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# <u>Auxin transport in the evolution of branching forms.</u>

C. Jill Harrison[†]

[†]Author for correspondence: jill.harrison@bristol.ac.uk, tel: +44 39411890117

Present address: School of Biological Sciences. University of Bristol, 24 Tyndall Avenue, Bristol, BS8 1TQ, UK.

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## Summary

Branching is one of the most striking aspects of land plant architecture, affecting resource acquisition and yield. Polar auxin transport by PIN proteins is a primary determinant of flowering plant branching patterns regulating both branch initiation and branch outgrowth. Several lines of experimental evidence suggest that PIN-mediated polar auxin transport is a conserved regulator of branching in vascular plant sporophytes. However, the mechanisms of branching and auxin transport and relationships between the two are not well known outside the flowering plants and the paradigm for PIN-regulated branching in flowering plants does not fit bryophyte gametophytes. The evidence reviewed here suggests that divergent auxin transport routes contributed to the diversification of branching forms in distinct land plant lineages.

**Keywords: land plant evolution, evo-devo, branching, auxin transport, PIN**

## I. Diversification of branching forms in land plants

Branching forms have evolved multiple times across the tree of life to optimise exchange between organisms and their environment. In land plants, shoot branching serves two main purposes (1) to optimize photosynthetic efficiency during indeterminate growth (Niklas & Kerchner, 1984) and (2) to determine reproductive output by affecting the number and position of **sporangia** (Niklas, 2004). Branching thus affects plant fitness and yield. Land plants have biphasic life cycles in which a **gametophyte** phase alternates with a **sporophyte** phase, and branching forms have arisen with independent evolutionary trajectories in each life cycle stage (Figure 1) (Harrison, 2016). Current hypotheses of plant interrelationship suggest that vascular plants emerged as a **monophyletic** group from an ancestor shared with bryophytes, but the relationship of bryophytes to vascular plants is contested- bryophytes either form a **paraphyletic** grade at the base of the land plant tree of life (Wickett *et al.*, 2014), or form a monophyletic sister group to vascular plants (Figure 1) (Wickett *et al.*, 2014; Cox *et al.*, 2014). These alternative toplogies bear on

45   inferences of the number and direction of branching innovations in land plant evolution in both life cycle
46   stages.

47   A general trend in land plant evolution has been the progressive elaboration of the sporophyte stage of the
48   life cycle at the expense of the gametophyte stage of the life cycle, and sporophytic branching innovations
49   may have contributed to this trend (Niklas, 1997; Niklas, 2004). Whilst the sporophytes of bryophytes are
50   small, comprising a single axis that terminates in sporangium formation, the sporophytes of vascular plants
51   can attain massive sizes as an outcome of branching and indeterminate growth (Figure 1C) (Harrison,
52   2016). Two fossils have forms that are intermediate between living bryophytes and vascular plants.
53   *Partitatheca* is a non-vascular fossil with tiny sporophytes that have stomata and branch (Figure 1E), a
54   character combination suggesting a close affinity to bryophytes (Edwards *et al.*, 2014) and *Cooksonia* fossils
55   reiterate the *Partitatheca* branching pattern but have vascular tissue (Boyce, 2009). These fossils suggest
56   potential steps in the elaboration of sporophyte branching (Figure 1). The number and direction of
57   branching innovations in gametophyte evolution is not yet clear, save to say that there have been multiple
58   origins of branching axial forms in bryophytes and monilophytes, and probably many reversals.

