roots of these plants. LC-MS showed that rice shoots contained only 4DO-type SLs, whereas sorghum shoots contained only 5DS-type with no 4DO stereoisomers. As the endogenous SL levels were below the limit for detection, the SLs recorded in the shoot must have been from the exogenous applications. This suggests that these species have to ability to selectively transport the strigolactone type they produce [95]. In tobacco where both SL types are produced, both are transported to the shoots, suggesting either that tobacco has a dual transportation system, or that the transportation system has evolved to allow transportation of both types [95]. Collectively, these data suggest R-S transportation of SL is a stereoisomer-selective process, which is not a likely property of xylem transport. Furthermore, R-S transport of SLs may be a slow process, since SL stereoisomers applied to roots were only detected in shoot tissue 20 hours post-treatment [95]. Using fluorescently labelled SL analogues applied to Arabidopsis seedling roots, Fridlender *et al*. (2015) showed that the analogue closest to natural SL structure was mainly present in the cell cytoplasm [96]. This suggests that a symplastic transportation method may be used for shootward transport [96] (Figure 3C), consistent with the slow and stereoselective transport of SLs.

Alternatively, a cell-to-cell transport mechanism could be provided at least in part by the PLEITROPIC DRUG RESISTANCE 1 (PDR1) ABC transporter family (Figure 3B). In petunia, *PDR1* is expressed in the hypodermal passage cells and cortex in the roots, and facilitates SL exudation into the rhizosphere; as a result, *pdr1* mutants have a significantly reduced ability to form mycorrhizal associations [97]. *PDR1* is also expressed in the stem in tissues adjacent to the vasculature, but is absent in axillary buds [97,98]. Intriguingly, *pdr1* mutants also have an increase in branching compared to WT, suggesting PDR1 function is important in the regulation of shoot development [97]. Following the application of GR24 to roots of *pdr1* mutants, there was significantly less GR24 in shoot tissues compared to WT controls [97,98]. This further suggests that PDR1 could also play a role in R-S SL transport (Figure 3B). Furthermore, as PIN-FORMED 2 (PIN2) and PDR1 are co-localised to the root tip cortex cells [99], PDR1 could have a similar function for SL transport as PIN2 does for shootward auxin transport [98].

However, there are also some major problems with the cell-to-cell model for SL transport. Aside from petunia and tobacco [97,100], there are no published reports of PDR1 functioning as a SL exporter in other plants [100], and the PDR1 family has been lost from the (non-mycorrhizal) Brassicaceae [97], suggesting it is more important for exudation than internal transport. Furthermore, given that no SL influx carrier has been identified in any species yet, there are still plenty of unanswered questions regarding SL transport. Other questions remain over the identity of the SLs (or SL-like molecules) that are actually transported to the shoot and regulate shoot development, given that root-applied strigol/GR5 can inhibit tillering with no apparent movement to the shoot [72,79,94]. The application of strigol to the roots may perhaps initiate the release of other SL molecules which then travel R-S to inhibit branching [72,95]. With the limited phylogenetic distribution of canonical SLs, and the lack of strigol transport in these experiments, this could mean that the long-distance signal inhibiting branching is more likely to be a non-canonical SL [72]. Thus, carlactone, or a non-canonical derivative of carlactone (Figure 3A), might be the SL R-S signal. Consistent with this, derivatives of carlactone have been identified in xylem of Arabidopsis and inhibit branch production in the shoot [90,91]. Testing this hypothesis however would be highly difficult due to the instability of these molecules, and application of these molecules to roots would be problematic for the same reason [72].

Even if the exact R-S signals are uncertain, there is little doubt that the SL synthesis and signalling pathways are involved in R-S signalling. In many ways, the situation is very comparable to CK, in which it is clear that the CK synthesis and signalling pathways are involved in R-S signalling, but only very specific CK molecules actually act as R-S signals. Unlike CK, there is currently no evidence that SLs move S-R, even though they can be synthesised in the shoot [101]. Elucidation of the mobile SL R-S signal remains a key goal, and similarly establishing whether all SLs are actually bioactive remains to be established; while many SLs have effects when applied to plants, they may require

conversion to active forms in plants (akin to gibberellins). Another incompletely resolved question is the overall purpose of the SL R-S pathway. It is clear that phosphate deficiency is one stimulus that promotes root SL synthesis, resulting in down-regulation of shoot growth to match resource availability [78]. However, it is less clear whether other rhizosphere stimuli also modulate the synthesis of SLs, and more work is thus needed to understand the exact function of SLs in R-S signalling.

Consistent with the main thesis of this review, there is now very strong evidence that the long-distance signalling role of SLs is indeed an innovation of vascular plants, and perhaps more specifically seed plants. All major land plant taxa possess the full complement of core SL synthesis enzymes, and where examined have been found to produce various SL molecules [102,103]. However, the specific SL signalling components defined in flowering plants, including the DWARF14 receptor family, and the SMAX1-LIKE7 proteolytic targets, are only present in seed plants [102,104]. While there are receptor proteins in lycophytes and ferns that could plausibly act in SL perception, there are no SL receptors in liverworts or hornworts, and possibly not in mosses either [104]. This has led to the suggestion that the ancestral role of SLs in land plants was in attraction of arbuscular mycorrhizal fungi, and that SLs have only been recruited as hormonal signals in seed plants (or possibly vascular plants more broadly) [102]. This recruitment of SLs as a hormonal signal would be entirely consistent with the need for new pathways for long-distance signalling in vascular plants, and especially in seed plants in which the shoot-root dichotomy reached its logical conclusion.

