DELVING INTO THE EVOLUTIONARY ORIGIN OF STEROID SENSING IN PLANTS

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Abstract: Brassinosteroids (BRs) are steroid hormones that play crucial roles in plant growth, development and adaptation to shifting environmental conditions. Our current understanding of the origin, evolution and functional significance of BRs is influenced by a double-edged bias: most we know stems from studies on a single species and, on the flip side, dearth of information from a phylogenetically broad and significant array of land plants precludes well-grounded comparisons. Here, we provide an update on BR presence and sensing along land plant evolution. Furthermore, a comprehensive search in all major plant lineages reveals the widespread presence of BR-receptor related sequences, suggesting that steroid-related signals may have been functional early in the evolution of land plants.

Keywords: brassinosteroids, receptor evolution, seed plants, LRR receptor-like kinase, hormone signaling, steroids.

Introduction

Steroids are essential regulators of growth and development in a wide variety of organisms. They were detected in plants, animals and fungi, pointing out to an ancient evolutionary origin [1]. Unlike animals, where steroid hormones are mainly sensed by intracellular receptors that relocate to the nucleus and regulate transcription, plant steroid hormones, brassinosteroids (BRs), are perceived extracellularly by receptors localized at the plasma membrane [2]. BRs binding triggers a cytosolic signaling cascade that ends up with the activation of transcription factors that regulate gene expression.

Both BRs synthesis and signaling have been thoroughly characterized in the model species *Arabidopsis thaliana* (Arabidopsis) and, to a lesser extent, in rice, both flowering plants. Because of a limited sequence information in other branches of land plants, the conservation of this pathway outside the seed-plant clade has remained controversial. Analysis in different species revealed that biologically active BRs, as well as intermediates of the biosynthetic pathway, can be detected already in algae [3,4] and that physiological responses to BRs are present in algae and non-seed plants [5,6,7]. Many components of the signaling cascade are indeed conserved along the plant kingdom; however, it has been assumed that BRs receptors, key players in the pathway, are absent in basalmost lineages, implying that BRs signaling evolved in angiosperms or, at most, seed plants. Thus, whether steroid phytohormones had any role at the origin of land plants remains unknown.

Comparative studies on several phytohormone signaling pathways have set up the basis to a better understanding of their evolutionary significance and their ecological and adaptive contexts [8,9*,10]. However, the analysis of only a few non-flowering species

have slowed the progress to unravel the history of BRs signaling. Here, we provide a comprehensive update of what is known about BRs perception in land plant evolution. Furthermore, a broad search that included many phylogenetically relevant species in all major plant lineages allowed us to confidently detect the widespread presence of BRs receptor-related sequences, redefining the appearance of this protein family early in land plant evolution.

BRs signaling, an assortment of well conserved modules along land plant evolution

BRs signaling that can be conceptually organized into three functional modules (Figure 1). For historic reasons, these modules appeared at first exclusive to BRs signaling, but have increasingly been shown to participate in other pathways [11,12*]. The first module involves the perception of brassinolide (BL), the most active BR compound, by the extracellular domain of the BRASSINOSTEROID INSENSITIVE 1 (BRI1) family of leucine-rich repeat receptor-like kinases (LRR-RLKs). BRI1-like family receptors (from now on BRLs) belong to group X of LRR-RLKs [13] and share the presence of an intervening sequence in the extracellular domain, between the 5^{th} and 4^{th} LRRs preceding the transmembrane domain, called the "island domain" (ID) [14]. The binding of BL to the pocket formed by the ID and adjacent LRRs creates a docking platform for the recruitment of RLK co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family. SERKs and cytoplasmic, membrane-associated receptor-like cytoplasmic kinases (RLCKs) mediate the activation of the BRL kinase domain and the relay of the signal on the cytoplasmic side of membrane compartments [15*]. SERKs and RLCKs are present in all land plants and participate in manifold processes related to the transduction of extracellular stimuli, such as male sporogenesis, separation of organs, immunity and cell death responses [16,17,18,19]. Specificity determinants must therefore

be in place in order to accurately convey the signals. A phosphocode at the SERK3 C-terminal extension has been suggested as a mechanism to discriminate between signaling outputs [20**]. The distribution of BRL sequences has led thus far to the conclusion that BRs signaling is restricted to seed-bearing plants [21]. We provide evidence in the following section supporting the widespread occurrence of BRL receptors in most land plants as well.