## II. Roles for auxin transport in branching

60   The mechanisms regulating branching are relatively well characterised in flowering plants, and transport of
61   the plant hormone auxin plays a key role (Domagalska & Leyser, 2011). This was first demonstrated by
62   surgical decapitation experiments in which the shoot apical **meristem** of *Vicia faba* was excised, a
63   manipulation that allows buds initiated in leaf axils to grow out as branches (Thimann & Skoog, 1933). If
64   excision was followed by replacement of the meristem with an agar block impregnated in auxin, the release
65   from suppression did not occur. This experiment suggested that auxin made at the shoot tip can move
66   through a plant to suppress branch outgrowth, a phenomenon known as apical dominance (Thimann &
67   Skoog, 1933). A requirement for long-range **basipetal** polar auxin transport was demonstrated by
68   application of auxin transport inhibitors to a region of the stem, and the subsequent outgrowth of branches
69   below the site of application (Panigrahi & Audus, 1966).  More recent work has demonstrated that auxin
70   transport is also required for branch initiation, a process that is intimately linked to leaf initiation (Galweiler
71   *et al.*, 1998; Blakeslee *et al.*, 2007; Bainbridge *et al.*, 2008; Wang, Q *et al.*, 2014; Wang, Y *et al.*, 2014). As
72   leaf **primordia** start to grow out from the shoot apical meristem, an auxin minimum forms in the crease
73   between the new leaf and the shoot apical meristem, and axillary branch meristems form in this region
74   (Wang, Q *et al.*, 2014; Wang, Y *et al.*, 2014).

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## III. Mechanisms for auxin transport in branching

Both the branch initiation and outgrowth processes above are dependent on **PIN** proteins, a class of auxin efflux facilitator with diverse roles in plant development (Petrasek & Friml, 2009). PINs generate directional auxin transport by inserting asymmetrically into plasma membranes (Galweiler *et al.*, 1998). PINs play a key role in leaf initiation by directing auxin to foci on the dome of the shoot apical meristem to determine the position of leaf initiation (Galweiler *et al.*, 1998; Reinhardt *et al.*, 2000). As leaf primordia start to grow out PINs direct auxin transport away from the crease forming between the shoot apical meristem and the new leaf, leading to the formation of auxin minima and axillary meristem initiation (Wang, Q *et al.*, 2014; Wang, Y *et al.*, 2014). PINs play a key role in branch outgrowth by generating and modulating long-range basipetal auxin transport in the stem as well as modulating the action of other hormonal cues on branching (Okada *et al.*, 1991; Shinohara *et al.*, 2013; Bennett *et al.*, 2016). **ABCB/PGP** auxin efflux transporters also effect long-range basipetal transport and modulate branch outgrowth, acting synergistically with PINs (Noh *et al.*, 2001; Blakeslee *et al.*, 2007), and **AUX/LAX** influx symporters effect auxin influx in leaf initiation, thereby modulating branching (Swarup *et al.*, 2004; Yang *et al.*, 2006; Bainbridge *et al.*, 2008). Cell to cell connectivity via **plasmodesmata** has recently been identified as a further potential route for auxin transport in the regulation of plant development (Han *et al.*, 2014), and deposition or break down of the cell wall polymer callose by *CalS* and *GHL* genes is one way that the size of plasmodesmatal openings can be actively regulated. All aforementioned families are conserved within the land plants and are therefore potential contributors to auxin transport in the evolution of branching forms (Del Bem & Vincentz, 2010; Carraro *et al.*, 2012; Bennett, T *et al.*, 2014; Gaudioso-Pedraza & Benitez-Alfonso, 2014; Lane *et al.*, 2016).

## IV. PIN-mediated polar auxin transport is a conserved regulator of branching in vascular plants

The bulk basipetal pattern of auxin transport that regulates **axillary branching** in flowering plants can be measured using radiolabelled auxin transport assays (Figure 1D, Table 1) (Goldsmith, 1966; Goldsmith, 1977). Tritiated auxin is applied to one cut surface of a stem segment, and its passage through the stem is monitored by extracting auxin from a portion at the other end of the segment in scintillation fluid. The rate of auxin transport is inferred by measuring radioactive decay using a scintillation counter in relation to time allowed for transport (Lewis & Muday, 2009). Such assays demonstrate that bulk basipetal auxin transport is a conserved property of vascular plant sporophytes (Steeves & Briggs, 1960; Wochok & Sussex, 1973; Wochok & Sussex, 1974; Walters & Osborne, 1979), and **PATI** sensitivity suggests that PINs provide the transport (Sanders & Langdale, 2013). The branching mechanism of basal vascular plant lineages involves **bifurcation** of the main shoot apex (in lycophytes and some monilophytes) or the establishment of new apices in leaf axils (in monilophytes; Figure 1) (Bierhorst, 1977; Harrison *et al.*, 2007; Schneider, 2012), but links between PIN-mediated **PAT** and branching are yet to be identified. However, decapitation