The emerging role of gibberellins as root-shoot signals

Gibberellins (GAs) are phytohormones regulating many aspects of plant development such as stem elongation, pollen maturation, seed germination, leaf expansion and flowering [105]. Over 130 GA species have been identified, but few have biological activity (which include GA_1, GA_3, GA_4 and GA_7), with non-bioactive GAs being either de-activated metabolites or precursors for the bioactive forms [106]. Gibberellins are mostly produced and used locally [107] but a few studies have suggested that endogenously produced GAs can move long-distance, both S-R and particularly R-S [108,109]. Studies in maize suggested that bioactive GAs from WT rootstocks are transported across graft junctions to complement GA synthesis mutant *dwarf1* and *dwarf5* scions [110]. Similar studies in pea showed that grafting scions of low-GA mutant plants to WT rootstocks also increased the shoot GA content [108]. More recently, to elucidate which GA species are mobile and transported long-distance, wild type Arabidopsis (Col-0) hypocotyls were micro-grafted to GA-deficient mutants which were defective in either early, intermediate or late steps of the biosynthesis pathway [109]. Developmental rescue was absent when WT rootstocks were grafted to late GA biosynthesis mutant scions, but present with early and intermediate mutants. This suggests the mobile compound is not an active GA, but a precursor that can be converted to bioactive gibberellin in the shoot. This mobile

intermediate was identified to be GA_{12}, which was found to be transported shootward in the xylem and rootward in the phloem [109].

The current evidence suggests that, like SLs, GA was probably only recruited as a hormonal signal in vascular plants (Hernández-García *et al.*, 2020, this issue), which would be consistent with the idea of significant innovation in hormonal signalling within vascular plants to allow long-distance communication. However, it is not currently clear what the relevance of GA R-S transport is. Grafting studies clearly show that substantial GA_{12} transport does occur, but the purpose of this movement, and its importance vis-à-vis shoot-derived GA are currently unclear. Further research into the nature of GA as a long-distance signal is thus highly warranted.

Is auxin a shoot to root signal?

Auxin (indole-3-acetic acid, IAA) is an absolutely essential plant hormone that is involved in most plant developmental processes [111–113]. IAA is widely synthesised across the plant body, although synthesis in the shoot is concentrated in new tissues and organs [114]. In flowering plants auxin undergoes highly directional, cell-to-cell transport in many tissues, a fact that has been clear since its first discovery [115]. This transport activity occurs on both small, local scales to drive patterning of tissues, and across long distances to connect tissues together in a coordinated manner [116–118]. The transport of auxin is exceptionally important for its activity, and generates developmental and signalling effects that cannot be generated simply by having more or less auxin in a given location [119]. Auxin transport requires three different families of membrane transport proteins, and operates under the so-called chemiosmotic theory [119–121]. At apoplastic pH (~5.5), auxin is largely protonated and can pass freely into cells through the plasma membrane. However, at cytoplasmic pH (~7), auxin becomes predominantly negatively charged, and can no longer diffuse through the plasma membrane. Thus, specific auxin efflux proteins are required to mobilize auxin from cells, and this role is fulfilled by apolarly-localised members of the ABCB family of ATP-BINDING CASETTE proteins [122], and by the PIN family of auxin efflux carriers, which can often adopt polar sub-cellular localizations [116]. It is the directional localization of PIN proteins that seems to be particularly important in generating directional cell-to-cell auxin transport. The AUX/LAX family of permease-like proteins form the third set of transporters, and promote auxin influx in regions of high auxin concentration [123,124].

PIN proteins are required in many tissues for the local, small-scale movement of auxin that is required to pattern tissues. For instance, in the shoot meristem, convergent movement of auxin driven by PIN proteins is required to generate new organs [99]. In flowering plants, PIN proteins are also required for the long-distance movement of auxin in the so-called ‘polar auxin transport stream’ (PATS) [117]. The PATS is created by the highly polarised auxin transport activity in cells of the

vascular-associated tissues in the shoot [117] (Figure 4A). These cells have PIN1 protein localised at their basal (rootward) faces, creating a channel for semi-quick (~1 cm per hour) rootward movement of auxin through the shoot system [117,125] (Figure 4A). All new organs in the shoot produce large quantities of auxin, and export this auxin into the PATS, which seems to be required for their growth. A less polar, lower-conductance activity of 'connective auxin transport', also driven by PIN proteins, allows organs to connect to the PATS and begin exporting auxin [117]. The auxin transport system thus ultimately connects all organs in the shoot system into one large rootward stream of auxin. Through the self-organizing properties of PIN protein localization, this system generates some interesting emergent properties, which allow organs to affect each other's growth through their indirect effects on the polar transport system [115].

Given this significant and unambiguous rootward transport of auxin through the shoot system, as well as the well-defined effects of auxin on root growth, it is easy to assume that auxin must be a S-R signal. Indeed, this view is supported by the expression of PIN proteins, which are present in vascular associated cells in an (apparently) continuous shoot-to-root auxin transport network. Furthermore, application of radiolabelled auxin to the root-shoot junction of Arabidopsis seedlings demonstrated that auxin did enter the root, and that this could be partially inhibited by the polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) [126]. However, while there is little reason to doubt that shoot-derived auxin does indeed enter the root system, the evidence that this has significant effects on root growth and development are surprisingly scarce.

In intact pea seedlings, radiolabelled ^{14}C IAA applied to shoot apical buds was transported slowly in the stem to the roots, and resulted in minor accumulation in lateral root primordia [127]. When epicotyls and cotyledons were removed from pea seedlings, this resulted in decreased numbers of lateral root primordia and decreased development of these into lateral roots [128]. Furthermore, this decrease can be partly rescued by application of IAA to the sites of excision in the shoot [129,130]. Similarly, when shoots were excised from Arabidopsis seedlings, this resulted in a 4-fold decrease in lateral root density and number compared to intact control plants [126]. Lateral root growth can be recovered by application of agar containing IAA at the root-shoot junction of shoot excised seedlings, with this response being dose-dependent, since greater concentrations of IAA result in greater lateral root number [126]. However, this shoot-derived auxin might only be important in early lateral root development. Bhalerao *et al.* (2002) identified a dramatic increase in IAA levels around day 5-7 post germination and the functionality of this was assessed by excision experiments [131]. When aerial tissues were excised from 4-day old seedlings the number of emerged and emerging lateral roots was much lower than intact plants; when only cotyledons were excised, lateral root number was intermediate between intact seedlings, and those where all the aerial tissues were excised [131]. When IAA was applied to excision zones at 4 days-post germination this allowed lateral root primordia (LRP) development to occur comparably to intact plants. Interestingly, when these

excisions were carried out at 7 days post germination there was no difference in LRP developmental stage between these seedlings and intact control seedlings [131]. Collectively, these data suggest that shoot-derived auxin is important for LR emergence in the first few days post germination but has little effect on the roots after 7 days post germination [131] (Figure 4C). On a related note, there are several mutants which have greatly elevated auxin transport in the shoot system, resulting in very high levels of auxin in the shoot-root junction [132]. However, these mutants typically have very mild root phenotypes [80], suggesting that the shoot-derived auxin may not be transported it into the root system in appreciable quantities.