A second BR signaling module involves the regulation of Glycogen Synthase Kinase-type kinases (GSK3). GSK3s are present in all eukaryotes and participate in fundamental processes like cytokinesis and environment-modulated developmental responses [22]. Active GSK3s block BRs signaling, and are inactivated upon BL perception. In Arabidopsis, GSK3s are inactivated by BRI1 SUPPRESSOR 1 (BSU1), a member of the BSU1 LIKE (BSL) small family of Kelch-containing protein phosphatases. BSLs likely perform important functions in plants, since they are highly conserved in all Viridiplantae and their absence causes early lethality in Arabidopsis [23*]. The signal emitted by the receptor complex at the membrane appears to activate BSU1 which, in turn, inactivates GSK3s, making BSU1 a promoter of BL signaling [24]. BSU1, the founding member of the family, is however a divergent member specific to the Brassicaceae, that appears to be subject to relaxed selective pressures probably related to a process of sub- or neo-functionalization [23*]. In contrast, in rice, which has the canonical set of BSLs shared by all other vascular plants, loss of function of one BSL results in phenotypes that suggest an inhibitory effect on BL signaling through the activation of GSK3s [25]. Whether the situation in rice is the norm and Arabidopsis the exception remains to be clarified.

Finally, the BRs gene regulatory output module is operated by transcription factors that belong to the BRASSINAZOLE RESISTANT 1 (BZR1) family, whose members can be found in Streptophyta from Coleochaetales to angiosperms. These proteins are

characterized by an idiosyncratic N-terminal variation of the bHLH motif [26*] and a regulatory C-terminal domain that recruits transcriptional co-repressors [27]. In angiosperms, the active phosphorylation of BZR1 and its homologs by GSK3s negatively affects their stability, intracellular localization and ability to bind to DNA, whereas inactivation of GSK3s leads to the accumulation of nuclear-localized, active forms. Functional studies on BZR1 family members in basal land plants have not yet been conducted.

Although GSK3s act in disparate processes in plants, BL was for some time the only signal identified to regulate their activity. In addition, BZR1 proteins were until recently supposed to be specific to the BRs pathway. However, these modules have recently been shown to act in other membrane-receptor dependent processes as well. Perception by the LRR-RLK PHLOEM INTERCALATED WITH XYLEM (PXY) of an extracellular CLAVATA3-ESR-related (CLE) peptide in ferns and seed plants maintains an undifferentiated state of procambium cells and prevents xylem differentiation[28]. In this case, the receptor directly interacts with and activates GSK3s, thus inhibiting the action of BZR1. On the other hand, the LRR-RLK EXCESS MICROSPOROCYTES1 (EMS1) in Arabidopsis [29*] or MULTIPLE SPOROCYTE1 (MSP1) in rice [30] also perceive extracellular peptides in anthers and ovules to control the developmental transit during sporogenesis, one of the defining features of land plants. It was recently reported that the co-expression of EMS1 and its cognate peptide TAPETUM DETERMINANT1 (TPD1) can partially complement *bri1* mutants and, conversely, the expression of a stabilized BES1 form in anthers can complement *ems1* mutants, showing that both pathways share downstream signaling components [31**,32**]. Significantly, the homologs of EMS1 and TPD1 from the moss *Physcomitrella patens* were able to complement Arabidopsis *bri1* mutants, indicating a strong functional conservation in land plants [31**]. Not

unexpectedly, both PXY and EMS1 also interact with SERK proteins, although they associate with SERK1 and 2 whereas BRI1 preferentially associates with SERK3 [18,33].

The evolutionary history of BRLs

Arabidopsis BRI1, the founding member of the BRL family, is composed of 25 extracellular LRRs interrupted, between the 21st and 22nd repeat, by the 70-amino-acid ID, followed by a transmembrane region (TM), a juxtamembrane region (JM), a kinase domain (KD) and a C-terminal tail (CT): LRR{20}-ID-LRR21-LRR{3}-TM-JM-KD-CT [34]. The ID folds back inside of the LRR-superhelix to create a surface pocket that is able to bind BL [35*,36,37]. This structural template is also found in the three BRI1 homologs in Arabidopsis, BRL1, 2 and 3. In a previous study, Wang and Mao [38] suggested that the BRL configuration is a consequence of a stepwise domain acquisition process: LRR-TM-KD predated the split between streptophytes and chlorophytes; basal land plants incorporated the JM domain and, finally, the ID appeared in the common ancestor of angiosperms and gymnosperms. Thus, the perception of BRs appears to be a relatively late acquisition.