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experiments in both lycophytes and monilophytes suggest that there is likely conservation. In the lycophyte *Selaginella*, apical decapitation can result in the outgrowth of angle meristems initiated at branch divergence points, mirroring the PIN-mediated plasticity of branching in flowering plants, and auxin applied at the tip counteracts this effect (Williams, 1937; Seidl, 1941; Jernstedt *et al.*, 1994). Similarly, decapitation can result in bud formation from the rhizome in ferns (Wardlaw, 1965). PATI sensitive basipetal auxin transport can be detected in the single-stemmed axes of moss sporophytes (Poli *et al.*, 2003; Fujita *et al.*, 2008), and disruption of *Physcomitrella patens (Physcomitrella)* PIN function can induce branching to generate a similar form to the most ancient branching fossils (Fujita *et al.*, 2008; Bennett, TA *et al.*, 2014). Current evidence suggests that PIN-mediated PAT is a conserved regulator of sporophyte branching in land plants.

## V. Axial auxin transport is non-polar or weakly polar in liverwort and hornwort sporophytes and bryophyte gametophytes

Although moss sporophytes show PATI sensitive bulk basipetal auxin transport (Poli *et al.*, 2003; Fujita *et al.*, 2008), other bryophyte sporophytes do not (Figure 1C, D). Transport assays in the hornwort *Phaeoceros pearsonii* detect low rates of PATI-insensitive transport in both directions in sporophyte axes, consistent with a diffusive mechanism (Poli *et al.*, 2003). Assays similarly detect low rates of bidirectional transport in the liverwort *Pellia epiphylla*, but transport is PATI-sensitive suggesting the involvement of membrane transporters (Thomas, 1980; Poli *et al.*, 2003). Transport in bryophyte gametophytes can be polar (Maravolo, 1976; Rose & Bopp, 1983; Rose *et al.*, 1983), bidirectional (Fujita *et al.*, 2008; Coudert *et al.*, 2015; [a]Piatowski *et al*., 2012), or have a weak directional bias (Gaal *et al.*, 1982), and transport may or may not be sensitive to PATI and **NOA** inhibitors (Maravolo, 1976; Rose & Bopp, 1983; Fujita *et al.*, 2008; Bennett, TA *et al.*, 2014; [a]Piatowski *et al*., 2012) that interfere with membrane transporter function (Geldner *et al.*, 2001; Parry *et al.*, 2001). The roles of PIN proteins and other auxin transporters have not yet been characterised in liverworts and hornworts, so the mechanisms for transport in these groups are not clear. In *Physcomitrella* **gametophores** PINs can target to the plasma membrane and are likely to generate short-range directional transport in a manner similar to the pattern observed in flowering plant shoot apices (Bennett, TA *et al.*, 2014). Disruption of PIN function and PATI application induces defects in gametophore apex function and leaf development (Bennett, TA *et al.*, 2014; Viaene *et al.*, 2014), but not gametophore branching (Coudert *et al.*, 2015). In combination, these data suggest that the roles of PIN-mediated auxin transport in branching are not conserved between bryophytes and vascular plants; the moss sporophyte exception discussed above may reflect convergence.