Although auxin has not been shown to have strong S-R signalling effects, some consideration must be given to the developmental stages used in these experiments. Since every shoot organ exports auxin, the quantity of auxin arriving at the shoot-root junction is an excellent proxy for the size of the shoot system. We might therefore expect the root system to respond to this auxin level to match root growth to shoot growth. However, crucially, the root system should only respond when there is a mismatch in the root system's ability to supply the shoot with water and nutrients. In other words, the effect of shoot-derived auxin on the root should be 'gated' by the nutritional status of the plant. Thus, it is very likely that the effects of shoot-derived auxin on Arabidopsis seedling root growth are so small because – under experimental conditions – the root system is completely adequate to supply the shoot system.

Pleasingly, very recent work on peptide signalling supports these ideas, and gives some indication of how this gating may work. C-TERMINALLY ENCODED PEPTIDEs (CEPs) are small peptide signalling molecules which are produced in the root system, and which have been shown to move R-S [14]. At least 7 of the family have been shown to be upregulated in response to local nitrate depletion in the root system [14]. These peptides are perceived through cognate CEP RECEPTORS (CEPRs), in both the root and shoot system [13,14,133–136]. In the leaves, CEP perception by CEPR1 leads to production of previously uncharacterised 'CEP-DOWNSTREAM' peptides, which move S-R and modulate nitrate uptake physiology in the root [13]. New work now shows that CEPR1 signalling in the shoot also modulates S-R auxin transport, thereby influencing root system architecture [133] (Figure 4B). Mutants in CEPR1 in Arabidopsis or the orthologous COMPACT ROOT ARCHITECTURE2 (CRA2) gene in *Medicago truncatula* have increased auxin transport from S-R, which cannot be downregulated by addition of CEP [133]. This increased auxin leads to lateral roots with decreased root gravitropic setpoint angle (i.e. more vertically oriented), and also leads to at least some increase in lateral root density [133]. These results suggest that when the root system is deficient in nitrate, the production and R-S transport of CEPs acts to minimize auxin transport into the root system. Effectively, since it is unable to fulfil demand, the root system stops responding to shoot demand for increased root growth by closing off auxin supply – and, as discussed above, also shuts off CK supply to the shoot, to bring shoot growth into line with root nutritional status.

From an evolutionary perspective, and again consistent with our central thesis, there is very good evidence that the role of auxin as a long-distance signal is a specific innovation of vascular plants. While there is clear evidence of local auxin transport activity in bryophytes [137,138], there is no apparent long-distance, polar movement of auxin [139]. However, long-distant polar auxin transport is present in the earliest diverging vascular plant lineage (lycophytes) [140] and throughout seed plants, suggesting it is a common feature of vascular plants. Indeed, given the well-established role of auxin in patterning linear vascular networks in flowering plants, it seems very likely that the evolution of self-organizing long-distance auxin transport networks was also key in the evolution of long-distance vascular networks that allow fully dichotomous root and shoot systems, and which also permit the long-distance exchange of signalling molecules between root and shoot (and *vice versa*)

Do sugars act as shoot to root 'hormonal' signals?

Recent work has clearly demonstrated that some photosynthetically-derived sugars (sucrose and various hexoses) have a dual role in plants as both nutritional metabolites, and as signalling molecules independent of their nutritional role [141–143]. Several sugar signalling pathways have been identified, including the glucose sensor HEXOKINASE1, the SNF1-Related Protein Kinase1 (SnRK1) pathway, and the trehalose 6-phosphate (T6P) pathway [142]. These pathways regulate a range of metabolic processes, and extensively interact with hormonal signalling pathways to influence growth and development [144,145]. Sugar signalling has been particularly associated with promotion of flowering [146], and regulation of shoot branching [147]. Photosynthetic sugars are nutritionally required for root growth and function, and since they are not produced in the root system, they are transported from the shoots to the roots via the phloem [148]. There is thus little ambiguity that shoot derived sugars do act as long-distance signals for root growth [149], but a more interesting question is whether sugars have a quasi-hormonal signalling role in the roots, which is independent of their nutritional quality. Does abundant photosynthate from the shoot act as signal to promote root growth and organogenesis, rather than just facilitating the root growth already in progress? As far as we are aware, there are currently no conclusive studies that address these possibilities, but recent studies on the regulation of shoot branching by sugar signals, which used non-metabolisable sugars such as palatinose to dissect out the signalling role of sugars, clearly point the way for future investigations [147].

Also unclear at the moment is the evolutionary trajectory of the different sugar signalling pathways. However, an intriguing possibility – albeit one currently lacking in evidence – is that sugar signalling pathways specifically arose in vascular plants to 'convert' a purely metabolic signal to a quasi-hormonal one. As the dichotomous root and shoot systems evolved, photosynthetic sugars would have been transformed from a locally-produced resource (as in bryophytes) to a commodity being

produced and moved in bulk from shoot to root. Since overall sugar supply is a good proxy for shoot system size and status, this bulk movement of sugar effectively acts as ‘free’ information about the shoot – so long as there is a system to ‘decipher’ the signal, and use this information to regulate growth and development (rather than to simply fuel growth). We thus hypothesise that the evolution of at least some of the sugar signalling pathways occurred during the dichotomization of plants into distinct shoot and root systems, to utilise this existing long-distance signal.