A recent study revealed that the KD of EMS1 and BRI1, both belonging to the same group of LRR-RLKs, are interchangeable in Arabidopsis and able to activate the same signaling pathway [31**]. In fact, the authors show that the EMS1-TPD1 pair is present in all land plants, whereas BRLs are apparently absent from non-flowering plants. Thus, EMS1 and BRLs seem to stem from a common ancestor which probably signaled through the GSK3 and BRZ1/BES1 modules. According to their hypothesis, BRLs evolved to bind a smaller, more diffusible molecule and acquired a broader expression pattern, whereas the short-range peptide signal perceived by EMS1 remained confined to specific tissues. These receptors would have subsequently undergone events of duplication and divergence giving rise to the angiosperm BRL family. As apparent in Arabidopsis and

rice, a first duplication gave rise to the BRI-BRL1,3 and BRL2 clades, and a second split originated the BRI1 and BRL1-3 clades [38,39*]. This timeline seems to have a functional correlate: AtBRI1, AtBRL1 and AtBRL3 perceive BRs, whereas AtBRL2 is not able to activate BRs signaling [39*]. BRL2 plays a role during the differentiation of provascular cells, but its ligand and downstream signaling events remain uncharacterized [40].

Our understanding of the evolution of BRLs has been heavily influenced by limitations in the phylogenetic sampling [38]. To address this question, we looked for the presence of the diagnostic ID and conserved positions therein in as many plant lineages as possible (Figure 2). Contrary to previous assumptions, we found *bona fide* BRL sequences in most land plants: BRLs are absent in streptophyte algae, but appear to be part of the innovation toolkit of land plants. We found BRLs in all hornworts and in many of the earliest diverging branches of mosses, but not in the more derived Funariales (to which, significantly, *Physcomitrella* belongs), Bryales and Hypnales. The recent clarification of the phylogeny of mosses has greatly helped to give context to our findings [41**]. Congruent with a selective loss in some lineages, no BRLs were found in liverworts. Although this might suggest that basal land plants lacked these proteins, it is increasingly clear that liverworts are not the direct descendants of the first land plants, but rather a derived and specialized group. Indeed, the recent suggestion that stomata may have been present in early land plants but subsequently lost in some lineages, namely liverworts, supports our claim [42]. Two main types of BRLs are found in vascular plants: the BRL2- and BRI1-types. BRL2-related sequences appear to retain ancestral features: proteins from bryophytes, lycopods and ferns are more closely related to seed-plant BRL2. On the other hand, BRI1-type sequences appear only, as previously observed, in seed plants. It seems that both angiosperms and gymnosperms underwent an early duplication event that originated two clades in each group. A diagnostic feature of the BRI1 clade is the presence

of a C-terminal tail involved in the final activation steps of BRI1 by SERK3 [43]. Significantly, both Arabidopsis EMS1 and all BRL2-like sequences lack this extension, suggesting that this might be the ancestral state. However, since the intracellular domains of BRI1 and EMS1 are functionally equivalent [31**], this tail is not absolutely needed for their signaling mechanism.

The inability AtBRL2 to bind BL and complement *bri1* mutants has made it the less studied member of the family [39*]. It is possible that the function of BRL2 in seed plants has been inevitably influenced by the appearance of BRI1 sequences: both genes may have suffered a subfunctionalization process after they split. Alternatively, BRI1 may have neofunctionalized whereas BRL2 retained an ancestral role, possibly related to a meristematic function that in angiosperms is expressed in procambial cells. It is interesting to note that, at least in Arabidopsis, BRL1 and BRL3 are preferentially localized in the vascular tissues [39*,44,45]. Most, if not all, players in the BR signaling pathway can be recruited to membrane-associated complexes by the TTL scaffolding proteins [46*]. TTL proteins were, in fact, long identified as BRL2-interacting proteins [47], an evidence that BRL family members share downstream signaling components; it can also be surmised that BRL2 interacts with SERKs. The overall features of the ID domain are also conserved in all BRL proteins. The analysis of a phylogenetically broad number of sequences reveals that this region, even among angiosperms, is a mosaic of features that may as well depend on function as on history, so that conclusions based solely on the Arabidopsis proteins should be taken with caution. A close inspection at the binding pocket formed by the ID and surrounding regions shows that during land plant evolution the inner surface of the cavity, lined by aromatic residues, has been subject to greater evolutionary constraints than its external rim (Figure 3). As noted previously in Arabidopsis and rice [37,48*], one of the few structural features that might account for

the absence of BL binding in BRL2 is the presence of a polar residue (Gln or Glu) at the external limit of the binding pocket. This position, conserved in BRL2-type sequences in ferns, hornworts and gymnosperms, is occupied by a hydrophobic residue in BL-binding BRLs but also, intriguingly, in lycophytes and mosses (Fig S1). This conservation pattern suggests that BRL2-type receptors might bind to a non-polar (also steroidal) ligand with different polar decorations than those found in BL, such as those discussed in the following section.