## VI. Apical dominance without PIN-mediated polar auxin transport in a moss

Recent work in *Physcomitrella* gametophores suggests an alternative route for auxin transport in the regulation of branching (Coudert *et al.*, 2015). Branches initiate by respecification of epidermal cells into

apical cells in leaf axils after the leaf has developed, and subsequent outgrowth is not delayed (Coudert *et al.*, 2015). Surgical decapitation experiments similar to the experiments undertaken in flowering plant sporophytes showed that the main gametophore apex is an auxin source that acts at a distance to suppress branch initiation (von Maltzahn, 1959; Nyman & Cutter, 1981; Coudert *et al.*, 2015). *Physcomitrella pinab* mutants initiate branches in a fairly normal pattern, and although there are two other PINs in *Physcomitrella*, application of **NPA** to *pinab* mutants does not disrupt branching further (Coudert *et al.*, 2015). These results suggest that PINs do not provide the auxin transport required to regulate branch initiation, and a computational modelling approach showed that the measured bi-directional property of auxin transport in moss gametophores is required to attain realistic branch initiation patterns (Coudert *et al.*, 2015). Modelling also predicted that variation in the rate of bidirectional auxin transport should perturb branch initiation patterns, and pharmacological treatments with the callose synthesis inhibitor **DDG** (Jaffe & Leopold, 1984) perturbed initiation patterns in a similar way to model predictions (Coudert *et al.*, 2015). These observations point to a role for auxin transport via plasmodesmata in branching in a moss, but links between auxin transport and branching have not yet been interrogated in other bryophytes.

## VII. Conclusions and future perspectives

As yet there is very scant data relating to the auxin transport properties of non flowering plants. The bryophyte data discussed here were obtained by different labs and have used different taxon sampling and techniques. In some instances experiments with the same species or different species within the same lineage have generated different results (Maravolo, 1976; Gaal *et al.*, 1982; Poli *et al.*, 2003; [a]Piatowski *et al.*, 2012). Branching patterns and their developmental basis are also poorly characterised in non-flowering plants (most notably in monilophytes), and land plant phylogeny remains contested at key nodes for inferences relating to branching evolution (Cox *et al.*, 2014; Wickett *et al.*, 2014). Despite these deficiencies in our knowledge, the data discussed suggest that divergent auxin transport pathways were recruited to regulate branching in land plant gametophytes and sporophytes during evolution. Current evidence suggests that PIN-mediated basipetal auxin transport is a conserved mechanism within vascular plants (Sanders & Langdale, 2013), and a conserved regulator of branching. Recent work shows that there is also bulk basipetal auxin transport in moss sporophytes, and demonstrates that perturbing PIN function can induce bifurcation (Fujita *et al.*, 2008; Bennett, TA *et al.*, 2014). In contrast bi-directional auxin transport regulates gametophytic branching patterns, potentially via plasmodesmata (Coudert *et al.*, 2015). The nature of auxin transporters in liverworts and hornworts, the ancestral mechanism for auxin transport within land plants, and links between auxin transport and branching in bryophyte gametophytes are open questions that are now amenable to experimental manipulation.

## Acknowledgements

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## Figure legend

**Figure 1: Auxin transport pathways in the evolution of branching forms.**

(A) Summary of axial gametophyte forms in bryophytes and monilophytes. Whilst hornwort and liverwort thalli branch dichotomously (Parihar, 1967), leafy liverwort and moss gametophores branch laterally (Buchloh, 1951; von Maltzahn, 1959; Crandall-Stotler, 1972; Berthier, 1973; La Farge-England, 1996; Coudert *et al.*, 2015). Monilophyte prothalli bifurcate or branch laterally (Nayar & Kaur, 1971; Schneider, 2012).

(B) Summary of documented auxin transport properties in bryophyte and monilophyte gametophytes. Whilst hornwort thalli have no detectable basipetal transport ([a]Piatowski *et al*., 2012), liverwort thalli may (Maravolo, 1976) or may not (Gaal *et al.*, 1982; [a]Piatowski *et al*., 2012) have basipetal transport and transport may or may not be PATI sensitive (Maravolo, 1976; Gaal *et al.*, 1982; [a]Piatowski *et al*., 2012) and NOA sensitive ([a]Piatowski *et al*., 2012). In moss gametophores the patterns of transport are consistent with bi-directional transport by membrane transporters or plasmodesmata (Fujita *et al.*, 2008; Coudert *et al.*, 2015), and a functional link between auxin transport and branching has been demonstrated in *Physcomitrella* (Coudert *et al.*, 2015). Although there is PIN-mediated auxin transport in *Physcomitrella* gametophores (Bennett, T *et al.*, 2014; Bennett, TA *et al.*, 2014; Viaene *et al.*, 2014), it is a minor contributor to the regulation of branching patterns. Experiments with DDG callose synthesis inhibitors (Jaffe & Leopold, 1984) support the hypothesis that a plasmodesmatal mechanism regulates branching (Coudert *et al.*, 2015). Monilophyte gametophyte development is sensitive to NPA (Gregorich and Fisher, 2006).