Conclusion: design principles of long-distance signalling

The long-distance signalling pathways that are present in seed plants to facilitate the coordination of growth between shoot and root systems are elegantly simple and, typically for plants, extensively recycled from existing components (Figure 5, Figure 6). We currently do not know enough about long-distance signalling in lycophytes and monilophytes to say whether the same, or similar, pathways are present in these taxa (Figure 6). Indeed, since root systems are thought to have evolved independently in lycophytes, monilophytes and seed plants, it is probable that different S-R and R-S signalling pathways have evolved in each group. At the very least, given the association between long-distance auxin transport and vascular patterning, and the evidence of polar auxin transport in lycophytes [116], it seems likely that long-distance auxin transport evolved at the base of vascular plants (Figure 6). Cytokinins are ancient signalling molecules in land plants, and could also plausibly have been recruited as long-distance signals at the base of vascular plants. We currently do not know enough about CK transport across the vascular plants to reach any clear conclusions on this, but the evolution of CKs as long-distance signals is clearly a very intriguing question, especially given the remarkable situation in flowering plants where different CK metabolites effectively act as separate signals, moving in opposite directions through the plant. One purely speculative hypothesis, given the rootless condition of the earliest vascular plants, is that the basipetal (i.e. equivalent to S-R) transport of iP is an ancestral feature of vascular plants, with the R-S transport of tZ being a specific innovation in seed plants (Figure 6). In contrast to auxin and CK, which are certainly ancient signalling molecules, SLs and GAs were specifically converted into hormonal signals within the vascular plants by the evolution of novel signalling pathways for existing metabolites (Figure 6). In both cases, the repurposing of members of existing receptor families allowed the new hormonal signals to exert effects through pre-existing signalling pathways. And – we speculate – in the case of sugar signals, the pre-existing long-distance transport of a key metabolite may have acquired S-R signalling functions precisely because it was a readily available source of ‘information’ on shoot system status.

The ‘design principles’ of long-distance signalling in plants revolve around the simplicity of signal transmission. In both the root and shoot system, a large number of external environmental and internal developmental stimuli are ‘pre-integrated’ into a small number of signals [150]. Because of this pre-integration, the signals carry no specific meaning; these signals can be considered to carry ‘generic information’, rather than ‘instructions’ [150]. This pre-integration greatly reduces the number of signals that need to be transmitted to the opposite part of the plant, and also prevents the need for each responding organ to ‘decipher’ the plethora of information. A second property of the system that we have not really discussed here, but which is nevertheless a key principle, is feedback regulation between these signals. These signals extensively regulate each other, allowing fine-tuning of the system to prevent ‘over-response’ to any given signal, and making sure the physiology of the whole plant is taken into account when responding. For instance, both SLs and CK regulate the S-

R transport of auxin [151,152], while auxin positively regulates the synthesis of both SLs and CK [153,154], creating an interlocking system which balances the respective strength of positive (CK) and negative (SL) R-S signals, whilst also refining the strength of the S-R auxin signal.

A third key property of the system is its output simplicity. Generally speaking, the responding organs only have a small number of growth options – maybe only 'grow' and 'no grow' – so that simple signals are entirely sufficient to induce the appropriate response. The precise nature of the stimuli that led to a signal being produced is not important for the responding organ, only the magnitude of the growth response it should undergo. It is worth pointing out that where specific information does need to be transmitted between shoot and root (stomatal closure in response to drought being an obvious example), there are specific additional long-distance signals to achieve this. But in the case of growth and development, there are only a small number of long-distance signals because that is all is required to make the system work. While there doubtlessly remain additional still to be discovered – the CEPs are an excellent example, which help to explain some previous obscure features of the system – long-distance signalling systems have remained streamlined through vascular plant evolution for the simple reason that they are highly effective and efficient.