Occurrence of BRs and biosynthetic pathways in land plants

BRs seem to appear early in the evolution of the plant kingdom. The active compounds BL and catasterone (CS) were detected in 24 algae species in the Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Charophyceae [4]. Responses to the application of BRs were detected in the green algae *Chorella vulgaris:* CS or BL treatments produced a stimulatory effect on cell proliferation and increases in nucleic acid and protein content [5,49,50]. Low concentrations of CS and BRs-related compounds were also detected in the bryophytes *Marchantia polymorpha* and *Physcomitrella patens* and in the lycophytes *Selaginella moellendorffii* and *Selaginella uncinata* [3,51]. The functional correlate of these compounds is still unknown. However, Cheon *et al*. [6] were able to demonstrate that BRs affect *S.moellendorffii* shoot growth. Indeed, treatments with a BRs biosynthetic inhibitor and an analogue of BL (epibrassinolide, eBL) resulted in delayed and increased growth, respectively. Many BRs-related compounds were also identified in ferns [7,52] and treatments with eBL affect spore germination in *Polystichum lonchitis* and *Pteridium aquilinum* [7]. BRs were also identified in a broad range of angiosperms and gymnosperms, with seemingly important functions related to growth and reproduction [53].

Intriguingly, only the pathway that leads to the synthesis of C-28 BRs in angiosperms is well described: reactions in this pathway are catalysed by 5α-steroid dehydrogenase (DET2) and enzymes belonging to the CYP85 clan (CYP85A and CYP90A/B/C/D subfamilies). The complete conservation of this pathway in gymnosperms has been also recently proposed [54]. Outside the seed clade, DET2 homologs have been detected in all land plants, and ferns seem to contain CYP90B1 and CYP90A1 sequences [54]. Although at first sight this might suggest that ferns are an intermediate state, a more biologically meaningful hypothesis is that groups other than seed plants have evolved, from common precursors, different biosynthetic pathways that are still unknown. The co-occurrence of both BRI1/BRL1-3 sequences and the complete set of enzymes involved in the C-28 BRs biosynthetic pathway suggests that this may indeed be the evolutionary path followed by seed plants; still, the ancient BRL2-like sequences may potentially bind steroids synthetized through alternative pathways still operative in other land plants, and also even in angiosperms.

CONCLUDING REMARKS

Our knowledge of BRs function and evolution is still profoundly skewed: almost all we know has been gained from a single species, and we inevitably tend to interpret other species under the light of this template. However, plant lineages carry long histories of stochastic sampling through extinctions and subsequent expansions. The bryophyte model species *Physcomitrella patens* and *Marchantia polymorpha* lack BRL sequences, which *a priori* might indicate that BRLs are an innovation of seed plants or, at most,

euphyllophytes. However, a broad phylogenetic survey shows that BRLs are present in hornworts, basal moss branches and lycophytes as well. An alternative, and more parsimonious hypothesis, would therefore state that BRLs were a common feature of the earliest land plants, but were subsequently lost in liverworts and crown moss groups. If that is the case, we may take full advantage of the genetic tools available in *Marchantia* and *Physcomitrella* to explore the alternative pathways that replaced BRLs, and ask whether associated signaling partners were modified in their wake. But at the same time we should get a better understanding of the primordial roles of BRLs. BRL2-type sequences apparently derive from the most ancestral forms of the BRL family and certainly deserve a fresh reassessment. Which are their ligands? and, if identified, how are they synthesized and what signals they convey? To this end, we should actively turn to alternative model species, like the hornwort *Anthoceros agrestis* or the fern *Ceratopteris richardii* [55,56], bearing in mind that they are not just intermediate steps that lead to Arabidopsis, but the result of independent evolutionary, ecological and developmental trails that we must strive to understand. The possibility to sample sequences from the full range of land plant lineages and their immediate ancestors gives us an unique starting point to foster biological inferences and eco-evo-devo sensible hypothesis. Actual functional evidence must follow suit.

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Figure Legends

Figure1. Conservation of the brassinosteroid signaling pathway. Three functional modules, Module 1: BL ligand sensing, Module 2: GSK3 regulation and Module 3: transcriptional regulation, are highlighted in blue, green and yellow boxes, respectively. Proteins in blue are present in Viridiplantae, proteins in brown are conserved in Streptophyta and proteins in green have its origin in Embryophyta.