(C) Summary of sporophyte forms in land plants. Whilst bryophytes have uni-axial sporophytes that terminate with sporangia (Parihar, 1967), vascular plants branch by bifurcation (Bierhorst, 1977; Harrison *et al.*, 2007) or branch laterally (Domagalska & Leyser, 2011).

(D) Summary of documented sporophytic auxin transport properties in land plants. Hornwort sporophyte axes do not directionally transport auxin and are PATI insensitive suggesting a diffusive mechanism (Poli *et al.*, 2003). Liverwort sporophyte axes similarly lack directionally biased auxin transport, but transport is PATI sensitive (Poli *et al.*, 2003), consistent with a contribution for membrane transporters and/or plasmodesmatal connectivity. Moss sporophytes have bulk basipetal auxin transport (Poli *et al.*, 2003; Fujita *et al.*, 2008) that is PATI (Poli *et al.*, 2003; Fujita *et al.*, 2008) and NOA (Poli *et al.*, 2003) sensitive. Disruption of PIN function causes sporophytic abnormalities including bifurcation (Fujita *et al.*, 2008; Bennett, TA *et al.*, 2014). The data suggest that bulk basipetal transport in mosses is PIN-mediated, and that the innovation of sporophytic branching forms may have involved changes in PIN function (Harrison, 2015). Lycophyte (Wochok & Sussex, 1973; Wochok & Sussex, 1974; Sanders & Langdale, 2013) and monilophyte (Walters & Osborne, 1979) sporophytes have bulk-basipetal transport that is PATI sensitive

392  (Hou *et al.*, 2004; Sanders & Langdale, 2013). In a lycophyte, PATI application disrupts bifurcation (Sanders

393  & Langdale, 2013), but as yet there are no functional data on the nature of auxin transporter involved. In

394  angiosperms, NPA sensitive (Geldner *et al.*, 2001) PIN-mediated bulk basipetal auxin transport is a major

395  determinant of branching patterns (Domagalska & Leyser, 2011) and NOA sensitive AUX/LAX auxin influx

396  carriers (Bennett *et al.*, 1996; Parry *et al.*, 2001; Bainbridge *et al.*, 2008), **BUM** sensitive ABCB/PGP efflux

397  carriers (Noh *et al.*, 2001; Geisler *et al.*, 2005; Blakeslee *et al.*, 2007; Cho *et al.*, 2007; Kim *et al.*, 2010) also

398  regulate branching.

399  (E) Current hypothesis of phylogenetic relationships between major land plant groups (Cox *et al.*, 2014;

400  Wickett *et al.*, 2014) showing potential steps involved in the innovation of branching forms as suggested by

401  the fossil record (Edwards *et al.*, 2014).