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FIGURES

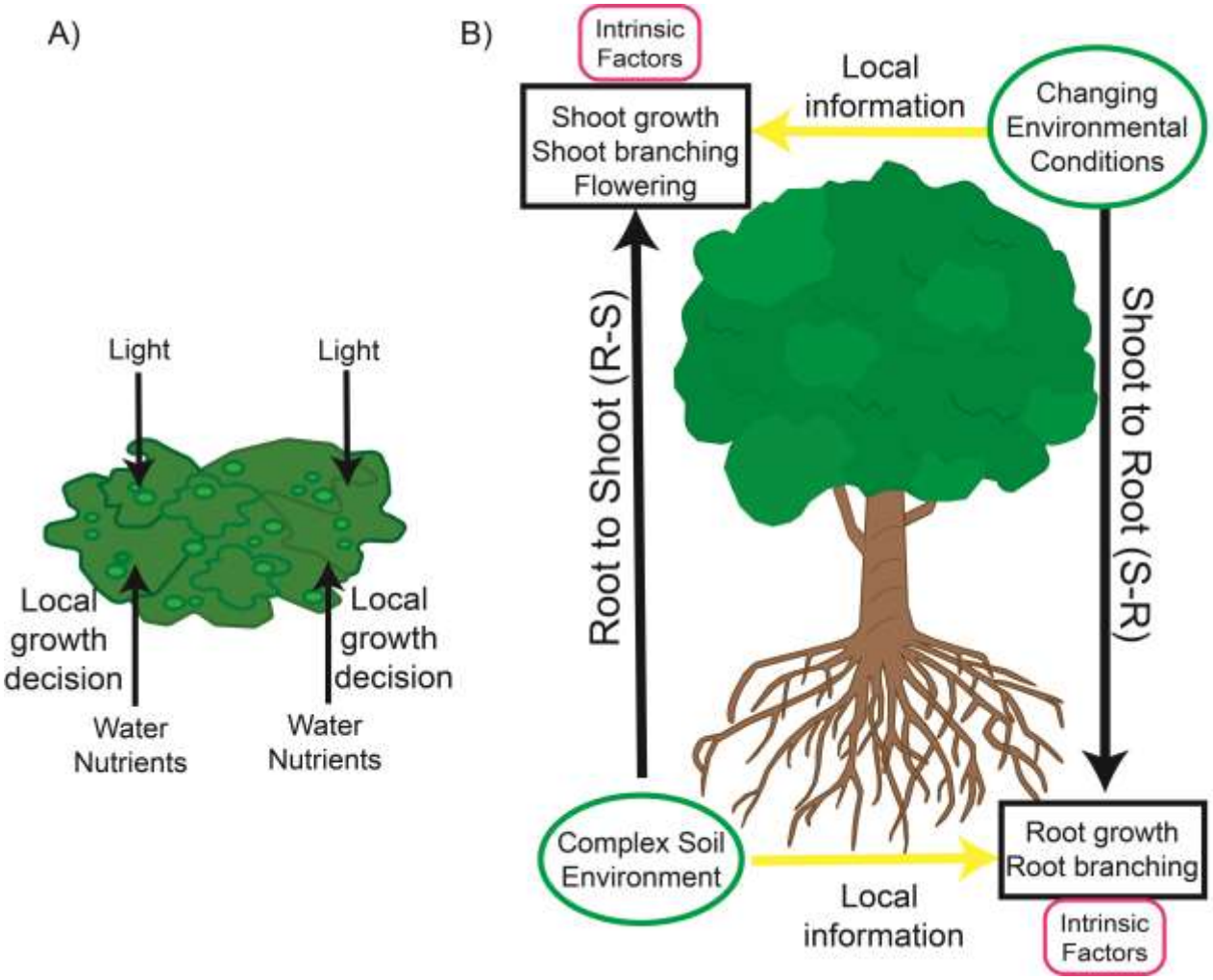

Figure 1: The Evolution of long distance signalling in plants

A) All parts of a bryophyte are able to locally acquire resources, and to make local growth decisions. B) The highly specialized shoot and root tissue systems of vascular plants exist in completely different environments, and only have access to certain resources. The growth decisions of both shoot and root systems must take into account local factors, but also the supply of, and demand for, resources in the other tissue system. The evolution of long-distance signalling permitted this coordination to occur.

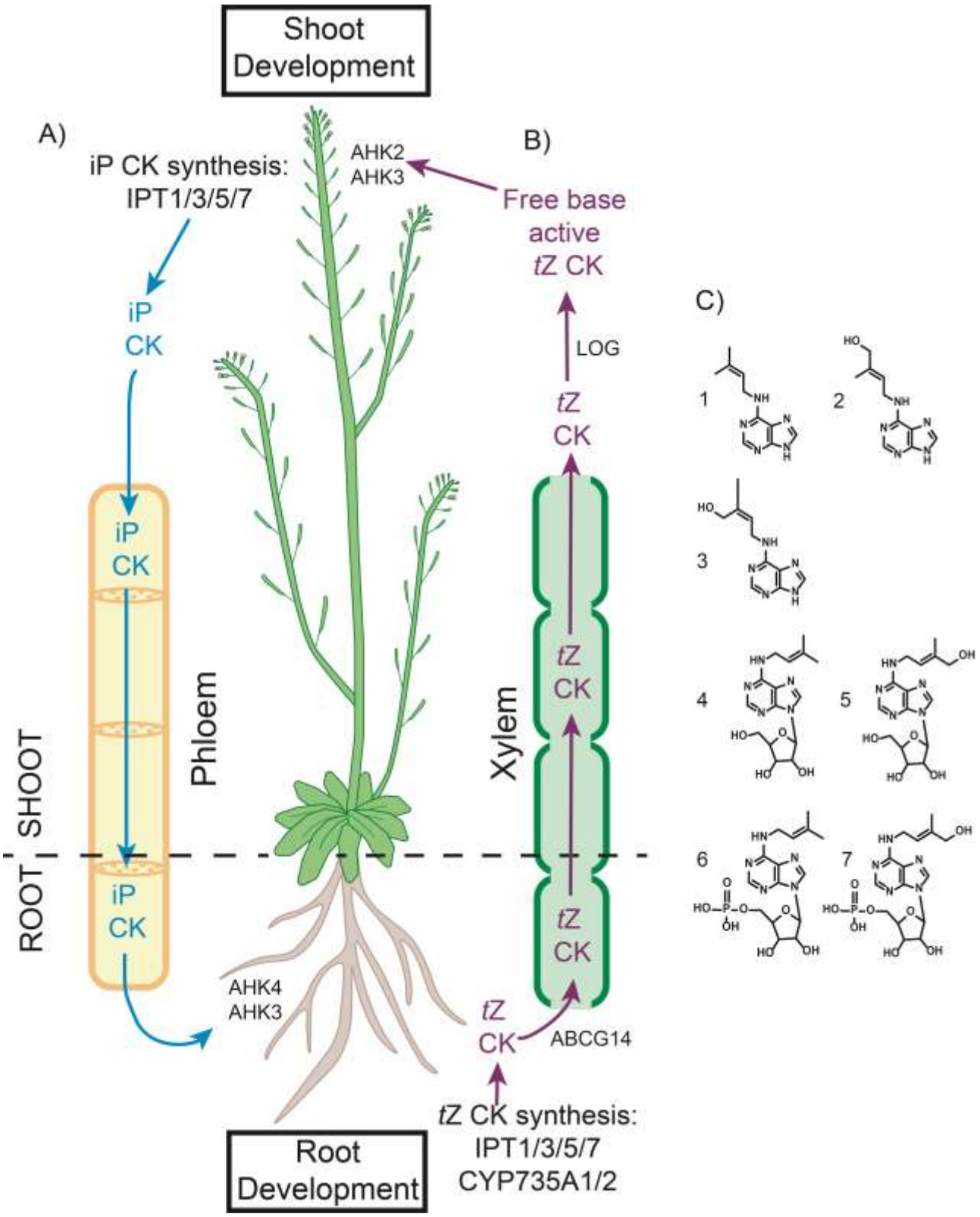

Figure 2: Cytokinins are key long-distance signals

A) Isopentenyl-adenine type cytokinin (iP) is synthesised in the shoot by IPT1/3/5/7 and transported to the roots via the phloem. In the roots, iP is perceived by AHK3 and AHK4 receptors, and promotes root development.