Figure 2. Phylogenetic relationships of the BRI1-like family members. Full genome sequences were searched for several angiosperms, *Selaginella moellendorfii* and *Sphagnum fallax* (phytozome.jgi.doe.gov), *Picea glauca* (congenie.org), *Ginkgo biloba* (CNP0000136), *Azola filiculoides* (fernbase.org) and *Anthoceros punctatus* (hornworts.uzh.ch). NCBI Whole-genome shotgun contigs, Transcriptome Shotgun Assembly and the OneKP project [57] databases were also extensively searched. Preliminary tBLASTn searches were performed using the sequence surrounding the ID in AtBRI1 and AtBRL2 as query; once hits in several species were found, these new sequences were used as queries for further searches in members of their clades. Sequences that showed conserved presence and spacing of the Cys residues in the ID as well as in other diagnostic positions were chosen for further analysis. Only high quality, full-length sequences were retained, except for some phylogenetically relevant species where we used what is available. Sequences were manually inspected for gaps or frameshifts and trimmed to encompass, whenever possible, from the start of the LRR N-terminal to the insertion of the ID, to the start of the kinase domain, including the TM and JM regions. 150 amino acid sequences from all major land plant lineages were used for the evolutionary analysis conducted in MEGA X [58]. Sequences were aligned with Muscle; the alignment manually curated, and gaps caused by single sequences removed. The

phylogenetic analysis was performed using the Neighbor-Joining method with 1000 replicates for the bootstrap (BS) test; during this analysis, positions with less than 80% site coverage were also eliminated. In the final representation, nodes with less than 50% BS support were collapsed, and branch lengths equalized. Sequences 3 and 4 from *Ceratopteris richardii* show an unstable position and do not match those found in other ferns; they can be specific novelties in response to a submerged lifestyle. Sequences are provided in Supplementary file 1.

Figure 3. Conservation model of BRLs binding pocket among land plant evolution. Representative BRL sequences from all land plant lineages spanning LRR(-5)/ID/LRR(-4_-1) from the transmembrane domain were aligned with Muscle. Conservation scores obtained with AL2CO [59] were mapped onto the same region of AtBRL1 (PDB ID:4j0m) using UCSF Chimera. Highly conserved regions are colored in red, more variable positions are in shades of pink and white. BL is colored in yellow.

Figure S1. Amino acid sequence alignment of representative BRI1-type and BRL2-type proteins from all major groups of land plants, a subset of those used for Figure 3. Sequences were aligned with Muscle; only residues less than 7Å from BL in the crystal structure of BRL1 (PDB 4j0m) are shown, numbered as in BRL1. Highly conserved residues are highlighted in red, more variable positions are colored in shades of pink and gray. The black arrowhead indicates the position (number 642 in BRL1) that may define the ability to bind BL or related molecules.