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**Figure 1**
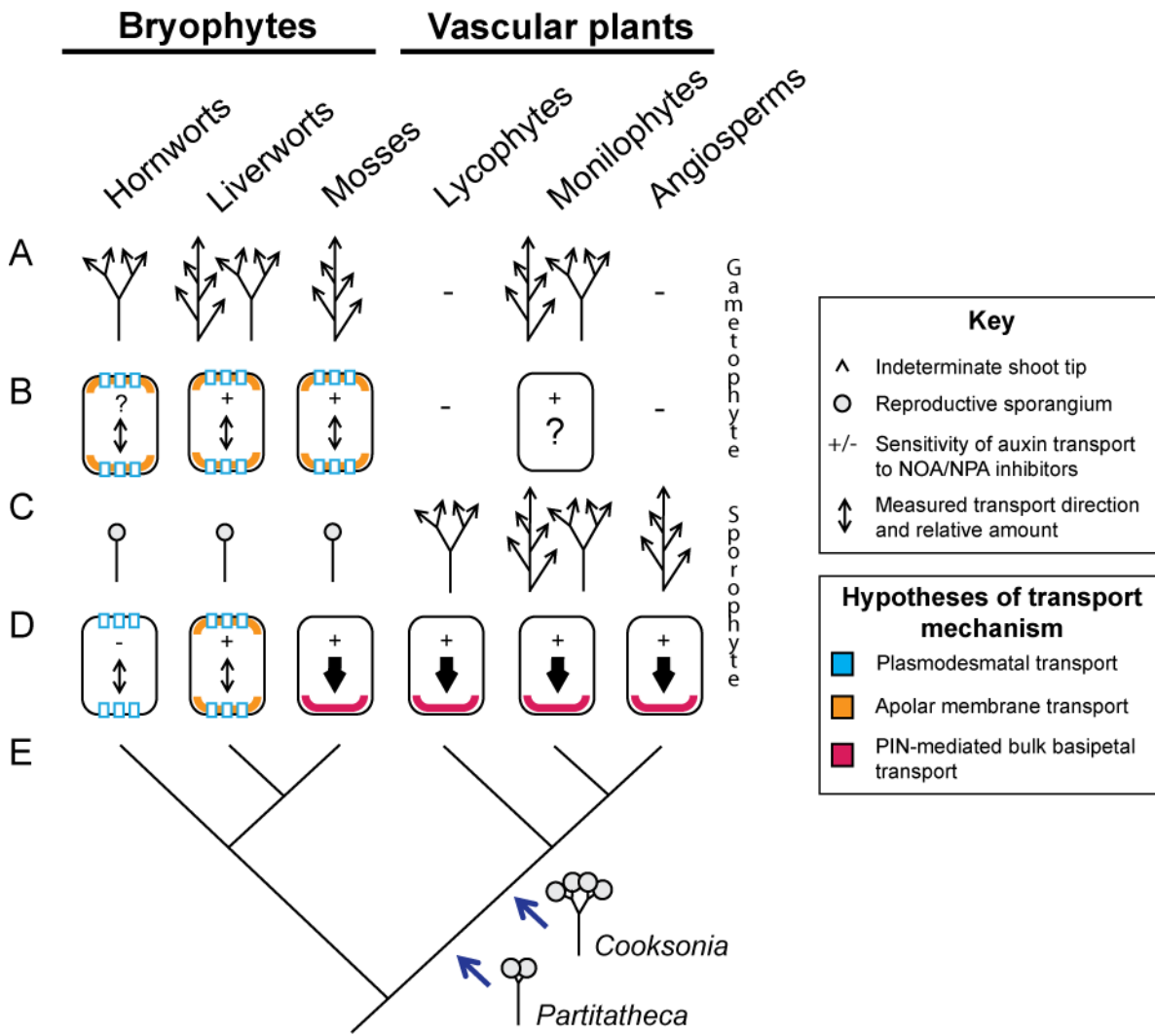
**Table 1**