B) *trans*-Zeatin type CK (*tZ*) is synthesised in the roots by IPT1/3/5/7 and CYP735A1/2. *tZ* type CK is loaded into the xylem by ABCG14 and transported to the shoots. *tZ* is converted to free base

active type CK by LOG genes in the shoot. In the shoots AHK2 and AHK3 receptors perceive *t*Z, which regulates shoot development.

C) Structures of different cytokinin molecules. 1: Isopentenyl-adenine (iP), 2: *trans*-Zeatin (*t*Z), 3: *cis*-Zeatin (*c*Z), 4: isopentenyl-adenine riboside (iPR), 5: *trans*-Zeatin riboside (*t*ZR), 6: isopentenyl-adenine ribotide monophosphate, 7: *trans*-Zeatin ribotide monophosphate.

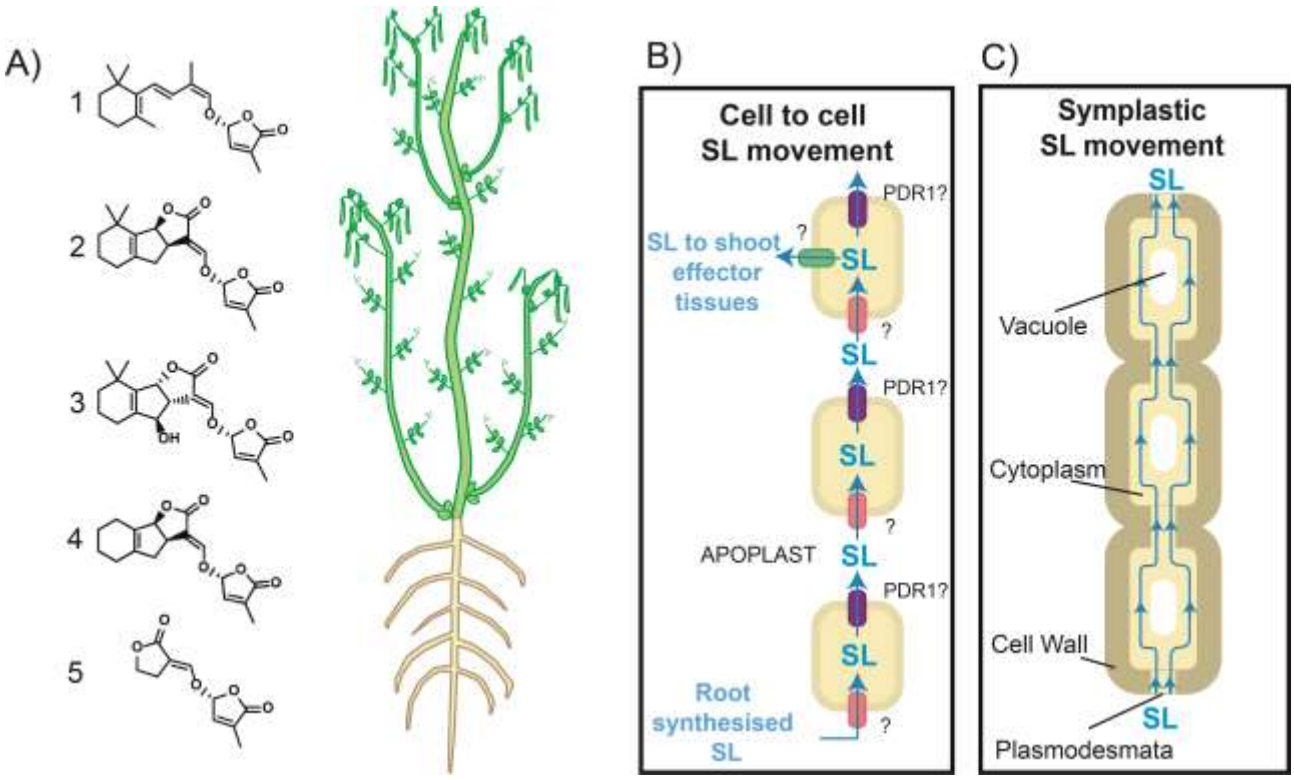

Figure 3: Strigolactones are key root-to-shoot signals

A) Chemical structures of SL molecules. 1: Carlactone, 2: (+)-5-deoxystrigol (5DS), 3: (-)-orobanchol, 4: (+)-GR24, 5: GR5.

B) Model for cell-to-cell transport of SLs from R-S. SL synthesised in the roots is imported into cells by an unknown transporter. SL is exported back into the apoplast by an exporter, possibly PDR1, although this protein is not present in Brassicaceae. In the shoot, the SL may additionally be unloaded by an additional exporter.

C) Model for symplastic movement of SLs from R-S.