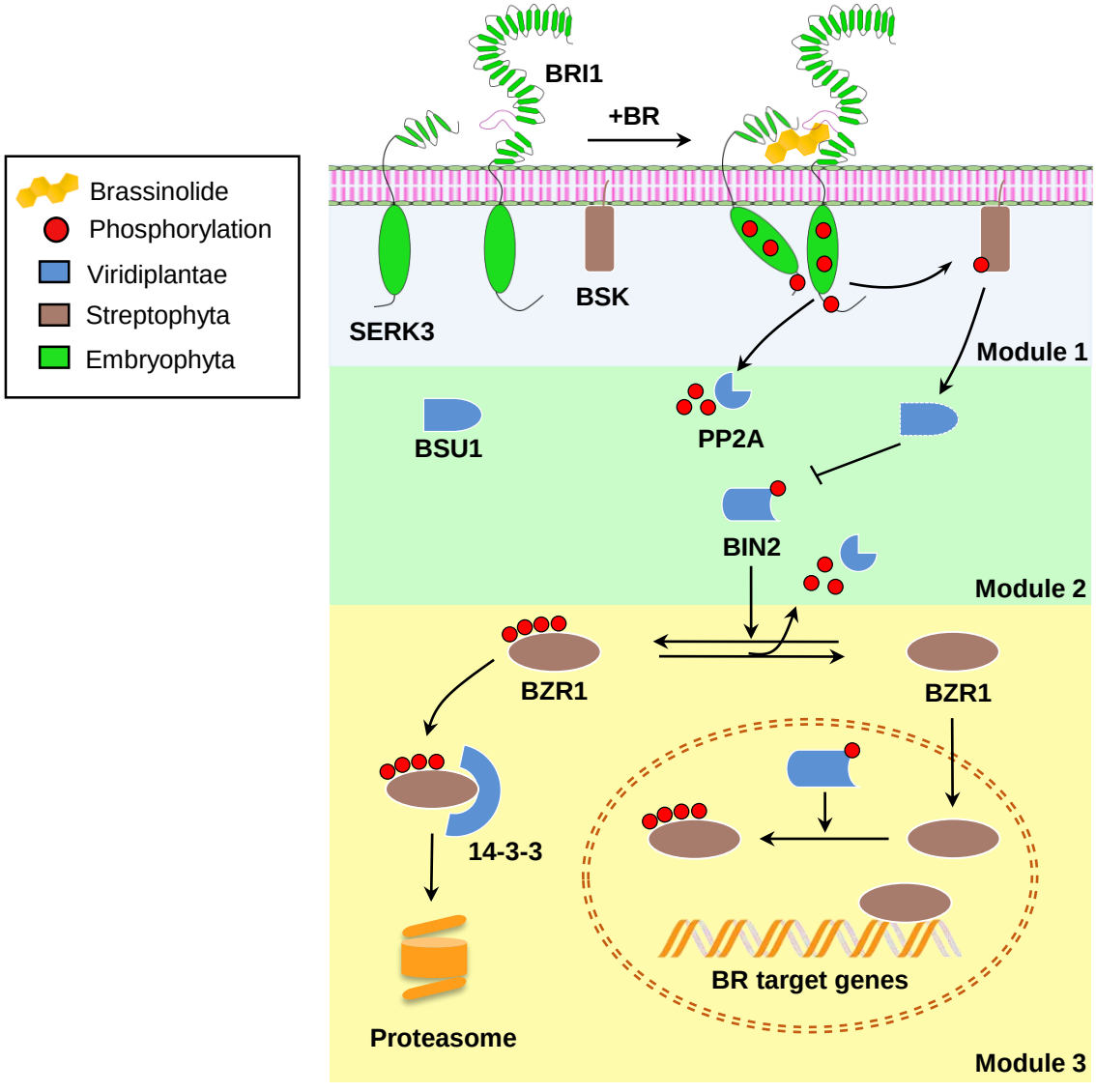

Figure1. Conservation of the brassinosteroid pathway.
Ferreira *et al.* 2020

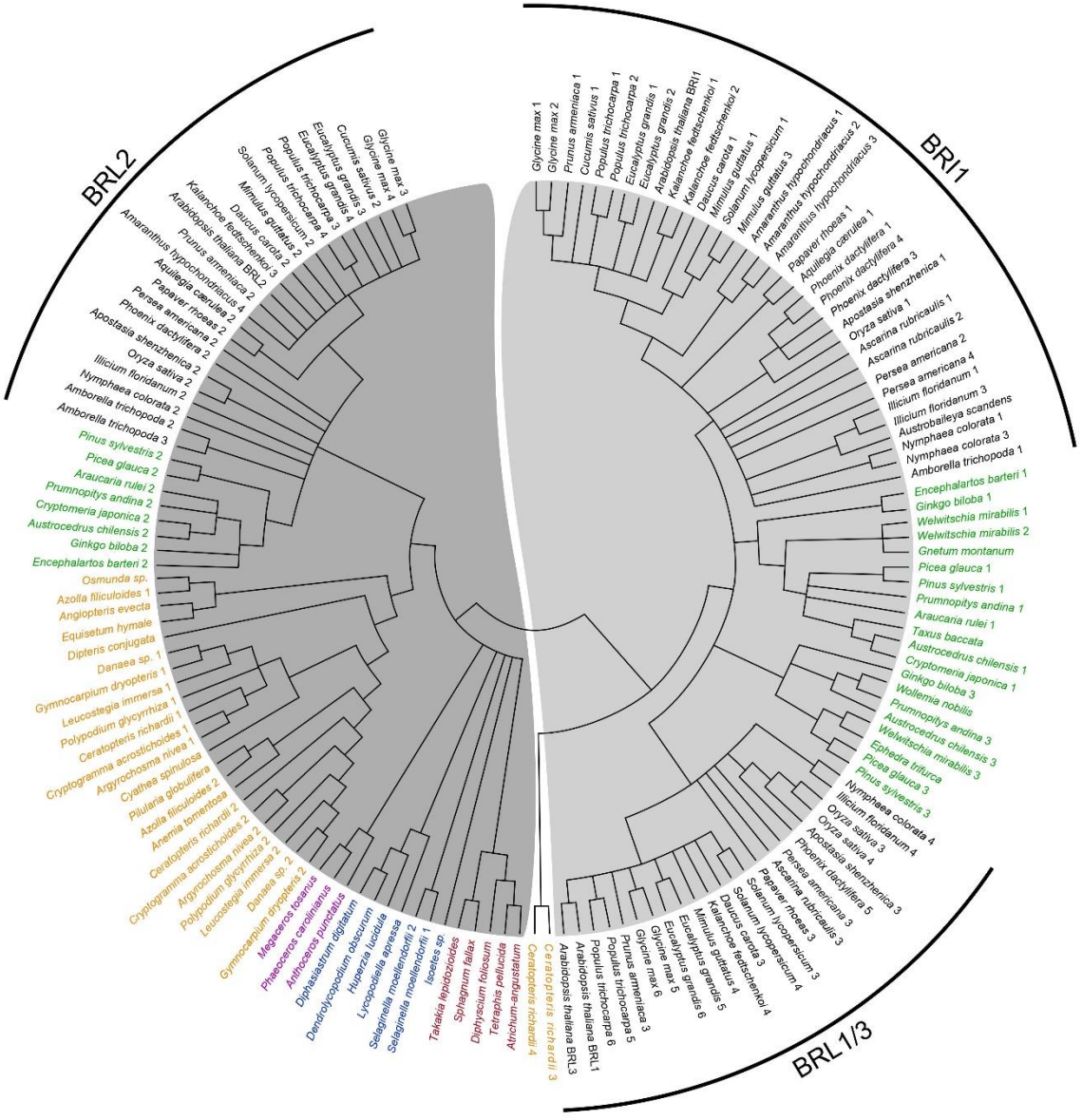

Figure 2. Phylogenetic relationships