| | Generation | Hornworts | Liverworts | Mosses | Lycophytes | Monilophytes | Angiosperms |
|---|---|---|---|---|---|---|---|
| **1. Bulk basipetal transport** | G | – [a] | +/– [2,3,a] | – [5,a] | n.a. | | n.a |
| | S | – [1] | – [1] | + [1,5] | +/– [10-12] | + [13] | + [1,5,18,19] |
| **2. Directional transport** | G | | +/– [2,3] | +/– [5-9] | | + [14,15] | |
| | S | – [1] | – [1,4] | + [1,5] | + [10-12] | + [13,15,16] | + [1,5,18,19] |
| **3. Polar ratio in axis** | G | | | | | | |
| | S | 1 [1] | 1.1 [1] | 9.3 [1] | c. 2 [10] | c. 100 [13] | 674 [1] |
| **4. Rate of transport mmh$^{-1}$** | G | | 14 [3] | | | | |
| | S | | 6.9 [1] | 8.9 [1] | 12 [10] | | 11 [1] |
| **5. NPA or TIBA sensitivity** | G | | + [2] | + [7-9,a] | | + [14] | |
| | S | – [1] | + [1] | + [1,5] | + [12] | + [17] | + [1,20] |
| **6. PIN-mediated Transport** | G | | | + [8,9] | | | + [21] |
| | S | | | + [8] | | | + [22] |
| **7. NOA sensitivity** | G | | | + [a] | | | |
| | S | | | + [1] | | | + [23,24] |

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**Table 1: Summary of auxin transport data from major land plant clades and data sources.**

G: gametophyte, S: sporophyte, +: presence, −: absence, blank: no data. Sources: [1]Poli *et al.*, 2003, [2]Maravolo, 1976, [3]Gaal *et al.*, 1982, [4]Thomas, 1980, [5]Fujita *et al.*, 2008, [6]Rose *et al.*, 1983, [7]Rose & Bopp, 1983, [8]Bennett, TA *et al.*, 2014, [9]Viaene *et al.*, 2014, [10]Wochok & Sussex, 1973, [11]Wochok & Sussex, 1974, [12]Sanders & Langdale, 2013, [13]Walters & Osborne, 1979, [14]Gregorich & Fisher, 2006, [15]Albaum, 1938, [16]Steeves & Briggs, 1960, [17]Hou *et al.*, 2004, [18]Goldsmith, 1966, [19]Goldsmith, 1977, [20]Geldner *et al.*, 2001, [21]Ding *et al.*, 2012, [22]Galweiler *et al.*, 1998, [23]Bennett *et al.*, 1996, [24]Parry *et al.*, 2001 [a]Piatowski *et al.*, 2012 (online conference abstract).

**Box 1: Glossary**

**ABCB/PGP:** A class of membrane targeted auxin efflux transporter.

**AUX/LAX:** A membrane targeted auxin influx symporter.

**Axillary branching:** Branching that arises due to the activity of meristems in leaf axils.

**Basipetal:** From the apex to the base.

**Bifurcation:** Branching that occurs by apex divergence.

**BUM:** 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid, a compound that inhibits ABCB/PGP-mediated auxin transport

**CalS/ GSL:** Callose Synthase/ Glucan Synthase enzymes that polymerize glucose to form Callose ($\beta$-1,3-Glucan).

**DDG:** 2-deoxy-D-glucose, a chemical inhibitor of callose synthesis.

**Gametophore:** A gamete producing leafy shoot in mosses and liverworts.

**Gametophyte:** A stage of the land plant life cycle during which gamete production occurs.

**GHL:** Glycosyl hydrolase enzymes that hydrolyze 1,3-beta-glucan polysaccharides such as callose.

**Meristem:** The growing tip of a plant.

**Monophyletic group:** A group containing all the species (or genes) derived from a shared ancestral lineage.

**NOA:** Naphthoxyacetic acid, a compound that inhibits AUX1-mediated auxin influx.

**NPA: 1**-N-Naphthylphthalamic acid, a compound that inhibits PIN-mediated auxin efflux.

**Paraphyletic group:** A group of species (or genes) that is not monophyletic and is basal with respect to a single monophyletic group.

**PAT:** Polar auxin transport.

**PATI:** Polar auxin transport inhibitor.

***Physcomitrella*:** A model moss.

**PIN:** An auxin efflux facilitator class.

**Plasmodesmata:** Plasma membrane lined channels that can provide cytoplasmic continuity between plant cells.

**Primordium:** An organ or tissue at the earliest stages of development.

***Selaginella*:** A model lycophyte.

**Sporangium:** A plant organ in which meiosis occurs to make spores.

**Sporophyte:** A stage of the land plant life cycle during which meiosis occurs.

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