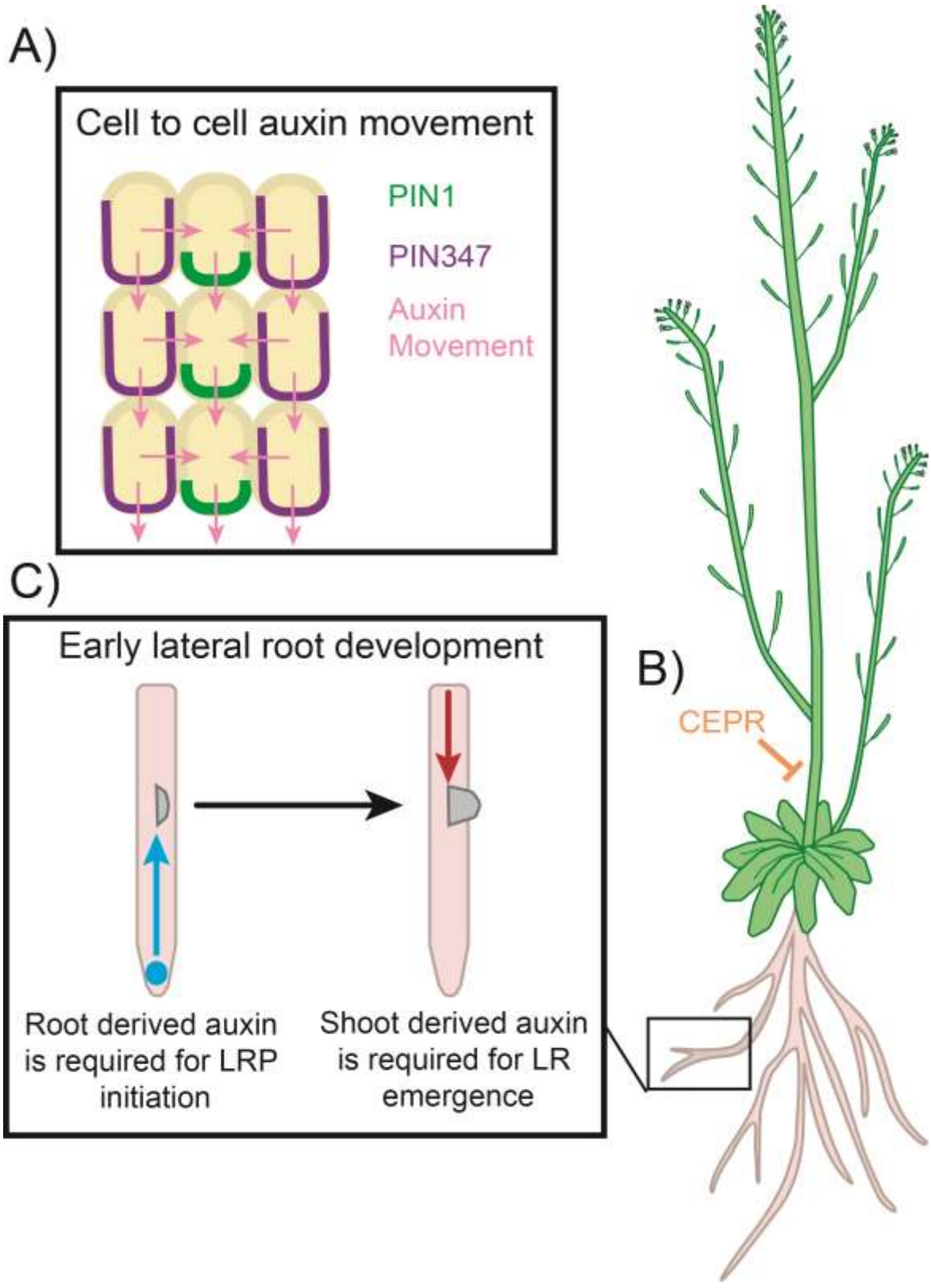

Figure 4: Auxin acts as a shoot-root signal

A) Auxin is transported on both local and systemic scales by cell-to-cell auxin movement. This movement requires auxin efflux carriers including non-polar ABCB transporters (not shown), and PIN efflux carriers which may have polar localizations. In the shoot, PIN3, PIN4 and PIN7 act to move auxin from tissues towards the 'polar auxin transport stream'. Vascular associated cells

transport auxin rootward in significant quantities in part due to the highly polarly-localized PIN1 protein.

B) CEP signalling peptides and their cognate CEP-RECEPTORS (CEPR) may act to ‘gate’ the transport of shoot-derived auxin into the root system in response to nitrate deficiency, helping to coordinate root and shoot growth.

C) Early lateral root development requires both root- and shoot-derived auxin.