of the BRI1-like family members. Ferreira *et al.* 2020

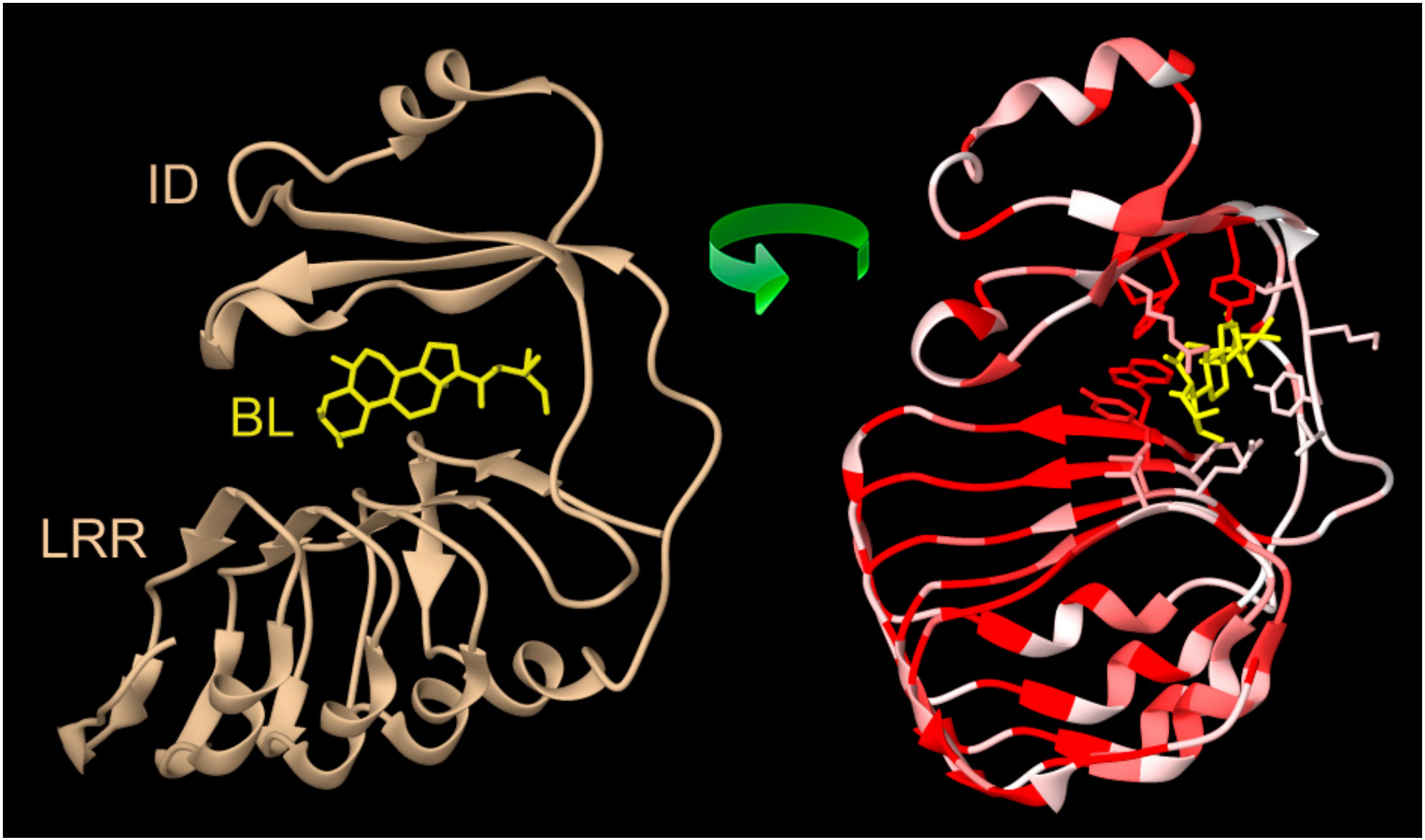

Figure 3. Conservation model of BRLs binding pocket among land plant evolution.
Ferreira et al. 2020