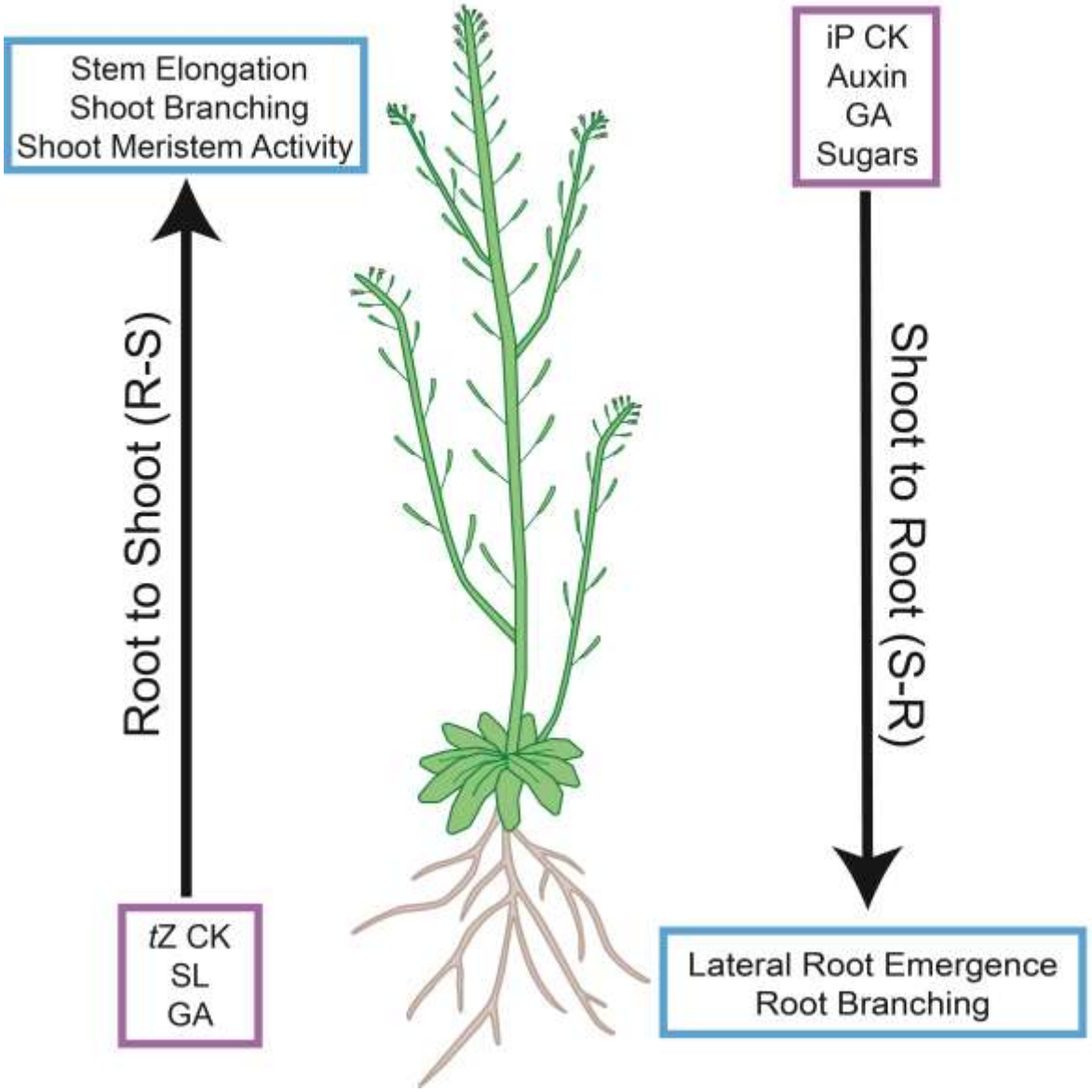

Figure 5: Summary of long-distance signalling pathways present in flowering plants.

Summary of the long-distance signalling pathways discussed in this review. *Trans*-zeatin cytokinin (*t*Z CK), strigolactone (SL) and gibberellin (GA) have been found to be transported from the roots to the shoots. Isopentenyl-adenine cytokinin (iP CK), auxin and sugars have been found to be transported from shoot to root.

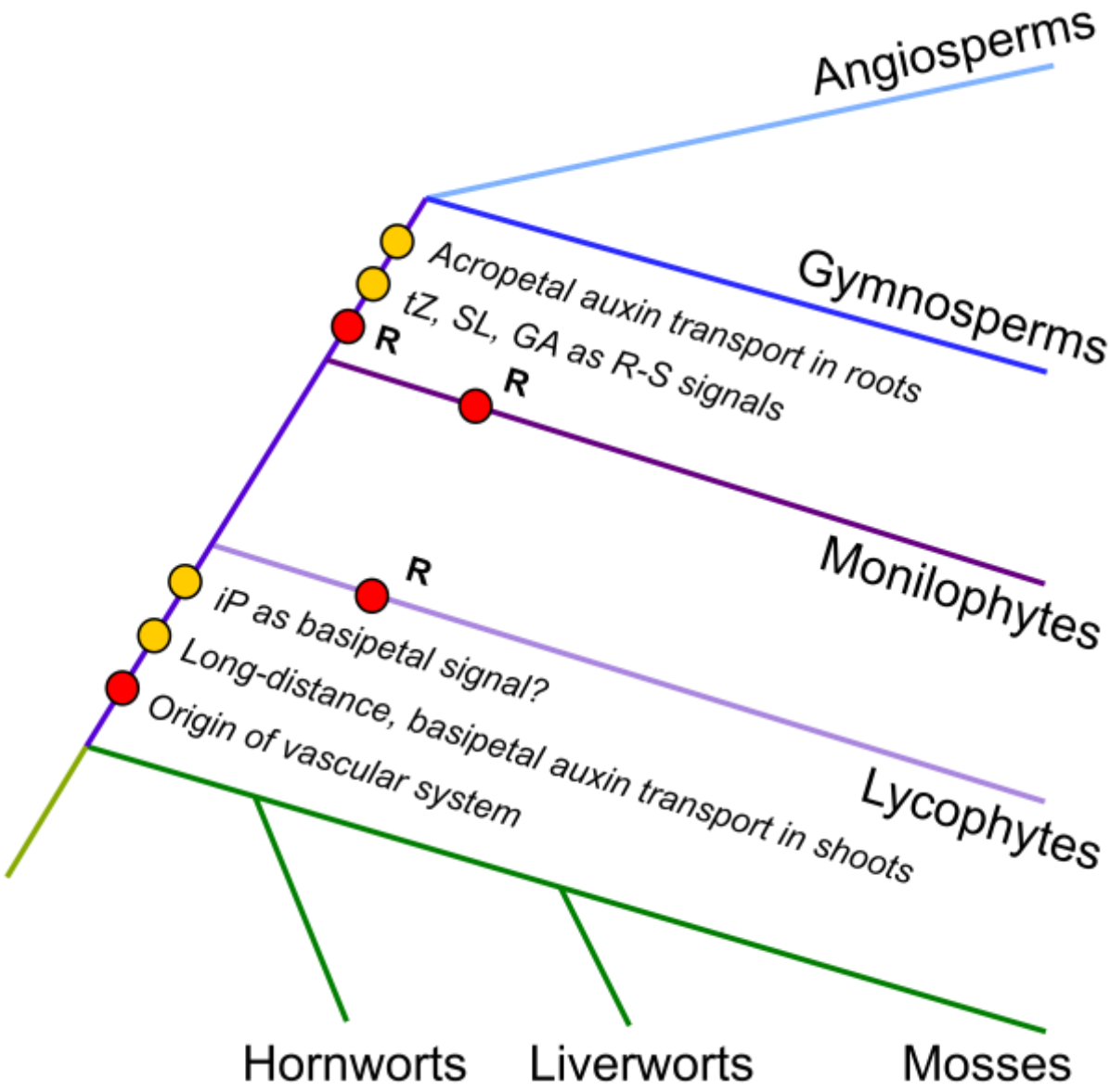

Figure 6: Distribution of key long-distance signalling innovations in land plants.

Phylogeny of land plants, showing major plant groups, and major innovations in body plan (red circles); R= independent origin of root system. Yellow circles show the inferred or possible positions of innovations in long-distance signalling in land plants.