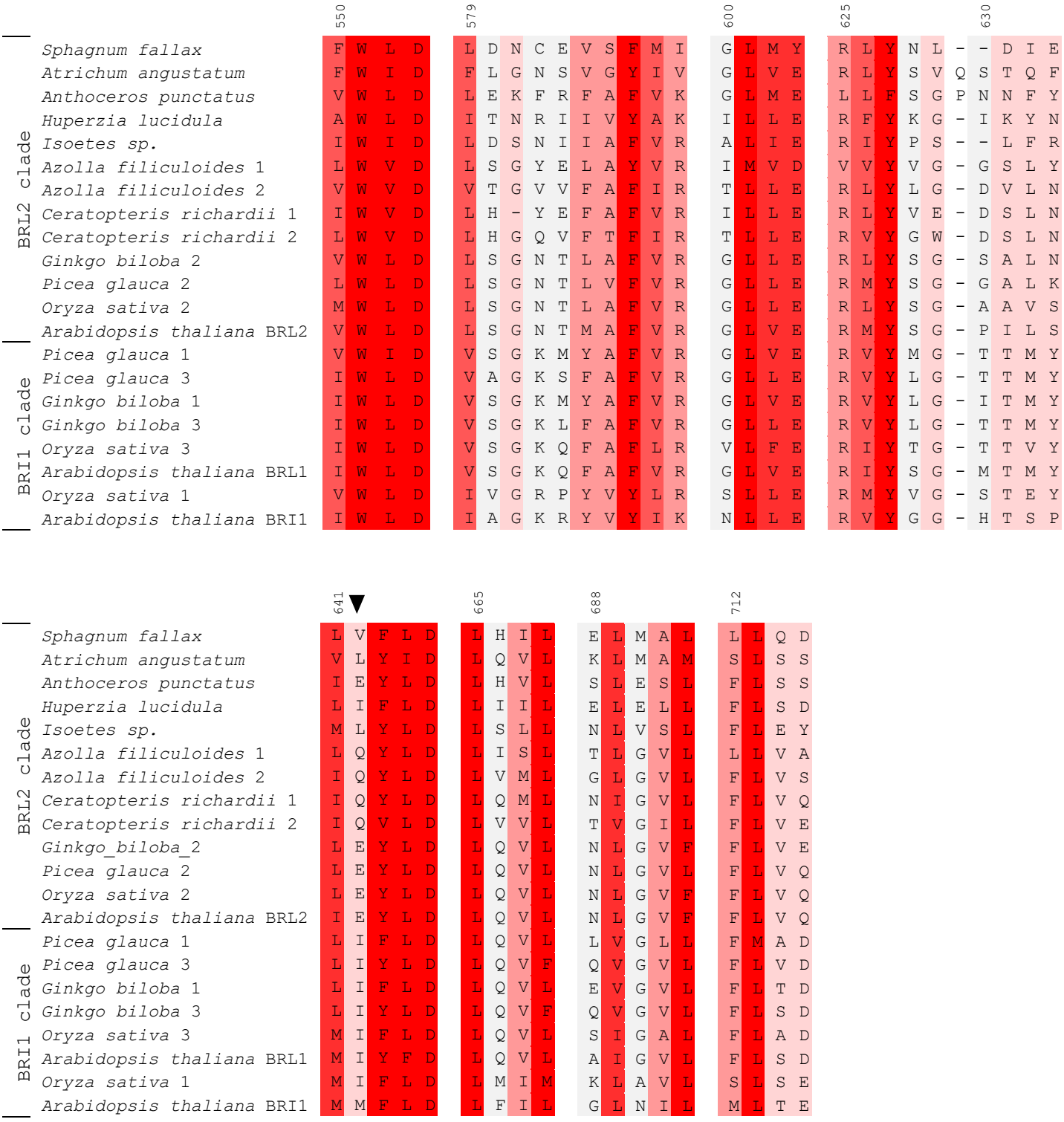

Figure S1. Amino acid sequence alignment of representative BRI1-type and BRL2-type proteins from all major groups of land plants, a subset of those used for Figure 3. Ferreira *et al.* 2020